THE REGENERATIVE AND ANTI-INFLAMMATORY CAPABILITY OF *PROSOPIS GLANDULOSA*

by

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Faculty of Health Sciences at Stellenbosch University

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DECLARATION:

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DEDICATION:

My loving husband, children, my family and friends,

for your continuous inspiration and support.

ABSTRACT

Introduction and aims: The use of herbal preparations for the treatment of various ailments has gained enormous prominence. The aim of this study was to evaluate the effects of a plant-derived product, consisting solely of dry-milled pods of the *Prosopis glandulosa* tree, on various altered metabolic demands placed on skeletal muscle. This study included the evaluation of (i) altered glucose uptake as a result of insulin resistance, (ii) exercise-induced fatigue and (iii) the inflammatory and regenerative process of skeletal muscle after a contusion injury, with particular attention paid to the infiltration of immune cells and the adaptation of regenerative markers.

Methodology: *P. glandulosa* (100 mg/kg/day) mixed into jelly, was orally administered daily to rats for a period of 8-10 weeks. *Aim 1:* Rats were rendered insulin resistant after being on a high caloric diet for 16 weeks, where after half the animals underwent a 120 min intra-peritoneal glucose tolerance test. The rest were fasted, body weight and intra-peritoneal fat weight determined, sacrificed, blood collected for blood glucose- and insulin level determination and soleus muscles removed for insulin sensitivity determination. *Aim 2:* Soleus muscles were excised, weighed, measured and mounted for isometric force determination. Muscles were vertically placed in Krebs Henseleit buffer solution in a water-jacketed organ bath (25˚C). Twitch- and tetanic force production, contraction time, half-relaxation time, force-frequency relationship and fatigue were measured. *Aim 3:* The gastrocnemius muscle was injured by a contusion injury (mass-drop model) and left for 1-, 3 hours, 1- or 7 days before further experimentation commenced. Following the different time periods, the gastrocnemius muscles were removed, divided and stored either in liquid nitrogen or 4% formaldehyde. Immune cell infiltration was analyzed with immunohistochemistry (neutrophils - His48-positive; macrophages - F4/80-positive). $ADAM_{12}$ (Western blotting) and desmin (immunohistochemistry) were used as markers to evaluate muscle regeneration.

Results: *Aim 1: P. glandulosa* treatment had no effect on body- or fat mass. Treatment significantly decreased the elevated blood glucose levels observed in the obese rats. *Aim 2: P. glandulosa* treatment had: (i) no effect on muscle mass or optimal muscle length; (ii) no significant

effect on muscle fatigue tolerance, as both treated and untreated groups fatigued at the same rate and (iii) *P. glandulosa*-treated rats generated significantly increased force when the muscle was stimulated to generate a single twitch and tetanus. This augmented effect disappeared after the fatigue protocol. *Aim 3:* Chronic *P. glandulosa* treatment as well as post-injury treatment led to a significant reduction in neutrophil infiltration into the injured area. Additionally, chronic *P. glandulosa* treatment significantly increased the expression of both $ADAM_{12}$ (day 1) and desmin (day 7) after injury, indicating faster muscle regeneration.

Conclusion: The data obtained from this study is novel, since there is no known literature on the effect of *P. glandulosa* on insulin resistance, force generation, fatigue tolerance or muscle recovery after injury. Given the current evidence, we conclude that *P. glandulosa* treatment might prove beneficial as supplement, aiding physical ability and assisting in the sooner recovery.

OPSOMMING

Inleiding en doelwitte: Die gebruik van plantaardige produkte vir die behandeling van verskeie siektes neem eksponensieel toe. Die doel van hierdie studie was om die effekte van 'n plant produk, wat uitsluitlik bestaan uit die droog-gemaalde peule van die *Prosopis glandulosa* boom, op veranderde metaboliese eise wat aan skeletspier gestel word, te toets. Hierdie studie sluit in die evaluering van (i) glukose opname, as gevolg van insulien weerstandigheid, (ii) oefengeïnduseerde moegheid en (iii) die inflammatoriese en regeneratiewe prosesse na 'n kontusiebesering, met besondere aandag aan die infiltrasie van immuun selle en die aanpassing van regeneratiewe merkers.

Metodes: *P. glandulosa* (100 mg/kg/dag) was daagliks oraal toegedien in jellieblokkies vir 'n tydperk van 8-10 weke. *Doel 1*: Insulienweerstandigheid is in die rotte geïnduseer deur 'n hoë kalorie dieet oor 16 weke. Helfte van die diere het 'n 120 min intra-peritoneale glukose toleransie toets ondergaan. Die res is gevas, hulle liggaamsgewig en intra-peritoneale vetgewig bepaal, geslag, bloed geneem vir bloedglukose- en insulien vlak bepalings en die soleus spiere verwyder vir insuliensensitiwiteits toetse. *Doel 2:* Soleus spiere is uitgesny, geweeg, gemeet en gemonteer vir isometriese kragbepalings. Spiere is in Krebs Henseleit buffer oplossing in 'n orgaanbad (25 ˚C) geplaas. Enkelkontraksies, tetanie, kontraksietyd, half-verslappingstyd, krag-frekwensie verhouding en moegheid is gemeet. *Doel 3:* Die gastrocnemius spier is beseer deur kontusiebesering (massa-val model) en vir 1-, 3 ure, 1- of 7 dae gelaat voor verdere eksperimentering. Na die verskillende tydperke, is die gastrocnemius spiere verwyder, verdeel en gestoor in vloeibare stikstof of 4% formaldehied. Immuun sel infiltrasie is ontleed deur immuunhistochemie (neutrofiele - His48-positief; makrofage - F4/80-positief). $ADAM_{12}$ (Westernblot) en desmin (immuunhistochemie) is gebruik as merkers van spierregenerasie.

Resultate: *Doel 1: P. glandulosa* behandeling het geen effek op die liggaamsmassa of vetmassa gehad nie. Behandeling het die verhoogde bloedsuikervlakke van die oorgewig rotte verlaag. *Doel 2: P. glandulosa* behandeling het: (i) geen effek op spiermassa of optimale spierlengte gehad nie; (ii) geen wesenlike uitwerking op spiervermoeienis gehad nie. (iii) Die spierkontraksiekrag in *P.*

glandulosa-behandelde rotte was aansienlik hoër wanneer die spiere gestimuleer is om 'n enkelkontraksie of tetanus te genereer. Hierdie verhoogde krag het erger na die vermoeienis-protokol verdwyn. *Doel 3:* Kroniese *P. glandulosa* behandeling sowel as post-beserings behandeling het tot 'n aansienlike vermindering in neutrofiel infiltrasie in die beseerde area gelei. Addisioneel het kroniese *P. glandulosa* behandeling die uitdrukking van $ADAM_{12}$ (dag 1) en desmin (dag 7) na besering aansienlik laat toeneem wat op versnelde spier-regenerasie dui.

Gevolgtrekkings: Die data verkry uit hierdie studie is nuut, want daar is geen gepubliseerde literatuur oor die uitwerking van *P. glandulosa* op insulienweerstandigheid, spier kontraksiekrag, spiervermoeienis of spierherstel na 'n besering nie. Gegewe die huidige bewyse, maak ons die gevolgtrekking dat *P. glandulosa* behandeling voordelig kan wees as ‘n aanvulling, dus as ondersteuning in die vermoë om fisies te presteer en om die terugkeer na besering vinniger te laat plaasvind.

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LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

Poster presentations:

- **George, C.**, Huisamen, B. 2010. The efficacy of Diavite™ (*Prosopis glandulosa*) as anti-diabetic treatment in rat models of Streptozotocin-induced type 1 diabetes and diet-induced obese insulin resistance. *10th AstraZeneca Health Science Research Day,* Tygerberg, Cape Town.
- **George, C.**, Huisamen, B. 2010. The effects of *Prosopis glandulosa* (Diavite™) on insulin sensitivity in a diet-induced insulin resistant rat model. 4th *Annual Medical Research Council (MRC) meeting,* Parow, Cape Town.
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- **George, C.**, Huisamen, B. 2012. The efficacy of *Prosopis glandulosa* as antidiabetic treatment in rat models of diabetes and insulin resistance. *SEMDSA/NOFSA congress,* Bantry Bay, Cape Town. Was awarded the prize for best poster in the < 35 oral poster competition.
- **George, C.**, Huisamen, B. 2012. The efficacy of *Prosopis glandulosa* as antidiabetic treatment in rat models of diabetes and insulin resistance. *6th Annual Medical Research Council (MRC) meeting,* Parow, Cape Town.

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- **George, C.**, Huisamen, B. 2011. *Prosopis glandulosa* as antidiabetic treatment in rat models of insulin resistance and diabetes. *University of Stellenbosch 55th Annual Academic Year Day,* Tygerberg, Cape Town.

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- **<u>George, C.</u>**, Lochner, A., Huisamen, B. 2011. The efficacy of *Prosopis glandulosa* as antidiabetic treatment in rat models of diabetes and insulin resistance. *Journal of Ethnopharmacology,* 137: 298– 304.

- Huisamen, B., **<u>George, C.,</u>** Dietrich, D., Genade, S., Lochner, A. 2013. Cardioprotective and anti-hypertensive effects of *Prosopis glandulosa* in rat model of pre-diabetes. *Cardiovascular Journal of Africa,* 24: 31-37.

Refereed full length papers in the proceedings of international symposia

- Huisamen, B., **<u>George, C.,</u>** Dietrich, D., Lochner, A. 2012. Cardioprotective and anti-hypertensive effects of *Prosopus glandulosa* in a rat model of pre-diabetes. 10th *Annual Scientific Sessions of the Society for Heart and Vascular Metabolism*, Oxford, UK.

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- Huisamen, B., **George, C.,** Dietrich, D., Lochner, A. 2012. Cardioprotective and anti-hypertensive effects of *Prosopus glandulosa* in a rat model of pre-diabetes. *13th Annual Congress of the SA Heart Association*, Sun City, Mpumalanga.
- **George, C.,** Huisamen, B. 2012. The efficacy of *Prosopis glandulosa* as an anti-diabetic treatment in rat models of diabetes and insulin resistance. 47th Congress of the Society for Endocrinology, Metabolism and Diabetes of SA. *JEMDSA,* 17:25.

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LIST OF ABBREVIATIONS

2-DG	- 2-deoxy-D-^{3}[H] glucose
ACE	- angiotensin-converting enzyme
$ADAM_{12}$	- a disintegrin and metalloprotease
ADP	- adenosine diphosphate
AMP	- adenosine monophosphate
AMPK	- adenosine monophosphate-activated protein kinase
ANOVA	- analysis of variance
AP	- action potential
ATP	- adenosine triphosphate
AU	- arbitrary units
AUC	- area under the curve
BC	- before Christ
bFGF	- basic fibroblast growth factor
BMI	- body mass index
BMP	- bone morphogenic protein
BSA	- bovine serum albumin
Ca^{2+}	- calcium

$CaCl_2$	- calcium chloride
CAP	- Cbl-associated protein
CARA	- Conservation of Agricultural Resources Act
CK	- creatine kinase
CO_2	- carbon dioxide
CoQ	- co-enzyme Q
COX	- cyclooxygenase
Cr	- creatine
CTX	- cardiotoxin
$CuSO_4$	- copper(II)sulphate
DFM	- diferuloylmethane
DIO	- diet-induced obesity
DMSO	- dimethyl sulphoxide
DMTU	- dimethylthiourea
DNA	- deoxyribonucleic acid
EC	- excitation-contraction
ECL	- enhanced chemiluminescence
ECM	- extra cellular matrix

EDL	- extensor digitorum longus
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethyleneglycoltetraacetic acid
eNOS	- endothelial nitric oxide synthase
ERK	- extracellular signal-regulated kinase
FAP	- fibro/adipogenic progenitor
FFA	- free fatty acid
FFR	- fructose-fed rat
FGF	- fibroblast growth factor
F_{max}	- maximum force
FST	- forced swim test
GLUT	- glucose transporter
GM-CSF	- granulocyte-macrophage colony-stimulating factor
GPx	- glutathione peroxidase
GSK	- glycogen synthase kinase
H&E	- haematoxylin and eosin
H^+	- hydrogen
H_2O_2	- hydrogen peroxide

HbA1c	- glycated hemoglobin
HCl	- hydrogen chloride
HDL	- high-density lipoprotein
HGF	- hepatocyte growth factor
HOMA-IR	- homeostatic model assessment of insulin resistance
HSPG	- heparin sulphate proteoglycan
Hz	- hertz
i.p	- intraperitoneal
ICAM	- intercellular adhesion molecule
IFN	- interferon
IGF	- insulin-like growth factor
IL	- interleukin
IMP	- inosinemonophosphate
iNOS	- inducible nitric oxide synthase
IPGTT	- intraperitoneal glucose tolerance test
IR	- insulin receptor
IRS	- insulin receptor substrate
JAK	- Janus activating protein kinase

JNK	- c-Jun N-terminal kinase
K^+	- potassium
KCl	- potassium chloride
KH_2PO_4	- monopotassium phosphate
KHB	- Krebs Henseleit buffer
LDH	- lactate dehydrogenase
LDL	- low-density lipoprotein
LLLT	- low-level laser therapy
LOOH	- lipid hydroperoxide
MAPK	- mitogen activated protein kinase
MCC	- Medical Control Council
MCP	- monocyte chemoattractant protein
MCT	- monocarboxylate transporter
Mg^{2+}	- magnesium
$MgSO_4$	- magnesium sulfate
MHC	- myosin heavy chain
MPO	- myeloperoxidase
MRC	- Medical Research Council

MRF	- myogenic regulatory factor
mtDNA	- mitochondrial DNA
N_2	- nitrogen
Na^+	- sodium
Na_2CO_3	- sodium carbonate
Na_2SO_4	- sodium sulfate
Na_3VO_4	- sodium orthovanadate
NAC	- N-acetylcysteine
NaCl	- sodium chloride
NaCMC	- sodium carboxymethyl cellulose
NADPH	- nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	- sodium bicarbonate
NaK^+	- sodium potassium
NaOH	- sodium hydroxide
NF-κβ	- nuclear transcription factor kappa-beta
NGF	- nerve growth factor
NH^{4+}	- ammonium
NO	- nitric oxide

Nrf	- nuclear factor erythroid 2-related factor
NSAID	- non-steroidal anti-inflammatory drug
NSB	- non-specific binding
NTX	- notexin
O_2	- oxygen
OH^-	- hydroxyl radical
PAI	- plasminogen activator inhibitor
PBMC	- peripheral blood mononuclear cells
PCr	- phosphocreatine
PDGF	- platelet-derived growth factor
PDK-1	- phosphoinositide-dependent kinase
PDTC	- pyrrolidine dithiocarbamate
PGC	- peroxisome proliferator-activated receptor gamma co-activator
P_i	- inorganic phosphate
PI3K	- phosphatidylinositide-3-kinase
PIC	- $PW1^+$ interstitial cell
PIP_2	- phosphatidylinositol (4,5) bisphosphate
PIP_3	- phosphatidylinositol (3,4,5) triphosphate

PKB/C	- protein kinase B/C
PMSF	- phenylmethyl sulfonyl fluoride
PPAR	- peroxisome proliferator-activated receptor
PTEN	- phosphatase and tensin homolog deleted on chromosome 10
PVDF	- polyvinylidene fluoride
RIA	- radioimmunoassay
RICE	- rest, ice, compression and elevation
RNS	- reactive nitrogen species
ROS	- reactive oxygen species
SDF	- stromal derived factor
SDS	- sodium dodecyl sulfate
SDS-PAGE	- sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	- standard error of the mean
SIRT1	- sirtuin 1
SOD	- superoxide dismutase
SR	- sarcoplasmic reticulum
STAT	- signal transducer and activator transcription protein
STZ	- streptozotocin

T2D	- type 2 diabetes
TBARS	- thiobarbituric acid reactive substance
TBS	- Tris-buffered saline
TGF	- transforming growth factor
T_H2	- T helper 2
TNF	- tumor necrosis factor
TRX	- thioredoxin
t-tubules	- transverse tubules
TxA_2	- thromboxane
VCAM	- vascular adhesion molecule
VEGF	- vascular endothelial growth factor
VLDL	- very low-density lipoprotein
WHO	- World Health Organization

LIST OF SYMBOLS

[]	- concentration
˚ C	- degree Celsius
µ	- micro
α	- alpha
β	- beta
γ	- gamma
θ	- theta

DISCLOSURE OF INTEREST

We hereby declare that there was no personal or financial gain for the researchers in this project. The researchers only retained the intellectual information that they generated through their studies and the right to publish these findings in peer reviewed scientific journals of their choice.

Signed on the ………………………….. day of …………………… 2014 at……………………………………………

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(Prof. B. Huisamen) (Mrs. C. George)

CHAPTER 1: BACKGROUND

1.1 WHAT IS TRADITIONAL MEDICINE?

According to the World Health Organization (WHO) (2002), traditional medicine refers to "health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose and prevent illnesses or maintain well-being." Additionally, terms such as "traditional medicine", "alternative medicine", "complementary medicine", "natural medicine", "herbal medicine", "phyto-medicine", "non-conventional medicine", "indigenous medicine", "folk medicine", "ethno-medicine" etc., all refer to a wide range of health care practices that are not essentially part of the dominant health care system of a country. Some of the most popular established systems that have been around for centuries include Chinese medicine dating back to 2800 BC [Borchers *et al.,* 1997], ayurveda, siddha, unani, kampo, jamu, homeopathy, acupuncture, chiropractic, osteopathy, bone-setting and spiritual therapies.

In the past, the practice of traditional medicine was seen as primitive, superstitious practices and even witchcraft. It was for that reason that little was done to scientifically investigate the legitimacy of these practices. However, during the last century, traditional/alternative medicine has gained increasing attention in both consumer and scientific arenas. According to the WHO, almost 65% of the world's population incorporates some form of traditional medicine into their primary health care [Fabricant and Farnsworth, 2001]. Fabricant and Farnsworth (2001) listed the numerous ways in which plants are currently used as "medicine". They indicated that, (a) the bioactive component can be isolated and used in manufacturing drugs such as digoxin, digitoxin, morphine, reserpine, taxol, vinblastine and vincristine, (b) by producing bioactive compounds of novel or known structures as the main compounds of manufactured drugs with higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon, taxotere, teniposide, verapamil and amiodarone, which are based, respectively, on galegine, Δ9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin and khellin, (c) by using it as a pharmacologic tool, e.g., lysergic acid diethylamide, mescaline and yohimbine and (d) by using the whole plant or part of it as a herbal remedy, e.g., cranberry, Echinacea, feverfew, garlic, *Ginkgo biloba*, St. John's wort and saw palmetto [Fabricant and Farnsworth, 2001]. However, even with the current research available, doctors and health practitioners, in most cases, still continue to shun traditional practice despite their contribution to meeting the basic health care needs of the population.

In the next few sections I will be discussing the role of traditional medicine in the treatment of diseases and also highlight the advantages and disadvantages of traditional plant use. I will also give a background on *Prosopis glandulosa*, which is our plant of interest and discuss the motivation for the current research.

1.2 THE ROLE OF TRADITIONAL MEDICINE IN DISEASE TREATMENT

1.2.1 The advantages

For centuries it has been known that plants have the intrinsic ability to synthesize and, at times, secrete a wide variety of chemical compounds. From accumulating scientific evidence it is apparent that these phyto-chemicals may have beneficial effects on long-term human health and they have been shown to effectively treat various diseases.

One of the benefits of plant-derived remedies is their powerful antioxidant effects, due to plant secondary metabolites. For the purpose of this review I will briefly discuss two well known plant species used for their medicinal value as wound healers, anti-inflammatory properties and anti-fatigue aids. Refer to comprehensive review articles for additional plant species used for their medicinal value [Borchers *et al.,* 1997; Winslow and Kroll, 1998; McKay and Blumberg, 2006]. The below-mentioned studies will be further elaborated on in the chapters that follow.

Chamomile (*Matricaria recutita*) is one of the most researched herbs. It is widely brewed in the form of a tea; however it is also used in soaps, detergents, perfumes, lotions, ointments, hair products, baked goods, confections and alcoholic beverages [McKay and Blumberg, 2006]. Traditionally, chamomile has been used as an anti-inflammatory agent, antioxidant, to treat wounds and a host of other ailments [Forster *et al.,* 1980; Crotteau *et al.,* 2006; Sakai and Misawa, 2005]. To date many of those traditional uses have been substantiated by scientific evidence. For example, the anti-inflammatory effects of chamomile have been thoroughly researched and one of the latest proposed mechanisms of action was reported by Srivastava *et al.* (2009). In this study the authors treated lipopolysaccharide-activated RAW 264.7 macrophages with an aqueous chamomile extract and found that this extract had the ability to inhibit the release of prostaglandin E2 from the LPS-activated macrophages. The authors speculate that the inhibitory activity of chamomile was due to a dose-dependent inhibition of cyclooxygenase (COX)-2 enzyme activity. They also found that chamomile treatment could reduce COX-2 messenger ribonucleic acid (mRNA) and protein expression, without affecting the activity or expression of the constitutive form, COX-1.

The term **Ginseng** refers to different species, all of the *Araliaceae* plant family, each having its own specific physiological effects. *Panax ginseng* (Chinese or Korean ginseng) is one of the most commonly used and highly researched species of ginseng. A total of 705 components have been isolated from ginseng, these include ginsenosides, polysaccharides, peptides and polyacetylenic alcohols, of which the main active agent, and the one that the majority of published research is on, are ginsenosides [Hui *et al.,* 2009]. *Panax ginseng* has traditionally been used as a “tonic”, performance enhancer, anti-cancer agent and aphrodisiac [O’Hara *et al.,* 1998]. To date it has been found that *Panax ginseng* has multiple effects, amongst others, anti-inflammatory, antioxidative, anti-diabetic and anti-fatigue effects [Kiefer and Pantuso, 2003]. Wang *et al.* (2010) evaluated the anti-fatigue effects of ginseng’s water-soluble polysaccharides, in an animal model of fatigue, the forced swim test (FST). They also tested the effects of these water-soluble polysaccharides on the biochemical markers for fatigue, such as glucose, triglyceride, lactate dehydrogenase, creatine phosphokinase, malondialdehyde, superoxide dismutase and glutathione peroxidase. In their article they reported that mice treated with the water-soluble polysaccharides displayed less time in an immobile state during the FST. In addition, the FST-induced reduction in glucose, glutathione peroxidase and increase in creatine phosphokinase, lactic dehydrogenase and malondialdehyde levels, all indicators of fatigue, were restored to baseline levels in the animals treated with the water-soluble polysaccharides. Wang *et al.* (2010) proposed two possible anti-fatigue mechanisms: (1) via prevention of lipid oxidation, by means of modifying several enzyme activities. Their finding coincide with that of Yu *et al.* (2006), which have demonstrated similar effects of polysaccharides from another plant species, the *Euphorbia kansui* (*Euphorbiaceae*), on malondialdehyde and glutathione peroxidase levels; and (2) via triglyceride (or fat) mobilization during exercise, as indicated by the decrease in triglyceride level and the simultaneous increase in glucose levels. However, further investigation is needed in order to identify the mechanism through which ginseng polysaccharide might affect fat mobilization. If fat mobilization is indeed ginseng's anti-fatigue mechanism, this would be advantageous during prolonged exercise, since better utilization of triglycerides allows the sparing of glycogen and glucose and therefore delays fatigue [Jung *et al.,* 2004].

Ginseng has also been reported to have anti-diabetic effects. These anti-diabetic effects have been investigated with both aqueous and ethanol ginseng extracts [Hui *et al.*, 2009]. Researchers such as Kim and Kim (2008) reported that *in vivo* treatment with ginseng resulted in the significant release of insulin from isolated rat pancreatic islets. In another study where ginseng was administered orally, a decreased serum level of glucose and glycated hemoglobin (HbA_1c) in streptozotocin (STZ)-induced diabetic rats was reported [Kim *et al.*, 2007]. Hypoglycaemia in KKAy mice was also reported in the study by Chung *et al.* (2001). In the latter study the researchers propose that the mechanism of action may be via ginseng possibly blocking intestinal glucose absorption and inhibiting hepatic glucose-6-phosphatase. Ginseng berry extracts have also been found to elicit anti-obesity effects in obese *ob/ob* and *db/db* mice, after a daily intraperitoneal (i.p) injection of ginseng extract, by reducing weight gain in these obese animals [Xie *et al.,* 2002]. Additional studies found the same anti-obesity effect in *ob/ob* mice as well as an anti-hyperglycaemic effect of ginseng berry juice [Xie *et al.,* 2007].

Another advantage in using traditional herbal medicine is that plant-derived products are perceived to be cheaper and are easier accessible than prescription drugs, especially for populations in developing countries. Since "modern medicine" is seen as costly and only available at medical facilities far away, most people continue to turn to traditional healers for help in combating disease.

Additionally, some cultural groups have preference to using natural remedies that are in line with their indigenous knowledge systems, a type of "man-earth" belief [Gesler, 1992]. By the "man-earth" relationship I'm referring to the interplay between environment and culture. According to Gesler's (1992) publication, there has been a long tradition that the physical environment provides "healing" to disease in many forms, medicinal plants being one.

Religion might have an additional impact, as the belief exists that, where an area gives rise to a particular disease, it will also provide the plants to cure. Due to this particular belief system, the use of plant-related medicines could result in better compliance in the taking of long-term medication, which would in turn have a positive outcome on disease treatment.

1.2.2 The disadvantages

Many herbal substances have scientifically been found to be beneficial, however something that is of great concern is that many of these herbal remedies can have serious and potentially lethal effects if not used appropriately. What is equally concerning is that many of these herbal remedies are marketed as "natural" or "homeopathic"- words that impart the perception that it must be safe for human consumption. Since herbal remedies fall under the category of "dietary supplements" they are exempt from the safety and efficacy requirements that the Medicine Control Council (MCC) have for prescription medication. For that reason, numerous herbal remedies have not been thoroughly evaluated on a large clinical scale and therefore little or no information is available on these substances, specifically in terms of optimal dosages that should be used to maximize desired effects while minimizing the deleterious effects of mega-dosing.

In the literature, there are instances described in which the use of herbal remedies has resulted in adverse effects. These adverse effects include mild to severe allergic reactions [Perharic *et al.,* 1993; Sandler and Aronson; 1993], toxicity [Aderson *et al.,* 1996], carcinogenic effects [Siegers *et al.,* 1993], or negative side-effects as a result of the combination thereof with other prescription medication [Heck *et al.*, 2000; Chang and Whitaker, 2001]. Examples of these include, royal jelly, which is secreted by honey bees and often used as a component in skin care products. This substance has been repeatedly linked with severe bronchospasm [Perharic *et al.,* 1993]. Another example is yohimbine (alleged aphrodisiac) that has been associated with allergic reactions with lupus-like symptom. There are other herbal remedies, such as camphor and the mixture of lavender, jasmine and rosewood used in aromatherapy, that have also been linked to allergic reactions [Sandler and Aronson; 1993]. Flavonoids are found to be present in many herbal preparations and they have been linked to many beneficial effects due to their antioxidant capabilities. However, they have been shown to have toxic effects too [Gandolfo *et al.,* 1992; Lin and Ho, 1994]. Germander, which is traditionally used for many different illnesses and to aid weight loss, has been associated with hepatitis [Larrey *et al.,* 1992; Mostefa-Kara *et al.,* 1992]. The long-term use (10–30 years) of plants such as aloe, cascara, frangula and rhubarb senna has also been linked to colorectal cancer [Siegers, 1992]. Additionally, the main component in chili powder, capsaicin, has been found to be carcinogenic when taken in high doses for extended periods of time [Surh and Lee, 1996].

The interactions of herbal medicines with prescription medicine are also a phenomenon that needs more research, as an inactivation or an enhancement of activity is possible [De Smet and D'Arcy, 1996]. In studies in which drug interactions were researched it was found that herbal substances such as feverfew, ginger, cranberry, St. John's Wort and ginseng can interact with the anti-clotting drug warfarin [Heck *et al.*, 2000] and potentially increase the risk of bleeding in patients using them in combined treatment. Many herbal medicines such as Ginkgo biloba, garlic, ginger, ginseng, feverfew and vitamin E were also found to increase risk of bleeding during dermatologic surgery [Chang and Whitaker, 2001]. They are known to have anti-platelet effects and thus add to the anti-platelet effect of drugs such as aspirin, non-steroidal anti-inflammatory drugs (NSAID) and other physician prescribed drugs. Valerian, which is used as a sedative, can intensify the effects of barbiturates, causing excessive sedation [Kaufman *et al.*, 2009]. St. John's Wort can interact with numerous conventional drugs such as cyclosporine, indinavir, irinotecan, nevirapine, oral contraceptives and digoxin [Hussain, 2011].

From the above research, it is clear that the use of herbal preparations can be beneficial, as the plant-derived remedy itself is not necessarily bad; however research into the optimal dosages and possible side-effects thereof is needed. Therefore, the careful optimization of plant-based products may result in fewer side-effects than traditional pharmaceuticals.

1.3 BACKGROUND ON *PROSOPIS GLANDULOSA* AND OTHER RELATED SPECIES

Prosopis is a genus of flowering plants in the *Fabaceae* (or legume) family. There are about 50 different species [Omidi *et al.,* 2013] of which *Prosopis glandulosa (P. glandulosa),* commonly known as Honey mesquite, is one. *P. glandulosa* is usually found in subtropical and tropical regions, since these trees thrive in arid soil and are resistant to drought. Their barks are usually hard, dense and durable and their fruits are nested in pods [Omidi *et al.,* 2013].

P. glandulosa trees are commonly used as animal feed, however it has been found that the ingestion of the young leaves, pods or beans of the *P. glandulosa* can cause toxicosis if it comprises the majority of the diet of the animals [Washburn *et al.*, 2002]. It appears that sheep are more resistant to the plant's toxic effects and they are thus able to consume a higher percentage of the young leaves, pods and/or beans in their diet than are cattle and goats [Washburn *et al.*, 2002]. The clinical signs of the toxic effects of this plant in animal models include weight loss, ptyalism (drooling), mandibular tremors, tongue protrusion, dysphagia (difficulty in swallowing) and episodes of hypoglycaemia [Washburn *et al.*, 2002].

In the past, the pods of *P. glandulosa* were used by the residents of the south-western regions of the North American deserts, because of its high protein content [Simpson, 1977; Zimmermann, 1991; Washburn *et al.*, 2002]. In South Africa, it was once one of the most common trees found in the dry north-western regions. Beginning in the 1880's numerous *Prosopis* species, including *P. glandulosa*, were introduced to South Africa from various sources in the Americas and became a common ornamental tree in many towns. For many years it was perceived to be a valuable source of shade, animal feed and fuel wood and these were the main reasons why *Prosopis* was introduced from the Americas to many parts of the world. However, in the 1960's this perception changed when the first alarming infestations appeared. During this time, hybridization between two dominant *Prosopis* species namely, *P. velutina* and *P. glandulosa,* started to occur and displayed what is known as "hybrid vigour". These hybrids proved to be very invasive [Simpson, 1977]. In 1983, *P. velutina* and *P. glandulosa* (including their hybrids), were declared category 2 invaders under the Conservation of Agricultural Resources Act of 1983 (Act No. 43 of 1983) (CARA) [Simpson, 1977]. Category 2 invader plants are plants with the proven potential of becoming invasive, but which nevertheless have certain beneficial properties that warrant their continued presence in certain circumstances. By demarcating and controlling the area in which these trees grow according to set regulations, producers can benefit from its resources. Since *P. glandulosa* is categorized as an invader tree it seems to be an ideal candidate for harvesting for natural medicine, as there is no risk of depleting natural resources of the plant. This scenario is ideal, in view of the fact that medicinal plant material is under extreme pressure due to increased demands for local and export markets. According to a publication by Wiersum *et al.* (2006), the excessive harvesting of plants for medicinal purposes is leading to a serious threat to the biodiversity in exploited regions.

In previous studies conducted in our laboratory [George *et al.*, 2011; Huisamen *et al.,* 2013] we have researched the anecdotal claims made by the consumers of the product consisting solely of *P. glandulosa* and found that treatment with *P. glandulosa,* modestly lowers fasting blood glucose levels, stimulates insulin secretion and leads to the formation of new pancreatic β-cells in rat models. It was also observed that *P. glandulosa* treatment could improve glucose uptake by isolated cardiomyocytes, elicit cardioprotection and decrease hypertension, without inducing hypoglycaemia in either of the rat models used [George *et al.*, 2011; Huisamen *et al.,* 2013]. In addition, a standard toxicology study was conducted at the primate unit of the Medical Research Council (Cape Town) under the supervision of Dr. Jurgen Seier, to determine the side effects, if any, of over-consumption of this product. It was found that after treating Vervet monkeys with a 1x, 5x and 25x the therapeutic dose, no clinically relevant changes were observed. The monkeys also did not show signs of hypoglycaemia at any stage over the 3 month experimental period. Data were compiled in a 66-page document [George *et al.,* 2011]. This data is not shown in this thesis, as the report obtained was too lengthy. Additional information can be obtained from the internet at: http://www.sciencedirect.com/science/article/pii/S037887411100376X.

To our knowledge, no other studies have been conducted on the mechanisms involved in the effects of this plant product and thus the active component of *P. glandulosa* has not yet been identified. We could find no literature on its medicinal value and health benefits. Thus, the mechanisms and effects of this plant are still largely unknown, except for anecdotal claims and the observations made in studies conducted in our laboratory. Despite the medicinal value of *P. glandulosa* not being documented previously, numerous studies have documented the medicinal value of other related *Prosopis* species [Sharma *et al.,* 2010; Pinto *et al.*, 2009; Adikwu *et al.,* 2003]. For example, Sharma *et al.* (2010) reported that a crude ethanolic extract of the bark of *P. cineraria,* administered for 45 days to alloxan-induced diabetic male Swiss albino mice, significantly lowered blood glucose levels, elevated hepatic glycogen content and resulted in the mice maintaining body weight and lipid-profile parameters towards near normal range. In addition, they found that treatment normalized the declined activity of antioxidant enzymes and the concentration of non-enzymatic antioxidants, thereby reducing the oxidative damage in the tissues of the diabetic animals. Pinto *et al.* (2009) evaluated the total phenolics antioxidant activity as well as the *in vitro* inhibition of α-amylase, α-glucosidase and angiotensin I-converting enzyme (ACE), which are potential sites in the management of hyperglycaemia and hypertension, linked to type 2 diabetes (T2D). In their study they found that the aqueous extracts from *P. pallida* had a high α-glucosidase inhibitory activity as well as a significant ACE inhibitory activity, reflecting its anti-hypertensive potential. Another interesting study was conducted by Adikwu *et al.,* 2003, in which they evaluated the anti-diabetic properties of the gum found in the seeds of the *P. africana* tree. In addition, they also tested whether this gum could act as a bioadhesive base for the delivery of metformin compared to other bioadhesive formulations, namely Carbopol 974-P and sodium carboxymethyl cellulose (NaCMC). In their study they found that *P. africana* gum released metformin at a higher rate, compared to the other bioadhesive formulations. This was validated by the shorter time period required to reach t(50) (the time required for 50% of the drug to be released) or t(20) (time required for 20% of the drug to be released). In addition, the gum showed moderate anti-diabetic properties when used in an aqueous solution and in combination with metformin in a bioadhesive form, the glucose lowering effect was found to be synergistic.

With these encouraging results obtained from other *Prosopis* species, it seems fitting to further evaluate the possible effects of *P. glandulosa*.

1.4 ISSUES TO TAKE FORWARD

Herbal medicine has been around for many years and the consumers thereof have testified to its beneficial effects. In the last few years, huge amounts of research have gone into investigating the validity of these claims. Without ignoring the possible negative effects that these plant substances may have on human health, many studies have reported on the positive therapeutic effects of certain herbal substances [Hui *et al.,* 2009; Hudson, 2012; George *et al.*, 2011; Huisamen *et al.,* 2012; Aggarwal, 2010; Cai *et al.,* 2010]. With this in mind, it is important to understand that firstly, each herbal substance contains thousands of components, only a few of which may have therapeutic value [Hui *et al.,* 2009]. Secondly, different parts of a plant have a different component profile, for example, while both ginseng root and ginseng berry possess anti-diabetic properties [Dey *et al.,* 2003], ginseng berry seems to have a more potent anti-hyperglycaemic effect, compared to the ginseng root [Dey *et al.,* 2002]. In addition, different methods of extracting active ingredients from the plant-based substance may also yield different components or the concentrations of these components. It is therefore important that natural health products be standardised [Shan *et al.,* 2007]. Finally, herbal formulae containing more than one herbal substance may elicit synergistic effects [Tan *et al.,* 2011; Liu, 2004]. It is therefore necessary to fully elucidate both desired and undesired effects and to develop appropriate dosing regimens to target specific ailments, ensuring both safety and efficacy of the product. Looking forward, natural products are likely to become even more important for development of drugs; due to the variety of functionally relevant properties these plant species possess [Ngo *et al.,* 2013].

In conclusion, it would be of great importance to produce high-grade pharmaceutical products at low cost in resource-poor communities, such as where there is a high burden of preventable disease, where existing medicines are either too expensive or where getting access to those medicines are difficult due to geographic location.

CHAPTER 2: LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

Skeletal muscles are the effector organs of the locomotor system and under voluntary control, however much of their activity is regulated subconsciously. One important aspect of skeletal muscle is that it has the ability to adapt in response to altered demand [Flück, 2006]. To understand the function of skeletal muscle one requires knowledge of its structure, both anatomically as well as its molecular organization.

In the following sections, I will be discussing the structure and function of skeletal muscle (refer to section 2.2 and 2.3 respectively) and how skeletal muscle adapts to various demands. For the purpose of this review, I will be focusing on altered glucose uptake in disease states, particularly focusing on insulin resistance and diabetes (refer to section 2.4), exercise-induced muscle fatigue (refer to section 2.5) and the repair process of skeletal muscle after a contusion injury (refer to section 2.6).

2.2 SKELETAL MUSCLE STRUCTURE

2.2.1 Summary of skeletal muscle architecture

Skeletal muscle is striated muscle tissue, accounting for between 40 and 45% of adult human body weight [Holloszy *et al.,* 2003]. It is an intricate structure that is composed of muscle cells, organized networks of nerves and blood vessels and an extracellular connective tissue matrix [Huard *et al.*, 2002]. The basic constituent of skeletal muscle is the muscle fiber (or myofiber), which is derived from the fusion of multiple myoblasts (embryonic progenitor cells). In short, numerous myoblasts fuse together and form long, cylindrical, multinucleated myotubes, with central nuclei; making the muscle fibers the largest cells in the human body [Silverthorn, 2004]. With time, the myonuclei shift from the central position to a subsarcolemmal position. These muscle cells are then termed myofibers. Myofibers in normal adult muscles have nuclei on the cell periphery, however when the nuclei present centrally it can be an indication of regeneration under certain conditions [Cabral *et al.,* 2008; Charge´ and Rudnicki, 2004]. The individual muscle cells (muscle fibers) are grouped together into elongated bundles called fasciculi. The size of the fasciculi reflects the particular muscle. Muscles that are responsible for fine, highly controlled movement have small fasciculi, whereas muscles that are responsible for gross movement have large fasciculi [Silverthorn, 2004]. The cytoplasm of the myofiber is called the sarcoplasm*;* it contains a cellular matrix and organelles, including the Golgi apparatus, mitochondria, sarcoplasmic reticulum (SR), lipid droplets, glycogen and myoglobin. The endomysium is the connective-tissue layer that surrounds individual myofibers, whereas the perimysium surrounds fascicles (bundles of myofibers) and the epimysium surrounds the skeletal muscle. The sarcolemma is the plasma membrane that surrounds each myofiber unit. The basal lamina or basement membrane is composed of an inner layer, an intermediate lucida and the outer lamina densa [Huard *et al.*, 2002] (Fig 2.1).

In addition to the multitude of nuclei on the periphery of the myofiber, separate cells called satellite cells are located between the basal lamina and plasma membrane and play a vital role in the process of muscle regeneration [Bischoff, 1994] (refer to section 2.6.3.1 for the role of satellite cells in muscle regeneration). Satellite cells are known to proliferate following muscle trauma to form new myofibers through a process comparable to muscle histogenesis in the embryo.

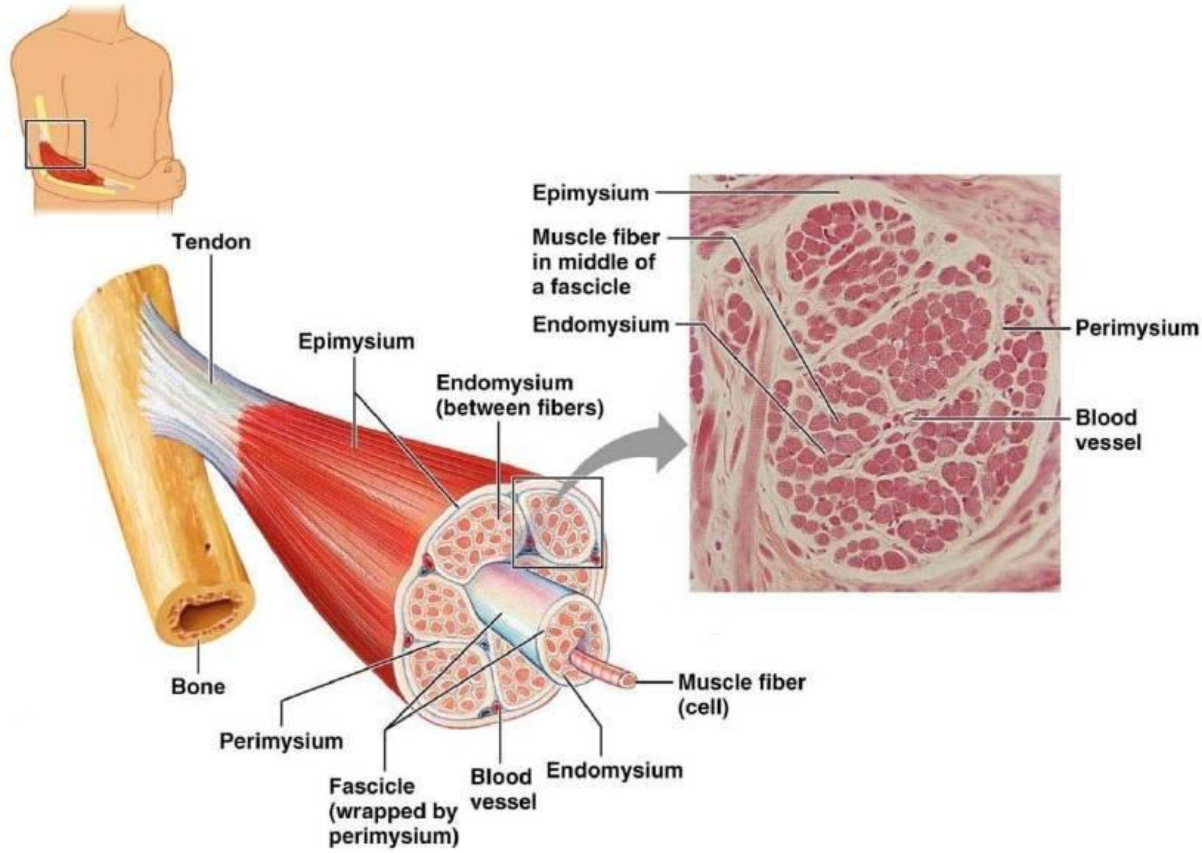


Figure 2.1: Schematic representing skeletal muscle architecture. Sketch obtained from internet. Website: http://satellitecell.files.wordpress.com/2012/07/skeletal-muscle-2.jpg

2.2.2 Different fiber types

During the late 1800 a French anatomist, Louis Antoine Ranvier, documented that a number of muscle groups of the rabbit were a deeper red colour and that these groups contracted at a slower, more sustained rate, than paler muscles of the same animal [Zierath and Hawley, 2004]. This observation formed the basis of the classical terminology of red and white muscle fibers.

For a detailed review on fiber types in mammalian skeletal muscle refer to Schiaffino and Reggiani (2011). In short, skeletal muscle has to perform a large range of activities, from keeping an individual in an upright position, to performing explosive movements in response to a threat. It is for that reason that skeletal muscle comprises of a mixture of different fiber types that have unique metabolic profiles, contractile characteristics and cellular Ca^{2+} handling [Spangenburg and Booth, 2003]. There are different classification systems for mammalian skeletal muscle; the dominating one being based on the myosin heavy chain (MHC) isoforms [Westerblad *et al.,* 2010; Allen *et al.,* 2008b]. In this system the major fiber types are classified as type I, IIa, IIx and IIb. Rodents express all four fiber types, whereas IIb MHC is not expressed in human muscle [Smerdu *et al.,* 1994]. Another classification system is the "speed of contraction" of the fiber. This classification system is more explanatory and therefore this is the classification system I will be referring to throughout this thesis. Muscle fibers can be divided into three main categories, namely fast-twitch oxidative, fast-twitch glycolytic and slow-twitch oxidative muscle fibers [Silverthorn*,* 2004]. These groups differ on the basis of their speed of contraction and their resistance to fatigue with repeated stimulation. Fast-twitch fibers can develop tension 2 to 3 times faster than slow-twitch fibers [Allen *et al.,* 2008b]. The reason for this increased speed lies in the isoform of the myosin adenosine triphosphatase (ATPase) present in the thick filaments of the fiber. Fast-twitch fibers have the ability to spilt ATP at a higher rate and can therefore complete more contractile cycles per time, which culminate into faster tension development in the muscle fiber [Silverthorn*,* 2004]. The duration of a contraction also varies according to the fiber type [Spangenburg and Booth, 2003]. The duration of a twitch is in essence determined by the speed at which the SR can remove Ca^{2+} from the cytosol. When the cytosolic [Ca^{2+}] decreases, Ca^{2+} bound to troponin is released, allowing tropomyocin to move back into its initial position and so-doing, block the myosin binding sites. This process blocks the power stroke action, which in turn leads to muscle relaxation. Fast-twitch fibers are able to pump Ca^{2+} into the SR more rapidly than slow-twitch fibers and therefore they are able to generate a faster contraction-relaxation cycle. These fast-twitch fibers are very useful for processes such as piano playing. Conversely, the contraction of slow-twitch fibers may last ten times longer than fast-twitch fibers. These muscles are therefore used for sustained movement, such as lifting a heavy load, standing, walking and maintaining posture [Silverthorn, 2004]. Another key difference between fast-twitch glycolytic fibers and slow-twitch muscle fibers is their ability to resist fatigue [Silverthorn, 2004]. Fast-twitch glycolytic fibers produce ATP primarily by anaerobic glycolysis, which in turn produces lactic acid, thought to contribute to the process of fatigue (refer to section 2.5 for more detail on the mechanism of fatigue). Slow-twitch fibers on the other hand depend largely on oxidative phosphorylation for the production of ATP and do not produce large amounts of lactic acid. As mentioned previously, fast-twitch fibers are divided into two subcategories, fast-twitch glycolytic fibers and fast-twitch oxidative fibers. They are divided into these two groups based on their relative diameter and resistance to fatigue [Silverthorn, 2004]. Fast-twitch glycolytic fibers are the largest in diameter and rely on anaerobic glycolysis for ATP production. Fast-twitch oxidative fibers are smaller in diameter, contain some myoglobin and use a combination of oxidative phosphorylation and glycolytic metabolism to produce ATP. As a result of being able to synthesize ATP via oxidative phosphorylation, these fibers are more fatigue-resistant than the fast-glycolytic fibers.

As stated above, human skeletal muscle comprises of a mixture of these different fiber types and the ratio in which they are present varies according to the function of the muscle. During the 1970's and 1980's numerous studies were conducted on muscle fiber composition of athletes excelling at different sports. The results of these earlier studies revealed that successful endurance athletes have more slow-twitch fibers in their trained muscles [Costill *et al.*, 1976; Fink *et al.*, 1977; Saltin *et al.*, 1977] and sprinters have predominantly fast-twitch fibers in their trained muscles [Costill *et al.*, 1976]. Recent studies have reported that muscle fibers can switch from one type to another under certain conditions, such as changes in physical activity, environment and pathological conditions [Schiaffino *et al.*, 2007]. For example, endurance exercise training induces a fast-to-slow fiber type transition, transforming the myofibers to an increased oxidative metabolism [Demirel *et al.,* 1999; Pette and Staron, 2001; Yaun *et al.,* 2011]. Additional factors leading to fiber type transition includes mechanical loading and unloading, hormones and aging

[Pette and Staron, 2001]. The debate is still ongoing as to what extent muscle fibers can shift from slow-twitch to fast-twitch fibers (and vice versa) in humans. Nevertheless, it has been shown that the relative number of slow-twitch fibers decreases and the relative number of fast-twitch fibers increases or remains unaltered with anaerobic training [Iaia and Bangsbo, 2010]. In addition, Stuart *et al.* (2013) reported fewer type I (slow-twitch) fibers and more mixed (type IIa, fast-twitch) fibers in subjects diagnosed with the metabolic syndrome. In their study they found that an individual's insulin responsiveness and maximal oxygen uptake correlated with the proportion of type I fibers. They also found that the insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and glucose transporter 4 (GLUT4) expressions were not different in whole muscle but all were significantly less in the type I fibers of metabolic syndrome subjects when they adjusted it for fiber proportion and fiber size.

In summary, skeletal muscles are composed of muscle fibers which exhibit marked differences in their metabolic profile, ranging from slow, energy conserving and highly oxidative fibers that are optimized for prolonged low-intensity activities to fast, highly energy-consuming fibers that depend mainly on anaerobic metabolism and are suited for short explosive movements. In addition, the studies aimed at elucidating the phenomenon of fiber type switch have not yet led to one being able to draw firm conclusions, but by examining the literature it appears that certain events, for example endurance training, leads to a switch in muscle fiber types. However, more research is necessary to determine exactly how this switch occurs and whether there are any other scenarios that are able to elicit this switch in fiber type, such as certain drug treatment, as this switch in fiber types might be beneficial in delaying muscle fatigue.

In the following sections, I will be discussing skeletal muscle function under normal physiological conditions and how muscle adapts to certain demands.

2.3 SKELETAL MUSCLE FUNCTION

2.3.1 Excitation-contraction (EC) coupling

The process of excitation-contraction (EC) coupling in normal muscle is well understood. Refer to Dulhunty (2006) for a more in-depth review. In short, under normal physiological conditions, the initial step in muscle contraction involves the release of acetylcholine by the presynaptic axon into the synaptic cleft. The released acetylcholine binds to its receptors in the post-junctional folds of the myofibers (postsynaptic area) and in turn depolarizes the cell. Muscle contraction consequently results from the depolarization triggering an action potential (AP) that moves along the length of the myofibers. Once depolarization of the end plate has occurred, the electric impulse then propagates along the surface membrane, by means of transverse tubules (t-tubules) and reaches the interior of the muscle. The t-tubular membrane expresses voltage-sensing receptors that are mechanically linked to Ca^{2+}-release channels in the adjacent SR (ryanodine receptors), which change their conformation during the AP, resulting in charge movement [Schneider and Chandler, 1973]. This action results in a brief release of calcium from the SR [Silverthorn, 2004] into the cytosol. The released calcium causes the contractile proteins, actin and myosin, to interact and generate force in a stepwise manner. In short, the calcium is first released from the SR, then binds to troponin and causes a conformational change thereof. Troponin is a component of the actin filament to which myosin binds. This conformational change allows the interaction between actin and myosin to occur and eventually lead to muscle contraction. At the end of contraction, the enzyme acetylcholinesterase deactivates acetylcholine, allowing the muscle to relax. The intracellular calcium is then transported into the SR within the myofibers while troponin prevents the interaction between actin and myosin molecules to occur [Silverthorn, 2004].

As can be seen from the paragraph above, the EC coupling process is a very intricate one and any of the above mentioned steps can be affected during intense muscle activity as a result of metabolic alterations. Muscle fatigue is perceived as a decrease in isometric force production, reduced shortening speed, altered force-velocity relationship and a slowed relaxation [Allen *et al.*, 1995]. A combination of these factors results in decreased power output and impaired muscular performance, whereas slowed relaxation decreases the frequency at which altering movement can be performed. Therefore many researchers have focused their attention on the effects that EC coupling processes have on fatigue development in skeletal muscle (refer to sections 2.3.1).

2.3.2 Different types of muscle contractions and mode of stimulation

Different types of muscle contraction can occur, namely isometric contraction, concentric contraction and eccentric contraction (Fig 2.2). Briefly, isometric contractions refer to when the force that is generated by the muscle, is equal to the resisting load of the muscle. In this type of contraction the length of the muscle does not change (Fig. 2.2 (a)). Concentric contraction is when the force generated by the muscle is larger than the resisting load and causes the muscle to shorten (Fig. 2.2 (b)). Finally, eccentric contraction occurs when the resisting load is larger than the force generated by the skeletal muscle and causes the muscle to lengthen (Fig. 2.2 (c)).

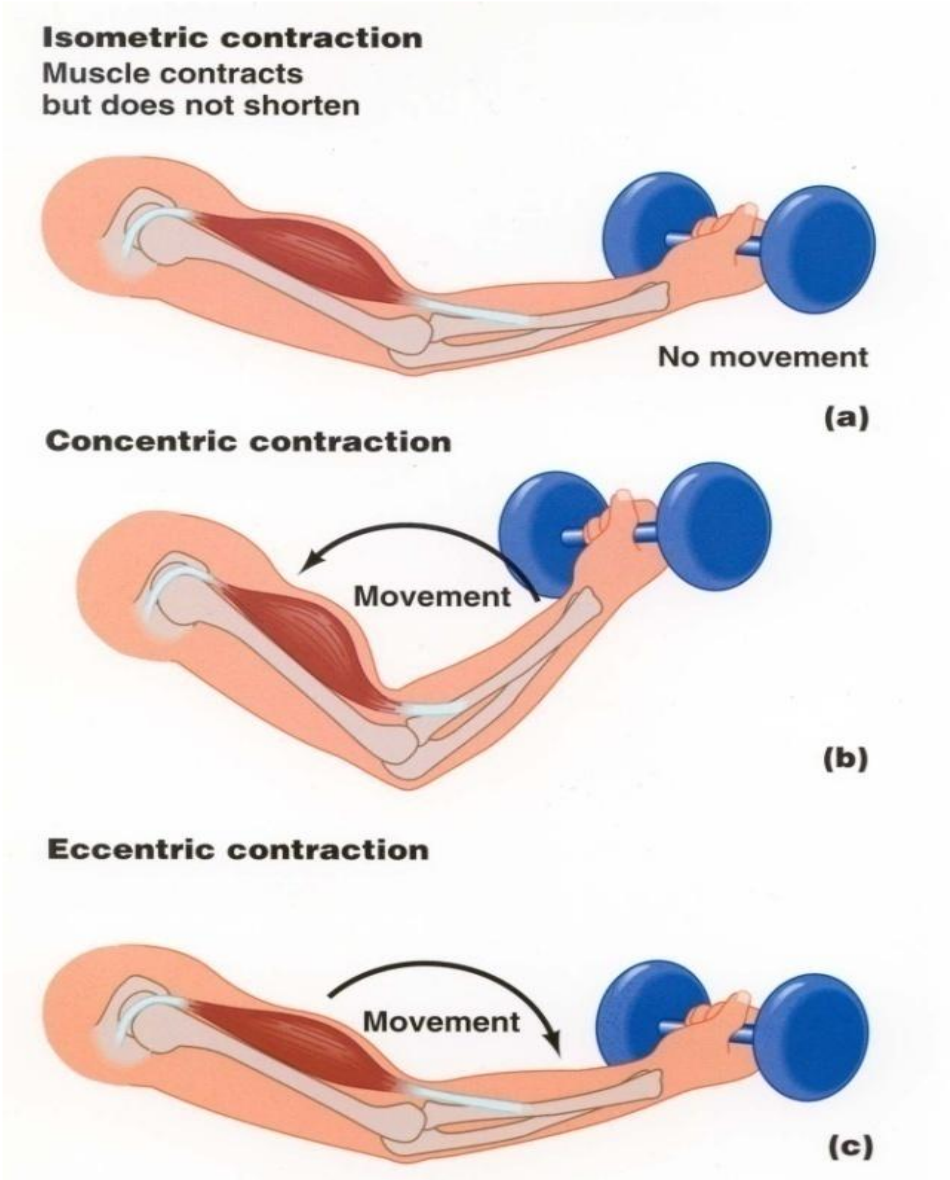


Figure 2.2: Schematic representation of the different types of muscle contractions. Sketch obtained from internet. Website: http://blog.corewalking.com/how-do-muscles-contract/

When skeletal muscle is stimulated with a single electric impulse at sufficient voltage, it will quickly contract and then relax. This response is called single twitch (Fig. 2.3). However, when skeletal muscle receives increasing frequency of electrical stimuli, the relaxation time between successive twitches gets shorter and shorter as the strength of the contraction increases in amplitude. This response is termed incomplete tetanus (summation) (Fig. 2.3). If the conditions of incomplete tetanus persist there will come a point at which there is no visible relaxation between successive twitches, leading to sustained muscle contraction. This response is termed complete tetanus (Fig. 2.3) [Huard *et al.*, 2002].

In the next few sections, I will be focusing on four different events that impact the normal function and/or structure of skeletal muscle, namely, skeletal muscle insulin resistance as a result of diet-induced obesity, exercise-induced muscle fatigue, muscle injury and recovery after a contusion injury.

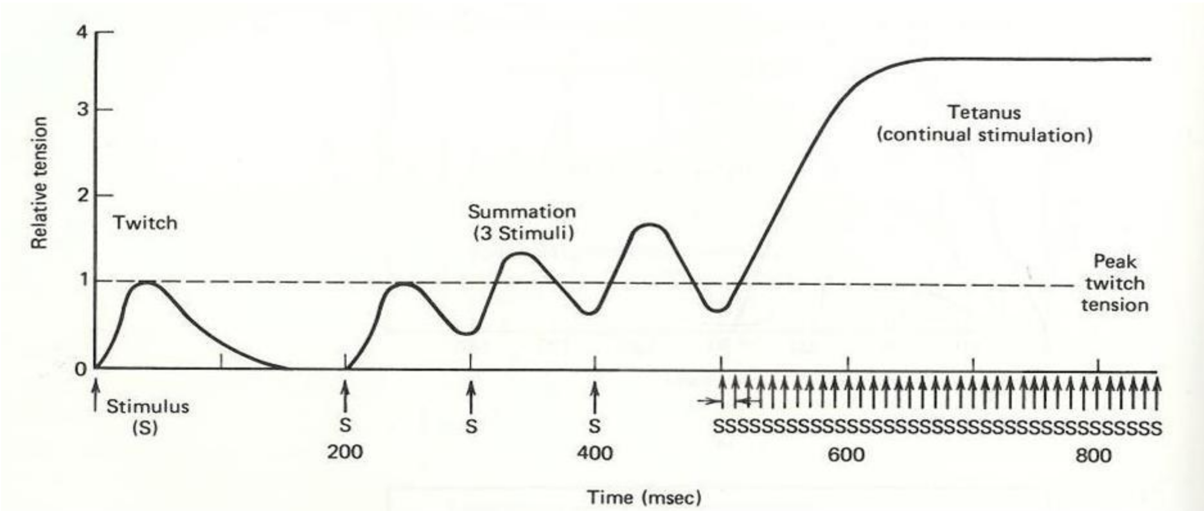


Figure 2.3: Schematic representation depicting the differences between a single twitch, incomplete tetanus and tetanus. Repeated stimuli, each of a given strength (S) can produce a tension that sums to greater than the twitch tension. Continual stimulation results in a tetanic contraction three to five times stronger than twitch tension. Sketch obtained from Brooks and Fahey, 1984

2.4 SKELETAL MUSCLE INSULIN RESISTANCE AS A RESULT OF DIET-INDUCED OBESITY

2.4.1 Global statistics

The prevalence of obesity has focused attention on a worldwide problem, now reaching epidemic proportions. Obesity is associated with various co-morbidities, amongst others, insulin resistance, T2D, hypertension and cardiovascular disease [WHO, 2013]. It has become a serious public health issue, escalating in countries with low and middle income [WHO, 2013]. In addition to being very prevalent in developed countries it is also quite common in parts of the developing world [Hossain *et al.,* 2007]. This growing prevalence is primarily attributed to an increase in sedentary lifestyle and the growing reliance on convenient and often processed foods, lacking nutritional value and that are rich in fats and sugars [Reeds, 2009; Varady and Hellerstein; 2008]. In 2008, more than 1.4 billion adults were overweight (body mass index: BMI ≥ 25 kg/m^2), of which over 200 million men and nearly 300 million woman were obese (BMI ≥ 30 kg/m^2) [WHO, 2013]. South Africa has also not been spared in this global increase [Puoane *et al.*, 2002]. It is estimated that more than 366 million people worldwide are diabetic and that this figure is expected to rise to 552 million by 2030 [Tabatabaei-Malazy *et al.,* 2012].

In the sections that follow, I will be discussing the link between obesity and insulin resistance, why research into obesity and its complications is imperative, as well as why the need for new and improved therapies are so important.

2.4.2 The link between obesity and insulin resistance

Insulin resistance is defined as the reduced responsiveness of the target organ (i.e. adipose tissue, liver and muscle) to the insulin concentration to which it is exposed, resulting in fasting hyperinsulinaemia in the quest to maintain euglycaemia [Shanik *et al.*, 2008; Kumar and Dey, 2003]. Insulin is a key hormone in maintaining glucose homeostasis. With regard to adipose tissue, insulin is responsible for reducing free fatty acid (FFA) efflux from adipocytes, by decreasing lipolysis; with regard to the liver, insulin inhibits gluconeogenesis, by reducing key enzyme activities and with regard to skeletal muscle, insulin predominantly induces glucose uptake by stimulating the translocation of the GLUT4, via phosphatidylinositide 3-kinases (PI3K)-dependent and PI3K-independent pathways [Pessin *et al*, 1999; Lizcano and Alessi, 2002] to the plasma membrane (Fig. 2.4).

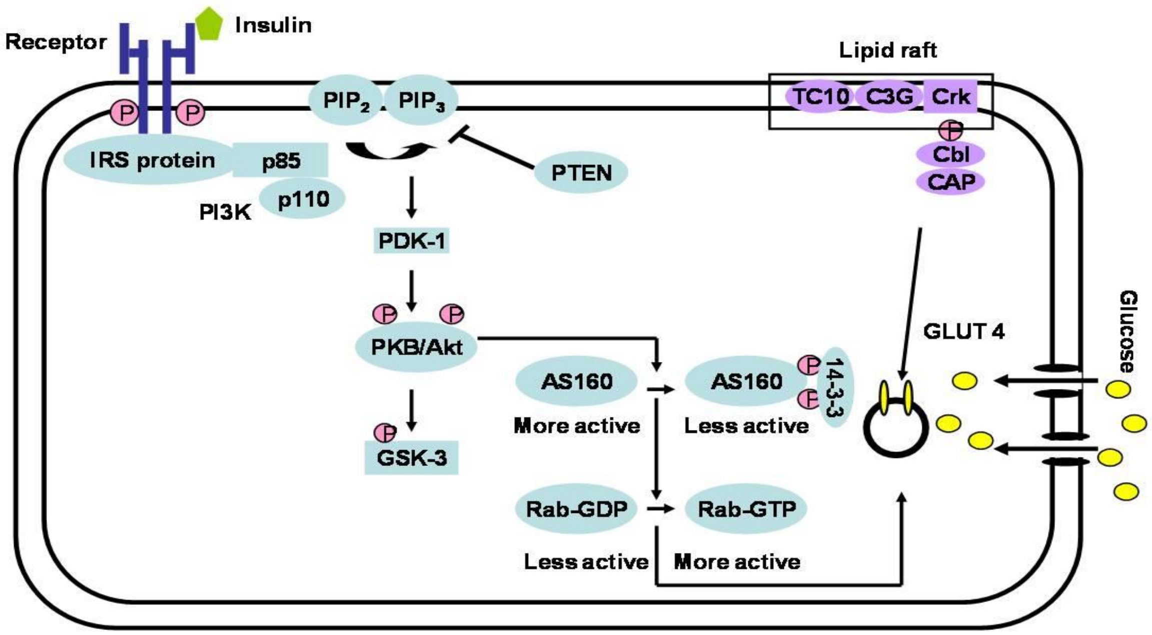


Figure 2.4: Simplified overview of insulin stimulated GLUT4 translocation and glucose uptake. Adapted from Saltiel and Kahn (2001) and Watson and Pessin (2007). IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; PIP_2: phosphatidylinositol (4,5) bisphosphate; PIP_3: phosphatidylinositol (3,4,5) triphosphate; PTEN: phosphatase and tensin homolog deleted on chromosome 10; PDK-1: phosphoinositide-dependent kinase 1; PKB/Akt: protein kinase B; GSK-3: glycogen synthase kinase 3; GLUT4: glucose transporter 4; CAP: Cbl-associated protein

The influence of body fat on insulin action is very important and the relationship between obesity, especially when it is centrally located [Kissebah *et al*, 1989], insulin resistance and the risk of developing T2D (T2D) is well recognized. The major contributor to the development of insulin resistance is an overabundance of circulating FFA in overweight and obese individuals (referred to as the FFA hypothesis). Skeletal muscle, responsible for up to 80% of the glucose disposal from the peripheral circulation, is particularly vulnerable to increased levels of saturated FFAs [Mazibuko *et al.,* 2013]. Consequently, insulin-mediated glucose uptake is reduced and FFA uptake and oxidation is increased [Barsotti *et al.*, 2009; Zeyda and Stulnig, 2009]. This decreased glucose uptake leads to increased insulin release into the bloodstream, leading to increased glucose production by the liver, resulting in hyperglycaemia. During the development of insulin resistance, there is an increased amount of lipolysis, which produces more fatty acids, continuing the cycle.

Accumulating literature has emerged stating that obesity is also associated with inflammation, which has been found to be causally involved in the development of insulin resistance [Zeyda and Stulnig, 2009] (Fig. 2.5). These studies have revealed that obese individuals experience chronic low-grade inflammation, validated by their increased plasma levels of C-reactive protein and inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8) [Zeyda and Stulnig, 2009]. The first compelling evidence that inflammatory mediators can cause insulin resistance was a study conducted by Hotamisligil *et al.* in 1993. In this study they found that the neutralization of TNF-α in obese *fa/fa* rats significantly increased the peripheral uptake of glucose in response to insulin. The results of the Hotamisligil *et al.* (1993) study is in accordance with other studies conducted on knockout mice deficient of TNF-α (*Tnf-/-*) or TNF-α receptor 1 gene (*Tnfr-1*). In these studies the obese mice (both diet-induced and genetic obesity (*ob/ob*)) were protected from insulin resistance [Uysal *et al.*, 1997]. In an *in vitro* study on cultured murine adipocytes it was found that TNF-α treatment induced serine phosphorylation of IRS-1 and converted IRS-1 into an inhibitor of the IR tyrosine kinase activity. Myeloid 32D cells, which lack endogenous IRS-1, were resistant to TNF-α-mediated inhibition of IR signaling, whereas transfected 32D cells that express IRS-1 were very sensitive to the effect of TNF-α. An inhibitory form of IRS-1 was observed in muscle and fat tissues from obese rats. These results indicate that TNF-α induces insulin resistance at the level of IRS-1 to attenuate insulin receptor signaling [Hotamisligil *et al.,* 1996]. In addition, TNF-α has been shown to affect insulin sensitivity, by altering the expression of the genes for the insulin receptor, IRS-1, GLUT4, adiponectin and peroxisome proliferator-activated receptor (PPAR)γ [Zeyda and Stulnig, 2009]. Furthermore, when adipose tissue of obese rodents was compared to their lean counterparts, increased levels of gene expression were observed for multiple pro-inflammatory cytokines [Weisberg *et al*, 2003; Xu *et al*, 2003; Kern *et al*, 2001] and weight loss reduced the TNF-α levels. Because the above mentioned effects appear before the development of insulin resistance and during high-fat feeding, it further supports the belief that adipose-derived inflammatory factors may have a causal role in the development of high-fat diet-induced insulin resistance.

Various mechanisms to explain the effect of TNF-α on the development of obesity-related insulin resistance have been proposed. Possible mechanisms include increased release of FFA by adipocytes through additional lipolysis of adipose tissue triglyceride stores [Eckel *et al*, 2005], reduction in adiponectin synthesis [Bruun *et al*, 2003] and impairment of insulin signalling [Greenberg and McDaniel, 2002; Hotamisligil and Spiegelman, 1994]. As mentioned previously, MCP-1, IL-8 and IL-6 have also been reported to have an effect in insulin resistance. The main proposed means by which these chemokines affect insulin sensitivity, is by attracting macrophages into tissue. Macrophages are the major source of inflammatory mediators as well as a target of inflammatory mediators. It is for that reason that they are thought to be central players in the cycle driving inflammation and insulin resistance. The link between IL-6 and insulin resistance is supported by epidemiological studies as well as genetic studies. There is a positive correlation between plasma IL-6 levels and human obesity and insulin resistance [Vozarova *et al*, 2001; Kern *et al*, 2001; Pradhan *et al*, 2001]. As with TNF-α, IL-6 is thought to exert its adverse effects by increasing circulating FFA, with its well described adverse effects on insulin sensitivity [Boden and Shulman, 2002], enhancing hepatic glucose production and decreasing adiponectin secretion [Fasshauer *et al*, 2003]. Weight loss is found to significantly reduce IL-6 levels in both adipose tissue and serum [Bastard *et al*, 2000]. In the clinical set-up, elevated levels of IL-6 are used as a predictor of T2D development [Pradhan *et al*, 2001] and risk of future myocardial infarction [Ridker *et al*, 2000].

The adipokines leptin and adiponectin have been found to have a significant effect on insulin resistance development. Leptin is an adipokine secreted by the adipocytes and has assumed a vital role in energy homeostasis [Pittas *et al*, 2004]. Although the main target of leptin is the appetite centre in the brain, it also seems to have effects on insulin action in peripheral tissues, as well as on blood vessels and pancreatic β-cells [Crowley, 2008; Ronti *et al*, 2006; Seufert, 2004]. There is some evidence that obesity is associated with a state of peripheral leptin resistance [Mark *et al*, 2002]. Furthermore, hyperinsulinaemia promotes both insulin resistance and stimulation of leptin production and secretion from adipose tissue. This may in turn enhance leptin resistance by further desensitizing its signal transduction pathways [Seufert, 2004]. Insight into the physiology of leptin, such as its relationship to insulin resistance, comes from studies of deficiency syndromes. Leptin deficient mice (*ob/ob*) are found to exhibit hyperphagia, obesity, hypercortisolemia, infertility and diabetes [Zhang *et al*, 1994]. However, once exogenous leptin is administered, these abnormalities are reversed [Pelleymounter *et al,* 1995]. Adiponectin is another anti-inflammatory cytokine that has been shown to both improve insulin sensitivity and inhibit many steps in the inflammatory process [Nawrocki and Scherer, 2004]. Unlike most adipose tissue products, adiponectin is negatively related to fat mass, possibly as a consequence of inhibition by TNF-α or cortisol [Fallo *et al*, 2004; Keaney *et al*, 2003]. In the liver, adiponectin regulates cells to decrease gluconeogenesis [Sheng and Yang, 2008; Combs *et al*, 2001] and to increase fatty acid oxidation. In skeletal muscle, it increases glucose transport and uptake and enhances fatty acid oxidation [Xu *et al*, 2003]. Adiponectin is reduced in humans with T2D, which ultimately results in increased levels of blood glucose and fat [Hotta *et al*, 2000; Beltowski, 2003; Matsuzawa *et al*, 2004]. Insulin resistance is reversed after administration of adiponectin in rodent models of obesity and T2D [Yamauchi *et al.*, 2001]. Healthy individuals with reduced baseline plasma adiponectin are predisposed to future development of insulin resistance [Spranger *et al*, 2003]. Although most obese patients have low levels of adiponectin, the negative correlation between adiponectin and insulin sensitivity is not dependent on adipose tissue mass alone. Many studies report that adiponectin levels decrease with increasing BMI, plasma glucose and insulin, as well as serum triglycerides, in rodents and in humans [Rajala and Scherer, 2003].

In addition to the FFA hypothesis and the involvement of adipokines and pro-inflammatory cytokines, growing evidence links plasminogen activator inhibitor-1 (PAI-1) with obesity and insulin resistance. Adipose tissue has been found to be a key source of PAI-1, with the bulk production in visceral adipose tissue [He *et al*, 2003]. PAI-1 is a key regulatory protein in processes such as tissue fibrinolysis, cell migration, angiogenesis, and tissue remodelling [Lijnen, 2005]. It has been found that PAI-1 deficient mice have reduced adiposity and an improved metabolic profile [Schafer *et al*, 2001], and PAI-1 deficiency attenuated diet-induced obesity and insulin resistance in C57BL/6 mice [De Taeye *et al*, 2006]. Furthermore, in mouse models, the absence or inhibition of PAI-1 through genetic alteration in adipocytes protect against insulin resistance by promoting glucose uptake and adipocyte differentiation via increased PPAR-α expression [Liang *et al*, 2006].

In summary, research into obesity and its associated complications, such as insulin resistance is well characterized and a large amount of attention is paid to the amelioration of these types of diseases (refer to section 2.4.3 on the current treatment modalities available).

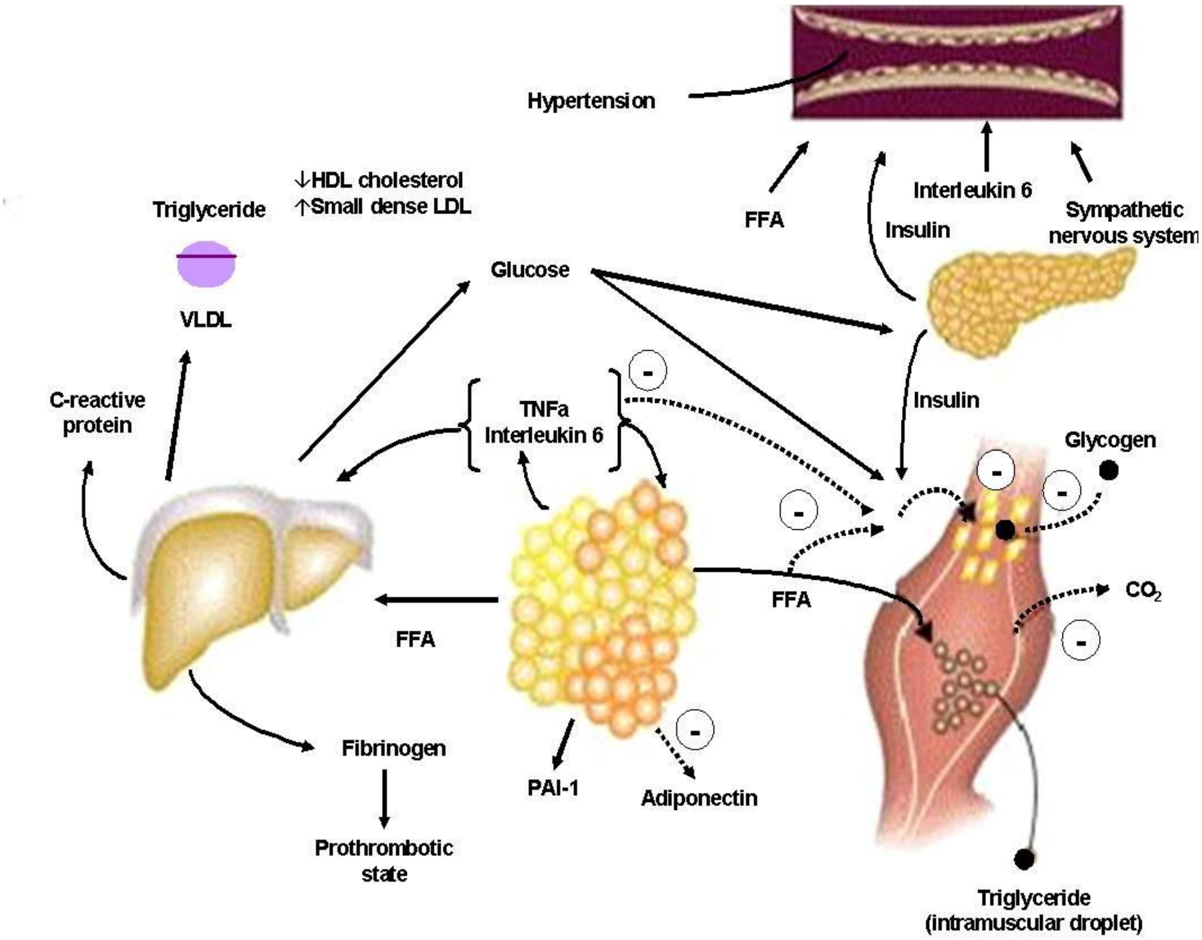


Figure 2.5: Simplified schematic representation of the inter-play between factors thought to be involved in the development of insulin resistance. Adapted from Eckel *et al.* (2005). VLDL: very low-density lipoproteins; HDL: high-density lipoproteins; LDL: low-density lipoproteins; FFA: free fatty acids; PAI-1: plasminogen activator inhibitor-1; TNFα: tumour necrosis factor-α

2.4.3 Treatment modalities for obesity, insulin resistance and type 2 diabetes

The mechanisms involved in the development of diet-induced insulin resistance and the consequent development of T2D are an active field of research. The therapies that are currently available do not always successfully enable patients suffering from hyperglycaemia to reach their glycaemic goals as even with intensive treatment, patients may still face spikes in blood glucose levels after meals, weight gain and a loss of effectiveness of their treatments over time [Pearson, 2009; Moller, 2001]. There are a number of treatment options available to overweight and obese individuals.

The most favourable and the one that has the least risk is simple lifestyle modification (healthy diet and exercise). There is strong scientific evidence that shows that even a modest weight loss (5 – 10%) can lead to a significant reduction in the risk of the co-morbidities of obesity [National Institutes of Health, 1998]. Additionally, exercise training also has beneficial effects on skeletal muscle insulin sensitivity, by amongst others, increasing the glucose infusion rate and plasma adiponectin [Ristow *et al.* 2009]. In this study by Ristow *et al.* (2009), they found an increased expression of reactive oxygen species (ROS)-sensitive transcriptional regulators of insulin sensitivity and ROS defense capacity (PPARγ and PPARγ co-activators (peroxisome proliferator-activated receptor gamma co-activator, PGC1α and PGC1β)). Molecular mediators of endogenous ROS defense (superoxide dismutases 1 and 2 and glutathione peroxidase) were also induced by exercise. Exercise-induced oxidative stress ameliorates insulin resistance and causes an adaptive response promoting endogenous antioxidant defense capacity.

There are other options in which there are more risks involved, such as pharmacotherapy and surgery [National Institutes of Health, 1998]. The present therapies for T2D mainly rely on the approaches to reduce hyperglycaemia, by means of oral hypoglycaemic agents such as sulphonylureas, which increase the release of insulin from pancreatic islets [Kar and Holt, 2008; Pearson, 2009]; metformin, which acts to suppress gluconeogenesis and thus reduce hepatic glucose production [Correia *et al*, 2008; Pearson, 2009]; PPARγ agonists (thiazolidinediones), which enhance insulin action primarily through indirect effects on lipid metabolism [Pearson, 2009; Edgerton *et al*, 2009]; α-glucosidase inhibitors, which interfere with gut glucose absorption and exogenous insulin injections, which suppress glucose production and augment glucose utilization. These therapies have limited efficacy, limited tolerability and significant side effects, which makes newer and safer therapies essential.

Due to the significant side-effects of prescription medication, the use of over-the-counter herbal products, nutritional supplements and meal replacements in the management of obesity, insulin resistance and T2D mellitus has gained increasing attention in consumer arenas, due to their natural origin and perceived fewer side effects [Aggarwal, 2010]. Herbal preparations, especially those rich in phenolic compounds, alkaloids, flavonoids, terpenoids, coumarins and glycosides, have been shown to have anti-obesity and anti-diabetic activities [Tabatabaei-Malazy *et al.,* 2012]. They have been shown to, amongst others, effectively prevent diet-induced-obesity (by preventing weight gain), significantly reduce body weight (in already overweight individuals) and have glucose-lowering abilities [Astell *et al.*, 2013; Hasani-Ranjbar *et al.,* 2009; Hui *et al.,* 2009]. Ginseng is an example of an herbal substance that has been reported to have anti-diabetic effects. When ginseng was administered orally (100 mg/kg body weight) for 20 days, decreased serum glucose level and HbA_1c levels in STZ-induced diabetic rats were reported [Kim *et al.*, 2007]. Similarly, in KKAy mice (a model of obese T2D), a decreased serum glucose level was reported [Chung *et al.,* 2001]. The authors of the latter study [Chung *et al.,* 2001], proposed that ginseng blocks intestinal glucose absorption and inhibits hepatic glucose-6-phosphatase, which would inevitably lead to the decreased serum glucose levels. Ginseng berry extracts have also been reported to have anti-obesity effects in obese *ob/ob* and *db/db* mice, by reducing weight gain in these obese animals [Xie *et al.,* 2007; Xie *et al.,* 2002] and anti-hyperglycaemic effect [Xie *et al.,* 2007]. Lee *et al.*, 2012 conducted a study in which they examined the anti-diabetic and anti-obesity effects of *Panax ginseng*, as well as the possible mechanism of action in high fat fed Sprague-Dawley rat model. They found that 18-week administration of Korean red ginseng was able to significantly reduce weight gain, reduce fat mass and increase insulin sensitivity, as demonstrated by an insulin tolerance and hyperinsulinaemic-euglycaemic clamp test. In addition, by means of Western blotting assays, they observed increased phosphorylation of the IRβ, IRS-1, PKB/Akt as well as increased membranous glucose transporter, GLUT4, in the muscle of the Korean red ginseng-treated group. From this data, they concluded that treatment with Korean red ginseng may have anti-diabetic effects and anti-obesity effects, due to partly increased insulin sensitivity, through increasing phosphorylation of IR-β, IRS-1, PKB/Akt and GSK3α/β and increasing GLUT4 translocation in skeletal muscle [Lee *et al.*, 2012]. Similar to the above-mentioned studies, Tan *et al.,* 2011 found that a Chinese herbal extract (denoted as SK0506), composed of *Gynostemma pentaphyllum*, *Coptis chinensis* and *Salvia miltiorrhiza* prevented weight gain and significantly reduced visceral fat mass during high-fat feeding of Sprague-Dawley rats. Muller *et al.,* 2012 also found that aspalathin (a component of green rooibos tea), dose-dependently increased glucose uptake (5×10^{-5} to 5 μg/ml) in C2C12 myotubules in an *in vitro* study. Likewise, in the *in vivo* portion of the same study, the extract sustained the blood glucose lowering effect in STZ-induced diabetic rats, validated by the decreased blood glucose levels. Mazibuko *et al.* (2013) too found that treatment with aspalathin increased glucose uptake and ATP production, down-regulated PKCθ activation, increased activation of 5' adenosine monophosphate-activated protein kinase (AMPK) and PKB/Akt and increased expression of GLUT4, in palmitate-induced insulin-resistance in C2C12 skeletal muscle cells. Mazibuko *et al.* (2013) proposed a mechanism of action via PKC-θ inhibition and increased activation of key regulatory proteins involved in insulin-dependent and non-insulin regulated signaling pathways, AMPK and PKB/Akt. Kawano *et al.* (2009) conducted an *in vivo* and *in vitro* study on aspalathin, which showed that aspalathin significantly increased glucose uptake by L6 myotubes, in a dose-dependent manner, at concentrations 1–100 mM [Kawano *et al.,* 2009]. They also found aspalathin treatment significantly increased insulin secretion from cultured RIN-5F cells at 100 mM. The *in vitro* study showed that dietary aspalathin (0.1–0.2%) suppressed increased fasting blood glucose levels and the intraperitoneal glucose tolerance test (IPGTT) showed an improvement in the impaired glucose tolerance of *db/db* mice. These results suggest that aspalathin has beneficial effects on glucose homeostasis through stimulating glucose uptake in muscle tissues and insulin secretion from pancreatic β-cells.

If medication is not successful, surgery, such as bariatric surgery, is recommended. One should keep in mind that surgery is the last option and it is only prescribed to a limited number of morbidly obese patients (BMI > 40 kg/m^2 or >35 kg/m^2 with co-morbid conditions). This is selective therapy, which is reserved for patients who are suffering from the complications associated with extreme obesity or are unresponsive to non-surgical treatment [Fisher and Schauer, 2002].

2.5 MUSCLE FATIGUE DURING EXERCISE

It is quite easy to recognize when one is fatigued, however it is quite difficult to identify the physiological mechanisms thereof. Since the early work of Mosso (1904) numerous studies have become available on the topic of muscle fatigue. Even though progress has been made in the study of muscular fatigue [Nordstrom *et al.* 2007; Nybo & Rasmussen, 2007] the exact reason why some individuals fatigue under certain conditions, are still fairly unknown. So the question remains, what is the mechanism(s) behind muscle fatigue?

2.5.1 Identification of fatigue

Anyone that has ever participated in any sporting discipline has experienced fatigue at some point. It is for this reason that the use of supplements to enhance athletic performance and delay the fatigue sensation is occurring at all sporting levels. When looking at the term muscle fatigue, it appears that in general, it is referenced to either as a motor deficit, a perceived decline in mental function, a gradual decrease in the force capacity of muscle or a decrease in sustained activity. This broad definition adds to the problem of identifying the cause of muscle fatigue as each of these descriptions has its own physiological mechanism. For the purpose of this review I will be discussing exercise-induced fatigue. Exercise-induced muscle fatigue is denoted as being the deterioration of muscle performance during prolonged activity [Roots *et al.*, 1985; Fitts, 1994]. This decrease in force is a reversible phenomenon as muscle performance can be recovered after sufficient rest and appropriate nutrition. It is also well documented that several factors, such as the types and intensity of exercise, the muscle groups involved and the biochemical environment, affect fatigue development [Weir *et al.,* 2006].

The observable existence of fatigued muscles has been recognized for many years. Since the early 1900's, numerous authors have contributed to our current knowledge of muscle fatigue. In 1904, Mosso illustrated that when a finger lifts a heavy load, fatigue of that muscle occurred, but that this fatigue could also occur when the nerves were stimulated electrically. From his data Mosso inferred that muscle fatigue occurs within the muscle rather than in the central nervous system. Authors such as Hill and Kupalov (1929) concluded from their earlier studies that the accumulation of lactic acid could be the cause of muscle fatigue. In their experiments they isolated frog muscles and stimulated these muscles in N_2 gas. These muscles rapidly fatigued and accumulated lactic acid; however when these muscles where removed from the gas and transferred to N_2-rich Ringer, the muscle performance was restored as the lactic acid was able to diffuse from the muscle. During 1963 Eberstein and Sandow published a paper in which they suggested that the malfunctioning of EC coupling was a contributing factor to muscle fatigue. They observed that when perfusing a fatigued muscle in caffeine, it could recover much of its force. Caffeine is known to directly facilitate the release of Ca^{2+} from the SR. Research by Burke *et al.* (1973) demonstrated that different fiber types fatigue differently. In their research they stimulated individual motor units in cat muscles to exhaustion and identified the muscle fibers that were involved by the depletion of glycogen. This research showed that fast-twitch fibers fatigued rapidly, whereas slow-twitch fibers were in essence "unfatigueable".

According to the literature, the mechanisms involved in fatigue vary with intensity, duration and mechanics (shortening, isometric and stretching) of the contractions involved adding to the complexity of the problem and, as a result, the process of fatigue is currently not completely understood. The process of muscle contraction-relaxation follows a complex pathway. In short, the contraction-relaxation process originates in the cortex and leads to the activation of lower α-motor-neurons in the spinal cord. The axons of the lower motor-neurons carry the action potentials to the neuromuscular junction of the muscle [Allen *et* *al.*, 2008b]. Fatigue can therefore potentially arise from processes in the spinal cord or above, or from processes in the peripheral nerve, neuromuscular junction or the muscle itself. Therefore the causes of fatigue are divided into either central or peripheral fatigue [Allen *et al.*, 2008]. The interplay between the nervous system and skeletal muscle is fairly complex, which makes it difficult to design an experiment to accurately assess the exact extent of central fatigue during intense exercise. For the purpose of this review, only components that lie within the muscle, i.e. peripheral fatigue, will be discussed in further detail.

2.5.2 Energy metabolism and peripheral muscle fatigue

Peripheral muscle fatigue relates to factors within the muscle that leads to impaired contractile function during intense exercise and therefore it is, in most cases, highly dependent on the capacity of the aerobic metabolic system. Hence, slow-twitch oxidative muscle fibers are notably more fatigue-resistant than fast-twitch glycolytic fibers under normal conditions (refer to section 2.2.2 on the difference between fiber types). In the sections below I will be discussing (i) the direct or indirect effects the accumulation of metabolites such as inorganic phosphate (P_i), adenosine diphosphate (ADP), magnesium ions (Mg^{2+}), (ii) the decrease in substrates, such as ATP, creatine phosphate and glycogen and (iii) the effect ROS have on muscle fatigue development.

As skeletal muscle contracts, the energy used is mostly spent on the cross-bridge action and the function of the ion pumps (mainly the SR Ca^{2+} pumps). The relative energy requirements between the cross-bridge action and the ion pumps depend on the type of contraction (refer to section 2.3.2 on the different types of muscle contractions). During all types of muscle contraction, ATP is the immediate source of energy for the muscle cells. Therefore the concentration thereof is important as it will affect the mechanics of the cell. At rest, skeletal muscle has an intracellular concentration of between 5 and 6 mM ATP [Sahlin *et al.,* 1998; Hochachka and Matheson, 1992] and during intense fatigue this concentration can drop to 1.2 mM in fast-twitch muscle [Allen *et al*,. 2008a]. This significant drop is due to the fact that during activity there are three ATPases that require ATP for their function. The Na^+/K^+-ATPase pumps Na^+ out and K^+ into the fiber after an action potential. The myosin ATPase uses ATP to generate force and the Ca^{2+}-ATPase pumps Ca^{2+} back into the SR to allow muscle relaxation [Homsher, 1987]. According to research done by Sahlin *et al.* (1998) in fully activated muscles, ATP can theoretically be depleted within 2 seconds [Sahlin *et al.,* 1998]. To counter this rapid depletion ability the cell utilizes both anaerobic as well as aerobic metabolism. Anaerobic metabolism is the dominating pathway during high-intensity activity of short duration as it yields ATP faster, but less ATP per glucosyl unit as compared to aerobic metabolism. Conversely, aerobic metabolism dominates during prolonged sub-maximal exercise [Sahlin *et al.,* 1998]. However slower generation of ATP is observed, more ATP per glucosyl unit is produced (theoretically 3 vs. 38 ATP/glucosyl unit).

During earlier research on muscle fatigue the debate centered on whether lactic acid accumulation or the accumulation of P_i, are the main contributors to muscle fatigue. It is well known that during intense exercise the energy consumption of a skeletal muscle cell can increase up to 100-fold, when compared to its resting condition [Westerblad *et al.*, 2002; Hochachka and Matheson, 1992], which leads to an increase in ATP demand. To compensate for the increased demand in energy a large fraction of the ATP required will come from anaerobic metabolism. Since muscle fatigue is the end result of a rapid decline in contractile function, it seems logical to assume that there is a causal relationship between anaerobic metabolism and muscle fatigue.

During **anaerobic metabolism** ATP is mainly produced through the degradation of phosphocreatine (PCr) and the breakdown of muscle glycogen, forming lactate and hydrogen ions (H^+) as byproducts. Creatine kinase (CK) is responsible for this phosphate exchange between ATP and PCr in a near-equilibrium reaction (PCr + ADP ↔ Cr (creatine) + ATP). A minor contribution to the energy pool is made by myokinase, which catalyses the near-equilibrium reaction *in vivo* (2 ADP ↔ ATP + adenosine monophosphate (AMP)).

When AMP increases sufficiently, it will be deaminated to inosine monophosphate (IMP) and NH^{4+}.

As stated above, during intense exercise, skeletal muscle can increase its rate of ATP use more than 100-fold. During periods of high ATP consumption, the reaction (PCr + ADP ↔ Cr + ATP) will be driven to the right. In other words, the net effects will be a reduction in [PCr] and an increase in [Cr] and [P_i], whereas [ATP] remains fairly constant initially. It has been found that Cr does not seem to have any effect on muscle fatigue [Allen *et al.*, 2008b], however the P_i does. It is thought that P_i may cause a marked decrease of muscle force production and myofibrillar Ca^{2+} sensitivity by reducing the number of force generating cross-bridges [Allen *et al.*, 2008b], as well as decreasing SR Ca^{2+} release. Additionally, it is thought that P_i enters the SR during fatigue, a process that will lead to the Ca^{2+}-P_i solubility product to be exceeded with consequent decreased free Ca^{2+} available for release [Fryer *et al.,* 1997]. An experiment that illustrates the importance of P_i in muscle fatigue was done on CK deficient muscle fibers [Dahlstedt *et al.*, 2000]. These genetically modified fibers cannot break down PCr and therefore display impaired contractile function at the onset of high-intensity stimulation, where PCr breakdown functions as an important energy source. Their study showed that the CK reaction contributed to fatigue development by increasing myoplasmic P_i during prolonged stimulation and that the absence of PCr breakdown resulted in a more fatigue resistant muscle [Dahlstedt *et al.*, 2000]. Consequently, when these deficient fibers were injected with CK, allowing PCr breakdown to occur, all features returned to near wild-type features [Dahlstedt *et al.*, 2003; Allen *et al.*, 2008a].

On the other hand, during periods of recovery (periods after high ATP consumption), the synthesis of PCr is favored. At the point at which [PCr] reaches low levels, [ATP] starts to drop and [ADP] and [AMP] start to increase. This is mirrored in the gradual accumulation of IMP [Zhang *et al.,* 2008]. In studies done on skinned fibers, it was found that the decline in [ATP] and [PCr] can reduce SR Ca^{2+} pumping and increase pump leakage, which inevitably results in elevated resting intracellular [Ca^{2+}], a phenomenon typically observed during muscle fatigue [Dutka and Lamb, 2004; Nakamura *et al.,* 2002; MacDonald and Stephenson, 2001], and in some cases a slowing of relaxation in fatigued muscle.

In a very detailed review by Allen *et al.* (2008b) on skeletal muscle fatigue, it is documented that the depletion of the intramuscular glycogen stores can limit muscle performance during prolonged exercise, such as long distance running. Under normal conditions the breakdown of glycogen is regulated by glycogen phosphorylase and the synthesis is catalyzed by glycogen synthase. Both these enzymes are controlled through phosphorylation. The phosphorylation of glycogen phosphorylase is catalysed by phosphorylase kinase at the Ser^{14} residue and the dephosphorylation is catalysed by protein phosphatase 1 [Johnson, 2009]. Glucose residues are released by glycogen with the help of phosphorylase to enter the process of glycolysis. This glucose is ultimately converted to pyruvate. During intense exercise, in a reaction upstream of glycolysis, NAD^+ is a necessary factor. Therefore, lactate dehydrogenase converts NADH + H^+ + pyruvate to lactate + NAD^+ to maintain the process of glycolysis [Katz and Sahlin, 1988]. In theory, lactic acid accumulates during periods of intense exercise when the demand for ATP is high. In earlier years it was thought that this build-up of lactic acid was the main contributor to muscle fatigue, however lactic acid accumulation alone may not be responsible for the decreased muscle performance found during muscle fatigue, as other factors may be involved as well. Parallel to this occurrence is the generation of H^+, which in essence leads to the decrease in muscle pH (acidosis) [Posterino *et al.*, 2001]. It has been found that the muscle's pH can drop from ~ 7.0 to ~ 6.5 [Fitts, 1994]. In addition to the accumulation of lactic acid, at some point acidosis was also thought to be the most important cause of impaired contractile function in fatigued muscles. However, in more recent studies it has been shown that acidosis is not the key cause of fatigue in mammalian muscle. In these studies the authors fatigued the muscles at physiological temperature and found that acidosis (at the magnitude observed in severely fatigued muscles (~ 0.5 pH-units) had no significant impact on force generation, contractile speed or the rate of fatigue development [Westerbad *et al.,* 2002; Allen *et al.,* 2008b].

During **aerobic metabolism**, oxidative metabolism of carbohydrates and lipids are the dominating ATP-producing mechanisms [Spriet and Watt, 2003]. As mentioned previously, this system is preferred over anaerobic metabolism where prolonged sub-maximal exercise is concerned. The major carbohydrate substrate for aerobic metabolism during prolonged exercise is muscle glycogen. Exactly how glucose uptake is regulated during exercise is still not fully understood. What is known is that it is by an insulin-independent pathway [Holloszy, 2003]. In the past few years it has come to light that the activation of AMPK might be involved in this process [Hardie and Sakamoto, 2006]. It is believed that AMPK is activated by the increase in AMP during exercise; however the increase in AMP is not that vast. It is therefore suspected that other mechanisms in addition to the increased AMP are involved [Westerblad *et al.,* 2010; Hardie and Sakamoto, 2006].

In addition to carbohydrate metabolism, lipid metabolism also contributes to the ATP-producing system. The substrate for lipid metabolism is free fatty acids, derived from triglyceride stores in the muscle and adipose tissue. Interestingly, the contribution of fatty acids to aerobic ATP production is in essence at its maximum at exercise intensities of ~ 60% of maximal oxygen uptake; however at higher intensities, fatty acid oxidation decreases [Sahlin *et al.*, 1998]. Additionally, amino acids, derived from muscle protein degradation, are also a substrate for aerobic metabolism. However they contribute but a minute portion of the overall energy pool during prolonged exercise [Lemon and Mullin, 1980].

An alternative mechanism that might induce muscle fatigue is thought to be ROS, as ROS is known to disrupt mitochondrial function, causing muscle depolarization and reduction in force [Nethery *et al.,* 2000]. There is a growing body of literature suggesting that the ROS produced during exercise plays a critical role in the modulation of muscle contractility. Numerous researchers have demonstrated that skeletal muscle cells continuously generate ROS throughout episodes of sub-maximal exercise [Powers and Jackson, 2008; Zuo *et al.,* 2011; Strobel *et al.,* 2011] and multiple potential sites for ROS generation in skeletal muscle have been identified, including mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes, phospholipase A2-dependent processes, and xanthine oxidase

[Jackson, 2009]. There are, however, large discrepancies in the literature regarding the role of ROS in fatigue development. Since the amount of ROS produced depends on the type and intensity of the exercise, there is evidence ranging towards it leading to fast development of contractile dysfunction to no effect at all [Allen *et al.,* 2008b; Powers and Jackson, 2008]. In unfatigued muscle, ROS are produced at low rates [Reid *et al.,* 1992a] and are essential for normal force production. Endogenous ROS are selectively depleted by antioxidant enzymes, leading to a reduction in force. This effect is reversed once these enzymes have been washed out [Regnier *et al.,* 1992; Reid *et al.,* 1993]. Under fatigued conditions, endogenous ROS appears to play a causal role. Exhaustive exercise increases ROS levels in the cytosol [Reid *et al.,* 1992a], extracellular space [Reid *et al.,* 1992b], and vascular compartment [Kolbeck *et al.,* 1997; O'Neill *et al.*, 1996] of exercising muscle. This increased ROS levels lead to elevated oxidative stress, observed as increased glutathione oxidation and malondialdehyde production [Powers and Jackson, 2008; Alessio, 1993; Packer, 1997]. The treatment with antioxidants have been found to inhibit exercise-induced fatigue of rat muscle [Diaz *et al.*, 1994, Reid *et al.,* 1992a], perfused mammalian muscle *in situ* [Barclay and Hansel, 1991], and intact human muscle [Reid *et al.*, 1994; Travaline *et al.,* 1997]. These observations strongly implicate ROS as mediators of fatigue.

2.5.3 Three phases of force decline during muscle fatigue

During a typical fatigue protocol the generated force will "fall" in three phases. Experiments conducted on isolated intact muscle fibers have brought to light that the decrease in isometric force during fatigue stimulation involves (i) a reduced ability of the cross-bridges to generate force, (ii) a plateau phase and (iii) a decrease in myofiber Ca^{2+} sensitivity.

The first phase involves a drop in force to about 80 to 90% of control values over ±1 minute (Fig. 2.6). This reduction is generally thought to be as a result of the inhibitory effects of P_i on the transition of the cross-bridge to its high-force state [Millar and Homsher, 1990]. During the second phase the force stays fairly constant (Fig. 2.6). It is thought that throughout this phase the ATP production (aerobic and anaerobic breakdown of glycogen) equals the rate of ATP consumption. Finally, during the third phase, the force starts to decline again [Allen *et al.,* 2008b]. This is principally caused by the reduction in SR Ca^{2+} release coupled to reduced sensitivity of the contractile proteins to Ca^{2+}. The mechanism of Ca^{2+} decline is still under debate [Allen *et al.*, 2008a]. However, this decrease in SR Ca^{2+} release can also be seen as a safety mechanism because it occurs at a stage at which the muscle fiber is exhausted [Westerblad *et al.,* 2010]. If SR Ca^{2+} release remains high, [ATP] might fall to dangerously low levels in which the cross-bridge enters rigor states and SR Ca^{2+} uptake fails. This will inevitably result in non-functional cells. However, these devastating events will be avoided if the reverse happens, i.e. the intracellular Ca^{2+} decreases, the cross-bridges will utilize less energy and the energy to pump back Ca^{2+} into the SR is reduced.

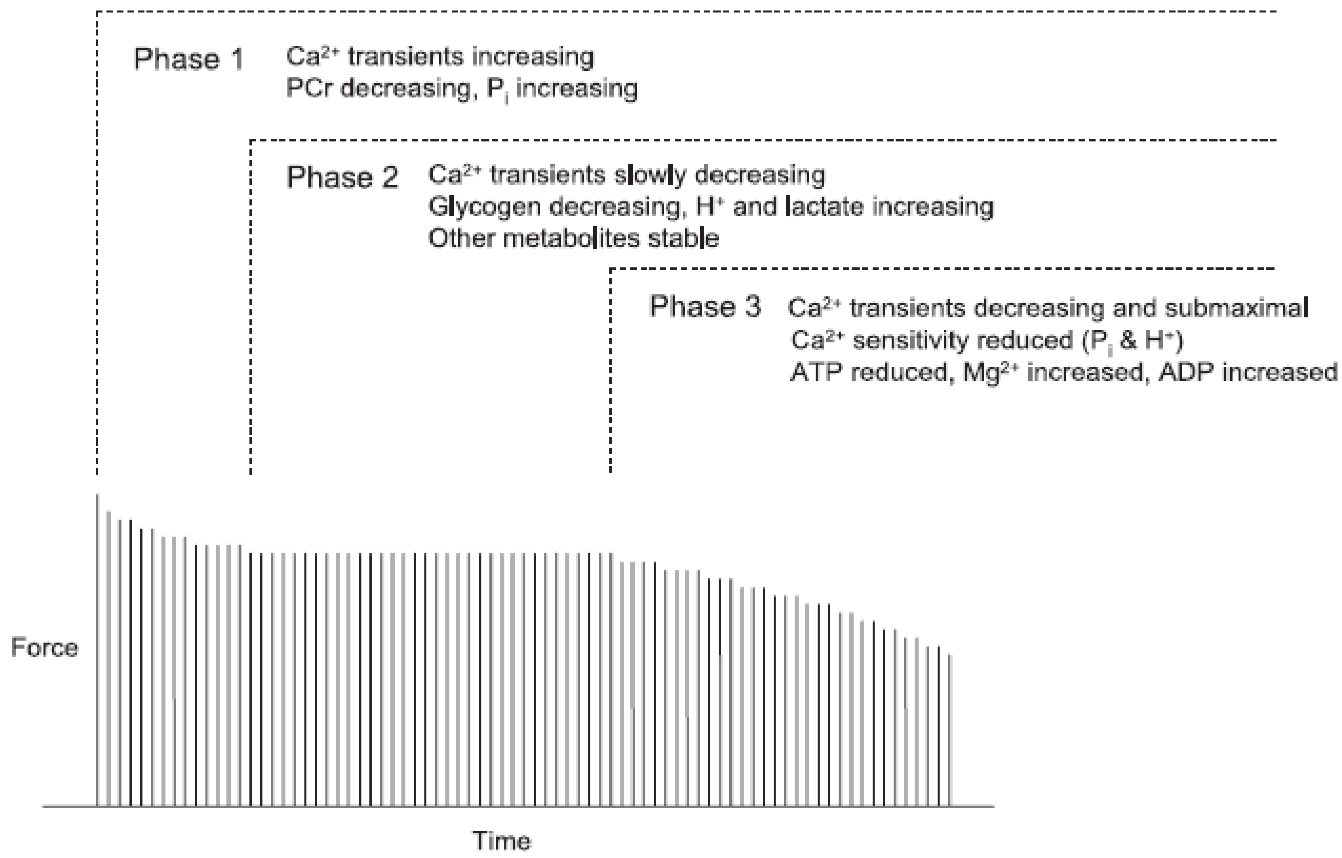


Figure 2.6: Schematic representation of fatigue in an isolated single fiber at room temperature stimulated with repeated, brief isometric tetani. The 3 phases of force decline are identified and relevant intracellular changes noted. Sketch obtained from Allen, 2009

2.5.4 Models of fatigue and their limitations

2.5.4.1 Models employed to investigate mechanisms of fatigue

The perceived "golden standard" for fatigue experimentation is the intact perfused muscle under the control of the central nervous system [Allen *et al.,* 2008b]. By utilizing the intact animal it is easy to measure the decline in force production, electromyography can be performed on selected muscle groups and biopsies are fairly easy to obtain. However, the difficulty that arises is that most muscles are of mixed fiber type, each type having its own unique set of properties (refer to section 2.2.2 on the different fiber types). In addition, as previously mentioned, development of muscle fatigue is also influenced by signaling from the central nervous system. Therefore, to avoid central complications, the nerve feeding the muscle is stimulated or alternatively, stimulating the muscle directly [Allen *et al.,* 2008b]. This largely eliminates the contribution by the central nervous system and, because the blood flow is intact, it will also eliminate the diffusion problems experienced with isolated muscles, allowing for studies focused on the fatigue-related processes in muscle fibers specifically.

Another approach of studying fatigue is to use isolated whole muscles [Allen *et al.,* 2008b]. One such approach consists of isometrically stimulating the isolated muscle with repeated tetani until force is substantially reduced while perfusing the muscle with physiological saline at room temperature in an organ bath. Obviously such methods do have limitations. Due to the absence of circulation, the muscle core tends to become anoxic and K^+ accumulates extracellularly. Barclay (2005) did a study in which he calculated the diffusion gradient of O_2 across isolated rat and mouse muscles and concluded that a whole soleus muscle of a mouse can only contract at a duty cycle of 0.5 for ± 60 seconds at 20˚C and only ± 12 seconds at 35˚C before an anoxic core develops. Active muscle fibers release K^+ and it accumulates in the extracellular spaces until a diffusion gradient develops which is sufficient to allow K^+ to diffuse out of the preparation. As a result of this active release of K^+, the concentration of K^+ will be much higher at the core of the muscle than it is in the perfusate.

In addition to K^+, CO_2, H^+ and lactate will also accumulate in the extracellular spaces in a similar fashion. All these factors complicate analysis of the mechanisms involved in muscle fatigue.

To overcome the issue of the anoxic core and the K^+ gradient, smaller preparations, such as smaller muscles and even single fibers are often used. With single fibers the extracellular accumulation of K^+ will not be observed. In research done by Roots *et al.* (1985) they isolated small bundles of fast twitch fibers from rat muscle and determined their performance at different temperatures ranging from 10 °C to 30 °C and they made use of isotonic contractions instead of isometric contractions. Their first interesting finding was that the rate of fatigue was much lower at 30 °C than at 10 °C, which is contradictory to previous studies which found that fatigue was faster at physiological temperatures [de Ruiter and de Haan, 2000]. The explanation that Roots and his colleague offered was that the efficacy of ATPases is elevated at higher temperature and therefore ATP consumption and P_i production will accelerate [Roots *et al.*, 1985]. However, one can argue that ATP resynthesis will also accelerate, so this argument alone does not suffice. Roots *et al.* (1985) also pointed to the fact that the sensitivity of the force production to P_i also falls with increasing temperatures [Debold *et al.*, 2004]. Though, to substantiate this fact, a full quantitative analysis will have to be conducted that includes P_i measurements at different temperatures and the sensitivity changes that go along with it. In addition, there are other factors that may also contribute to the temperature dependence of muscle fatigue, such as the increased ROS production that happens at higher temperatures [Arbogast and Reid, 2004]. The second interesting finding of the research done by Roots *et al.* (1985) was that the muscles in their experiments fatigued more rapidly when contraction involved shortening, compared to isometric contractions, that do not involve shortening of the muscle. A reasonable explanation could be that shortening contractions utilize ATP at a much higher rate than do isometric contractions, so P_i accumulation is therefore greater and the depressive effect of P_i on force production is also elevated.

It is very important to determine what type of stimulation protocol best suits your perceived outcome before embarking on your experiments. Refer to section 2.5.4.2 below on the different modes of stimulating muscle to fatigue.

2.5.4.2 Stimulation protocols employed to achieve muscular fatigue

Continual maximum activity: When a muscle is continually stimulated at a frequency that is at or close to its maximal force, it is called high-frequency fatigue. With this type of fatigue the force production shows a rapid decline, thus early onset of fatigue as well as rapid recovery of the muscle [Allen *et al.,* 2008b]. An example of this type of fatigue is when an individual lifts a very heavy object such as a piano. In laboratory based studies this type of experiment would constitute stimulating the muscle at a constant high frequency. However, this is not normal physiological conditions and therefore this type of experimentation is not a true representation of what happens in an intact individual.

Repeated short tetani: Unlike continual maximum stimulation, repeated short tetani is a more popular pattern of studying fatigue, however there is no consistency in the protocols in the literature. The two main discrepancies include the fraction of time during which the muscle contracts (this differs from between 0.1 to 0.5 duty cycles) and the stimulus frequency during the tetani. Repeated short tetani simulates most natural occurrences, such as walking and running, and it leads to a much slower rate of fatigue as compared to the high-frequency fatigue described above [Allen *et al.*, 2008b]. The fatigue protocol is usually stopped after a fixed set of tetani or when the force reaches a predetermined level (e.g. 50% of its initial tetanic force). Just as the protocol for this type of fatigue differs, so does the rate of recovery. Under normal circumstances there is “fast” recovery and “delayed” recovery. The “fast” recovery is complete in about 5 to 10 minutes; however the “delayed” recovery may take hours to set in. This slow component of recovery was first observed by Edward *et al.* 1977. They made use of voluntary contractions in humans under ischaemic conditions until the produced force was negligible. Doing this, they discovered that muscle recovery was relatively fast at higher frequencies (50 - 100 Hz) but very slow at lower frequencies (10 - 20 Hz) and that there was also still a component of weakness persisting after a day.

In summary, research into the mechanism of fatigue can be seen as being moderately well defined in some simplified preparations. It is important to perform research in which the preparation is close to physiological conditions for inference to be drawn. It is therefore best to determine what information you would like to gain from your experiment and then decide which model will suit your study best as there really is no "golden standard" in fatigue experiments, since all models have their short-comings. The important challenge is to define the mechanism of fatigue in human diseases so that necessary attempts can be made to ameliorate such conditions. Much research has gone into the mechanism of muscle fatigue and the current treatment modalities are discussed below in section 2.5.5.

2.5.5 Treatment modalities for skeletal muscle fatigue

Fatigue exists as a result of accumulating factors and therefore treatment is geared at different causal factors of fatigue. It is known that when exercise is exhaustive it causes tissue damage, muscle fatigue and inflammation [Cobley *et al.*, 2011; Peake *et al.,* 2007], due to increased ROS production in skeletal muscle [Powers and Jackson, 2008; Zuo *et al.*, 2011; Strobel *et al.*, 2011]. For many years athletes consumed antioxidant supplements as research stated that antioxidants would be a good treatment option for exercise-induced fatigue, as they may attenuate ROS-related oxidative damage and delay fatigue during exercise [Hathcock *et al.,* 2005]. For example, Reid *et al.* (1994) reported that N-acetylcysteine (NAC) pretreatment did not alter the function of unfatigued muscle of healthy human volunteers. NAC being an antioxidant compound that is commonly used clinically to treat paracetamol overdose and has been shown to scavenge ROS, including hypochlorous acid, hydroxyl radical and hydrogen peroxide (H_2O_2) [Aruoma *et al.* 1989]. However, during fatiguing contractions stimulated at low-tetanic frequency (10 Hz), NAC increased force output by approximately 15%, an effect that was evident after 3 min of repetitive contraction and persisted throughout the 30-min protocol. The researchers therefore concluded that NAC pretreatment can improve performance of human limb muscle during fatiguing exercise, suggesting that oxidative stress plays a causal role in the fatigue process and they identified antioxidant therapy as a novel clinical intervention. However, there is increasing evidence that exercise-induced ROS may act as signals regulating beneficial skeletal muscle adaptations, such as increased mitochondrial biogenesis [Strobel *et al.,* 2011], and that they are also necessary and integral regulators of redox-sensitive signal transduction pathways [Jackson, 2007]. Signaling cascades which are redox-sensitive and crucial for the adaptive regulation in skeletal muscle include the mitogen activated protein kinases (MAPK), which are necessary in growth, metabolism, differentiation, transcription, translation and remodeling [Petersen *et al.,* 2012; Qi and Elion, 2005]. Additionally, the nuclear transcription factor kappa-β (NF-κβ) complex is a major stimulator of the genes involved in processes such as inflammation and muscle protein turnover. The importance of exercise-mediated ROS production in skeletal muscle is demonstrated in studies where antioxidant supplementation attenuates or prevents adaptations to exercise [Marshall *et al.* 2002, Gomez-Cabrera *et al.* 2005, 2008]. Reactive oxygen and nitrogen species are produced during exercise, due at least in part, to the activation of xanthine oxidase [Gomez-Cabrera *et al.,* 2005]. This was validated by Gomez-Cabrera *et al.* (2005) as they found that exercise caused an activation of the MAPK, p38 MAPK, extracellular signal-regulated kinase (ERK) 1 and ERK 2, which in turn activated NF-κB in rat gastrocnemius muscle. This activation was found to up-regulate the expression of enzymes associated with cell defense (superoxide dismutase) and adaptation to exercise (endothelial nitric oxide synthase (NOS) and inducible nitric oxide synthase (iNOS)). These effects were abolished when xanthine oxidase-induced ROS formation was prevented by allopurinol. These results demonstrate that decreasing ROS formation prevents activation of important signaling pathways, predominantly the MAPK–NF-κB pathway. The practice of taking antioxidants before exercise therefore needs to be re-evaluated. In addition it was found that the up-regulation of the PGC-1α gene, which is a transcriptional co-activator involved in mitochondrial biogenesis, was attenuated by antioxidant treatment following incubation of muscle cells in H_2O_2 [Irrcher *et al.* 2009], electrical stimulation of skeletal muscle cells [Silveira *et al.* 2006] and exercise training in human skeletal muscle [Ristow *et al.* 2009]. In addition Petersen *et al.* (2012) investigated the effects of antioxidant supplementation during exercise on the MAPK or NF-κB cell signaling pathways in human skeletal muscle. In this study they found that NAC infusion blocked the exercise-induced increase in c-Jun N-terminal kinase (JNK) phosphorylation, however not ERK1/2, or p38 MAPK as previous studies have shown after antioxidant treatment [Gomez-Cabrera *et al.* 2005]. In the same study they also observed that NF-κB p65 phosphorylation was unaffected by exercise, but that NAC treatment reduced NF-κB p65 phosphorylation under fatigue conditions compared with pre-infusion. The above-mentioned studies show that antioxidant supplementation may attenuate the early adaptive response to exercise by inhibiting certain signaling pathways and exercise-induced gene expression.

Another form of therapy currently being used for exercise-induced muscle fatigue is low-level laser therapy (LLLT). The first clinical trial with LLLT, in which they investigated the effects of LLLT on rheumatoid arthritis, was published by Goldman *et al.* in 1980. Since then several positive effects of LLLT have been identified in different pathologies [Hegedus *et al.,* 2009; Bjordal *et al.,* 2006; Ozcelik *et al.,* 2008; Basford *et al.,* 1999; Gur *et al.,* 2004; Rochkind *et al.,* 2007; Lampl *et al.,* 2007]. Across all LLLT studies, it has been found that LLLT increases microcirculation [Tullberg *et al.*, 2003], enhances ATP synthesis, stimulates the mitochondrial respiratory chain [Silveira *et al.*, 2009] and enhances mitochondrial function [Xu *et al.,* 2008]. It has also been reported that LLLT reduces release of ROS, reduces creatine phosphokinase activity and increases production of antioxidants and heat shock proteins [Avni *et al.,* 2005; Rizzi *et al.,* 2006]. In recent years phototherapy has been used in the treatment of fatigue. In these studies it was found that LLLT enhances muscle performance in both animal and human studies [Leal *et al.,* 2010; De Marchi *et al.,* 2011; Vieira *et al.,* 2012; Lopes-Martins *et al.*, 2006]. In the animal studies, LLLT with 655 nm red [Lopes-Martins *et al.,* 2006] and 904 nm infrared [Leal *et al.,* 2010] wavelengths, and clinical trials employing red [Leal *et al.,* 2008], infrared [Leal *et al.,* 2009b] and mixed [Leal *et al.,* 2009a] wavelengths delayed the development of skeletal muscle fatigue. However, the variations in these studies make it difficult to conclude as to whether red or infrared wavelengths produce better results in delaying the development of skeletal muscle fatigue. In the studies where LLLT enhanced mitochondrial function, LLLT induced the formation of giant mitochondria, presumably produced from the fusion of adjacent lower mitochondria. This was found to provide higher levels of respiration and energy (in the form of ATP) to cells [Leal *et al.,* 2009b], contributing to the increase of cellular energy, consequently increasing the performance during aerobic exercise. Recent literature reports that when laser therapy is applied before exercise, it can significantly attenuate the increase in serum lactate, CK, inhibit inflammation and accelerate muscle recovery between exercise sessions [Silveira *et al.,* 2009; Xu *et al.,* 2008; Avni *et al.,* 2005]. These positive responses to LLLT could be of fundamental importance in muscle performance, as treatment might increase resistance to fatigue.

In the literature there are reports where researchers examined the anti-fatiguing effects of numerous traditional medicines in rat, mouse and human models. One example is *Cordyceps sinensis* (CS), which is a fungus used in traditional Chinese medicine. For years traditional Chinese herbalists recognized CS as having the ability to promote well-being and athletic power if consumed by humans [Kumar *et al.,* 2011], however the mechanism by which this occurs was first reported by Kumar *et al.* (2011). In their study they found that CS supplementation improved fatigue in rats when they were left to swim until reaching exhaustion. To study the molecular mechanism of the fatigue-resistance observed, they measured the expression levels of endurance responsive skeletal muscle metabolic regulators AMPK, PGC-1α and PPAR-δ and endurance promoting and antioxidant genes like monocarboxylate transporter (MCT)1, MCT4, GLUT4, vascular endothelial growth factor (VEGF), superoxide dismutase (SOD)1, nuclear factor erythroid 2-related factor (Nrf)2 and thioredoxin (TRX) in gastrocnemius muscle. Their results showed a significant upregulation of the skeletal muscle metabolic regulators and they observed better glucose and lactate uptake both in exercised and non-exercised rats after CS supplementation. In addition they also observed an increased expression of oxidative stress responsive transcription factor

NRF-2 and its downstream targets SOD1 and TRX after CS supplementation [Kumar *et al.,* 2011]. In another study Chen *et al.* (2011) examined the anti-fatigue effects of *Renshen Yangrong* Decoction (RYD) in mice. From their study they concluded that RYD has anti-fatigue effects. They based their conclusion on the fact that loaded swimming time was significantly increased in their treatment group [Chen *et al.,* 2011].

2.6 SKELETAL MUSCLE INJURY AND REPAIR

2.6.1 Overview of events after muscle injury

Muscle injury is a common problem encountered in traumatology. Injured muscles heal slowly and when the muscle repair process is inadequate, the once normal muscle architecture is substituted with fibrotic tissue [Serrano and Munoz-Canoves, 2010]. There are both direct and indirect ways in which muscle tissue can be damaged. Direct trauma includes lacerations, strains and contusion injuries, whereas indirect trauma is related to ischaemia and neurological dysfunction [Baoge *et al.*, 2012]. For the purpose of this review, only contusion injuries will be discussed in further detail. In section 2.6.4 the other models of investigating muscle injury, with their limitations, will be discussed in more detail.

Muscle contusions, which are produced by the direct impact of a non-penetrating object [Crisco *et al.*, 1994; Smith *et al.*, 2008], is very common in contact sports. The muscles that are most affected are those of the legs, arms, hands, feet and buttocks [Kearns *et al.*, 2004]. This blunt-force on the muscle belly can produce disability due to significant pain and impaired muscle function. The repair mechanism of skeletal muscle is similar in most types of muscle injuries. This process consists broadly of three distinct phases, namely the destruction and inflammatory phase (1 to 3 days), the repair phase (3 to 4 weeks) and the remodeling phase (3 to 6 months) [Järvinen *et al.*, 2005; Tidball, 2005; Arrington and Miller, 1995]. The last two phases tend to overlap. The exact detail within these processes depends on the mechanism of injury.

In contusion injury, the first phase (destruction and inflammatory phase) is characterized by destruction of the integrity of the myofiber plasma membrane and basal lamina, leading to the ingress of extracellular calcium [Järvinen *et al.,* 2005]. Local swelling at the injury site and the formation of a haematoma further promotes muscle degeneration [Hurme *et al.*, 1991]. Necrotic tissue is invaded by small blood vessels, neutrophils and mononuclear cells [Hurme *et al.*, 1991].

In short, the inflammatory response consists of the infiltration of polymorphonuclear leukocytes (neutrophils) [Fielding *et al.*, 1993] and later macrophages [Orimo *et al.*, 1991] into the injured tissue. Neutrophils recognize previously sequestered proteins spilling from damaged tissue, so their main function during a sterile injury, such as a contusion injury, is to phagocytose damaged cells. As part of their function, neutrophils release ROS, which contributes to damage. They also release chemotactic factors to strengthen the inflammatory response by attracting more neutrophils. Macrophages on the other hand, consist of different subtypes, which have two main functions. Firstly, the more pro-inflammatory sub-type removes the necrotic myofibers by phagocytosis, and secondly, they produce (along with fibroblasts) chemotactic signals such as growth factors, cytokines and chemokines. The nature of cytokines released by macrophages changes over time, as the macrophage phenotype gradually changes from a pro- to anti-inflammatory, with the same result in their secretory products, ultimately leading to the resolution of inflammation, so that regeneration can occur.

The healing process of skeletal muscle, in response to an injury, depends mainly on the type of injury (contusion, strain, laceration) and the severity of the injury. Under normal physiological conditions adult mammalian skeletal muscle is fairly stable, with only sporadic fusion of satellite cells to compensate for muscle turnover caused by daily wear [Silverthorn, 2004]. These minor lesions are repaired without causing cell death, inflammatory response or histological alterations. However, if skeletal muscle is damaged, it has a remarkable ability to regenerate itself [Chargé and Rudnicki, 2004]. This organized regenerative process relies greatly on the satellite- and inflammatory cells, of which the monocytes/macrophages play the greatest role in the repair process. After injury, the damaged cells send signals that activate quiescent satellite cells alerting them to begin to proliferate, differentiate and fuse into new myotubes. Not all the satellite cells will proliferate and aid in the repair process - some of them will undergo self-renewal and replenish the pool of quiescent satellite cells [Tidball and Villalta, 2010]. In addition to the satellite cells and the infiltrating inflammatory cells, there are also other cells that participate in the repair process, which include the PW1$^+$ interstitial cells (PICs), mesoangioblasts, fibro/adipogenic progenitors (FAPs) and other extra cellular matrix (ECM)-associated cells [Kharraz *et al.,* 2013]. Activated inflammatory cells produce growth factors, cytokines, inflammatory mediators and "damage" signals that have an impact on satellite cell behavior during the repair process [Tidball and Villalta, 2010; Huard *et al.,* 2002]. Another crucial step in the repair process is the restoration of the ECM. During this remodeling phase, the formation of a connective tissue scar by fibrin and fibronectin deposition occurs. It is very important that this step occurs as this will provide a new scaffold structure over which new myofibers will be formed [Cornelison, 2008]. However, excessive scar tissue deposition will lead to accumulating fibrosis and therefore a defective regenerative outcome and impaired muscle function [Huard *et al.*, 2002; Chan *et al.*, 2005]. Thus, effective muscle repair requires the promoted recruitment of myogenic cells to the injured area and the suppression of fast growth of fibroblasts.

According to the literature it seems that by limiting the extent of early-phase inflammation, by means of short-term anti-inflammatory treatment, one might be able to limit the extent of the secondary damage incurred, so-doing decrease pain from swelling of the injured area [Kruger and Smith, 2012; Myburgh *et al.,* 2012; Smith *et al.,* 2008]. This knowledge provides support for the use of acute anti-inflammatory treatments, usually with NSAID's (refer to section 2.6.5.1). However it is not that simple, since it has long been known that decreasing the macrophage infiltration during the late phase of inflammation, has negative effects on the healing process, including reduced regeneration, satellite cell differentiation and muscle fiber growth [Smith *et al.,* 2008].

In the following sections I will be elaborating on the inflammatory processes following induction of injury and the effects certain factors have on the healing process.

2.6.2 Inflammatory response to injury

As mentioned in the brief background above (section 2.6.1), the response to muscle injury follows a fairly consistent pattern, irrespective of the underlying cause of injury. The inflammatory process is initiated within seconds of tissue injury. As soon as the tissue is damaged, the arterioles within the damaged area dilate, as a result of histamine released from mast cells present in the injured area or via the vascular endothelial growth factor-nitric oxide (VEGF-NO) pathway [Frantz *et al.,* 2005]. This causes vasodilatation and subsequently increases blood flow to the injured site. The damaged muscle releases factors that activate resident inflammatory cells, which in turn provide chemotactic signals to circulating inflammatory cells, signaling them to invade the damaged areas [Robertson *et al.,* 1993]. Endothelial cells play a crucial role in regulating the inflammatory response. During the early steps of immune cell migration, the endothelial cells express adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule-1) to facilitate extravasation of the inflammatory immune cells from the circulation. Mainly IL-1β and TNF-α regulate expression of these adhesion molecules [Gotsch *et al.,* 1994]. In turn, stimulated endothelial cells secrete IL-1α [Kurt-Jones *et al.,* 1987], IL-1β [Warner *et al.,* 1987], IL-6 [Jirik *et al.,* 1989] and IL-8 [Gimbrone *et al.,* 1989]. The localized histamine also increases the permeability of the surrounding capillaries, by increasingly enlarging the endothelial pores of the capillaries. This leads to the finely orchestrated movement of phagocytic leukocytes and plasma proteins into the surrounding tissue [Tidball, 1995; Sherwood, 2007]. In the case of severe tissue injury, platelets become active by adhering to the exposed collagen, forming a platelet plug. In their activated state they release pro-inflammatory mediators such as serotonin (5-HT), histamine and thromboxane A_2 (TxA_2). Following the formation of the platelet plug, the infiltration of white blood cells (predominantly neutrophils) occurs

[Järvinen *et al.,* 2005]. Over the following few days the number of neutrophils will gradually decrease, while macrophage numbers increase [Li *et al.,* 2001b].

For the purpose of this review, specific attention will be paid to the specific roles white blood cells (neutrophils and macrophages) and cytokines have in the inflammatory process after injury.

2.6.2.1 Role of neutrophils in muscle damage and repair

Neutrophil granulocytes are the most abundant type of white blood cells in mammals. They represent 50 to 60% of the total circulating leukocytes and constituting the "first line of defense" against infectious agents. They originate in the bone marrow and migrate into the tissue upon injury. Within 1 hour after injury, neutrophil invasion begins and peaks at ± 24 – 48 hours [Fielding *et al.,* 1993; Tidball *et al.,* 2005]. Their numbers then start to decrease rapidly and they are essentially undetectable by day 3 to 4 post-injury [Tidball and Villalta, 2010]. Chemical intermediates from the injured area, such as complement components (e.g. C5), prostaglandins, leukotrienes and factors released by activated platelets (TxA_2, serotonin and histamine), injured muscle and resident immune cells, attract neutrophils to the injured area [Marder *et al.,* 1985; Sherwood *et al.,* 2007]. Neutrophils infiltrate the damaged tissue by processes of rolling, adhering and migrating through the capillary endothelium (diapedesis) [Menger and Vollmar, 1996]. These circulating neutrophils roll along the endothelium until they are slowed down by the interaction between the heparin sulphate proteoglycans (HSPG's), which are expressed on both neutrophil and endothelium [Djanani *et al.,* 2006]. This process triggers integrin-dependent adhesion of the immune cells to the endothelium [Luo *et al.,* 2007].

The question whether the infiltration of neutrophils is a friend or foe for muscle recovery after injury, has been researched extensively. There are studies showing that complete inhibition of neutrophil infiltration is detrimental to muscle recovery, as neutrophils are important for the removal of tissue debris and activation of satellite cells [Tiidus, 1998], but also that neutrophils contribute to secondary damage, by releasing ROS [Tidball and Villalta, 2010]. On the one side, the oxidants produced by the neutrophils are crucial in the process of damaged tissue clearance, as it allows phagocytosis of debris by neutrophils or macrophages. It has been found that muscle regeneration tends to be slower in older animals and animals with slower rates of phagocytic removal, which coincides with slowed phagocytosis by inflammatory cells [Grounds, 1987; Zacks and Sheff, 1982]. This type of research supports the expectation that phagocytosis is a necessary feature of muscle repair. However, these observations do not distinguish the contribution of neutrophils from other phagocytes, such as macrophages, that are also present. The more recent study by Hofling *et al.* (2003), reported slower muscle regeneration after injury by snake toxin, if neutrophils and monocytes were first depleted from the animals. These animals depleted of neutrophils and monocytes also showed more tissue debris in injured muscles, which suggested the possibility that the impaired capacity to remove tissue debris by phagocytes could slow the regenerative process. In addition, the pro-inflammatory cytokines (especially IL-1 and TNF-α) released by the neutrophils are required during the adhesion process, which assists with the influx of neutrophils and macrophages at the site of injury [Cannon and St. Pierre, 1998 Dubravec *et al.*, 1990; Rosenberg and Gallin 1993; Tidball, 2005].

Conversely, pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) stimulate neutrophils to generate superoxides via a “respiratory burst”, which is catalyzed by the enzyme NADPH oxidase (located in the plasma membrane of neutrophils). In activated neutrophils, NADPH shuttles electrons from cytosolic NADPH to dissolved oxygen in the extracellular fluid, forming superoxide (O_2^-) [Tiidus, 1998]. Superoxide can be converted to H_2O_2, which in-turn reacts with superoxide in the presence of a transition metal, which results in the formation of the highly reactive hydroxyl radical (OH^-) [Tiidus, 1998]. Studies which are perhaps the most compelling in arguing that neutrophils are involved in the skeletal muscle damage, are two studies conducted by Nguyen and Tidball (2003a; 2003b). In one study, in which they utilized the hindlimb suspension model, they found that mice deficient in NADPH oxidase (null mutation of gp91^{phox}) demonstrated a significant reduction in muscle fiber damage during reloading without changes in the concentrations of neutrophils and macrophages of the reloaded muscles. Gp91^{phox} is the peptide subunit of NADPH oxidase [Nguyen and Tidball, 2003b]. In their second study, they demonstrate the importance of muscle-derived nitric oxide (NO) in inflammation and muscle damage. In this *in vitro* study, they demonstrated that muscle-derived NO reduces neutrophil-mediated lysis of muscle cells and decreases superoxide concentration [Nguyen and Tidball, 2003a]. This protective effect could occur as a result of NO scavenging superoxide and so-doing prevent its conversion to a more cytotoxic oxidant [Rubanyi *et al.,* 1991] or by inhibiting the activity of NADPH oxidase and reduce superoxide production [Clancy *et al.*, 1992]. In addition, muscle-derived NO may also protect muscle from damage by inflammatory cells by inhibiting the expression of adhesion molecules that are necessary for leukocyte interactions with the vascular endothelium [Niu *et al.,* 1994; Almekinders and Gilbert, 1986]. In addition to the superoxides generated by neutrophils, they also generate hypochlorous acid, via myeloperoxidase (MPO), a peroxidase enzyme, which is most abundantly expressed in neutrophils. Processes such as injury or exercise typically produce an increase in the activity of MPO in the muscle and is therefore a good indication of neutrophil invasion. It is thought that MPO can generate hypochlorous acid, a highly reactive oxidizing agent [Winterbourn, 1986]. Thus injury leads to increased neutrophil representation, leading to increased MPO levels and therefore increased cytolytic capacity.

By examining the literature it seems plausible that if one can identify and inhibit the molecule(s) responsible for promoting neutrophil migration into damaged skeletal muscle, it may be possible to develop treatment options to alleviate neutrophil-mediated muscle injury. An example is methylprednisolone, which have been found to decrease the expression levels of adhesion molecules and integrins on the leukocyte surface, consequently resulting in less neutrophils infiltrating the injured muscle area [Droogan *et al.,* 1998]. However, with this in mind, the neutrophil response should also not be completely inhibited, given its role in satellite cell processes, which is crucial in muscle structure restoration. Therefore it is necessary that treatment be developed that will counter the negative effects that neutrophils elicit but that do not hinder the repair process.

The solution is that neutrophil action should be allowed, but limited in magnitude and duration.

2.6.2.2 Role of macrophages in muscle damage and repair

Monocytes, which are the late precursor form of macrophages, originate from the bone marrow hematopoietic stem cell precursors. During a local insult, monocytes are recruited to the site of injury by migrating into the damaged areas, in a somewhat similar fashion to neutrophils, where they settle and differentiate into tissue macrophages [Tidball, 2005]. Apart from macrophages recruited from circulation, the macrophage population at the site of injury is further increased by cytokine-mediated migration of the resident macrophage population from surrounding tissue [Geissmann *et al.*, 2010; Pillon *et al.,* 2013]. The macrophage phase in inflammation follows the neutrophil phase and it was previously thought that the neutrophils were responsible for the macrophage chemotaxis. However, it has since been found not to be the case as macrophages can accumulate in muscles that are depleted of neutrophils, as validated by Pizza *et al.* (2005), in which they demonstrated this in an injury model induced by lengthening contraction. The neutrophil and macrophage phases overlap and cell numbers are inversely related, with significant numbers of macrophages appearing after day 2, when neutrophil numbers start to decline [Tidball and Wehling-Henricks, 2006]. In conjunction with neutrophils, macrophages initially contribute to phagocytosis of the necrotic material. They also have other functions, including antigen-presentation and production of reactive nitrogen species (RNS) and ROS as well as cytokines and growth factors involved in chemotaxis [Pillon *et al.,* 2013; Tidball, 2005].

There are two waves of monocyte/macrophage infiltration. The first wave, constituting the macrophages recruited initially by the damaged muscle, are of phagocytotic, pro-inflammatory phenotype, whereas the second wave is of anti-inflammatory phenotype, releasing growth factors to support muscle repair and regeneration [Tidball and Villalta, 2010; Tidball, 2005]. In the review article by Kharraz *et al.* (2013) they describe how macrophages are sub-divided into different populations depending on their location and the way they are activated. These populations appear at different time-points after injury, suggesting the multi-functionality of macrophages. The macrophage populations can either have a M1 (classical activation) or M2 (alternative activation) phenotype, as they are either geared towards pro-inflammatory processes or anti-inflammatory processes [Arnold *et al.*, 2007]. Similar to neutrophil accumulation [Tidball, 2005], the accumulation of type M1 macrophages exacerbates injury, while type M2 (in particular M2c) macrophages are associated with tissue repair [Arnold *et al.*, 2007]. M1 macrophages are recruited to the site of injury by increased expression of interferon gamma (IFN) and TNF-α, where they play a pro-inflammatory role [Mantovani *et al.,* 2004; Sica and Mantovani, 2012]. These macrophages are usually observed during the earlier stages of muscle injury, where they are thought to be involved in phagocytosis [McLennan 1996] and presenting of antigens. In addition to releasing pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12 and IL-23 [Mantovani *et al.,* 2004], M1 macrophages also promote muscle damage both *in vitro* and *in vivo* via the production of cytotoxic levels of NO generated by iNOS [Sica and Mantovani, 2012; Villalta *et al.,* 2009; Nguyen and Tidball, 2003a]. The study by Nguyen and Tidball (2003a) reported that macrophages lysed muscle cells by a NO-dependent, superoxide-independent pathway and that this cytolytic ability is propagated by the presence of neutrophils. This same muscle membrane lysis capacity was observed in an *in vivo* study with *mdx* mice [Petrof *et al.*, 1993]. The *mdx* mouse model of muscular dystrophy, consist of mice that are null mutants for the membrane-associated protein, dystrophin. In this study they found that these muscle cells were more susceptible to cellular damage during contraction, which led to inflammation and muscle damage [Petrof *et al.*, 1993]. However, when the preparation was depleted of macrophages, muscle membrane lysis was decreased by 80% [Wehling *et al.,* 2001].

As the process of muscle regeneration advances, M1 macrophages reach their peak concentration in injured and regenerating muscle. When this occurs, the phenotype of M1 macrophage is switched to M2 macrophage (alternatively activated) phenotype, in order to resolve the inflammatory process. This claim is substantiated by *in vivo* trace studies [Arnold *et al.*, 2004]. The M2 macrophage group is more complex as it has three known subtypes, namely M2a, M2b and M2c, each with its own physiological role. M2 macrophages are generally activated by T helper 2 (T_H2) cytokines, namely IL-4, IL-10 and IL-13 [Gordon, 2003].

Numerous studies have reported that macrophages are associated with skeletal muscle regeneration [Bosurgi *et al.*, 2012; McLennan, 1996; St Pierre and Tidball, 1994] and that they actively participate in the muscle repair process [Tidball and Wehling-Henricks, 2006; Arnold *et al.,* 2007; Shireman *et al.,* 2006] and not just in merely removing tissue debris. In these studies the authors have observed a delayed appearance in regenerating fibers, if the number of monocytes/macrophages entering the injured area were reduced [Tidball and Wehling-Henricks, 2006; Arnold *et al.,* 2007; Shireman *et al.,* 2006]. Furthermore, they found that when monocyte/macrophages were totally abolished during the first 24 hours after injury, by diphtheria toxin injection, total prevention of the muscle repair process occurred [Arnold *et al.,* 2007]. Researchers have also found that regeneration by myogenic cells are impaired if monocytes and macrophages are depleted [Lescaudron *et al.,* 1999]. Additionally, the study by Lesault *et al.* (2012) reported that macrophages promoted survival and proliferation of the myogenic precursor cells that were introduced into *mdx* skeletal muscle. These studies emphasize that macrophage infiltration, during inflammation, is beneficial for muscle regeneration in the context of inflammation. The study by Massimino *et al.*, 1997, demonstrates that M2 macrophages played a vital role in the activation of satellite cells and myoblast proliferation, thereby contributing to skeletal muscle regeneration and tissue repair.

In summary, M1 macrophages play a predominant role in removing debris but also contributing to further muscle injury, whereas M2 macrophages aid muscle recovery. Figure 2.7 demonstrates the inter-play between neutrophils and macrophages.

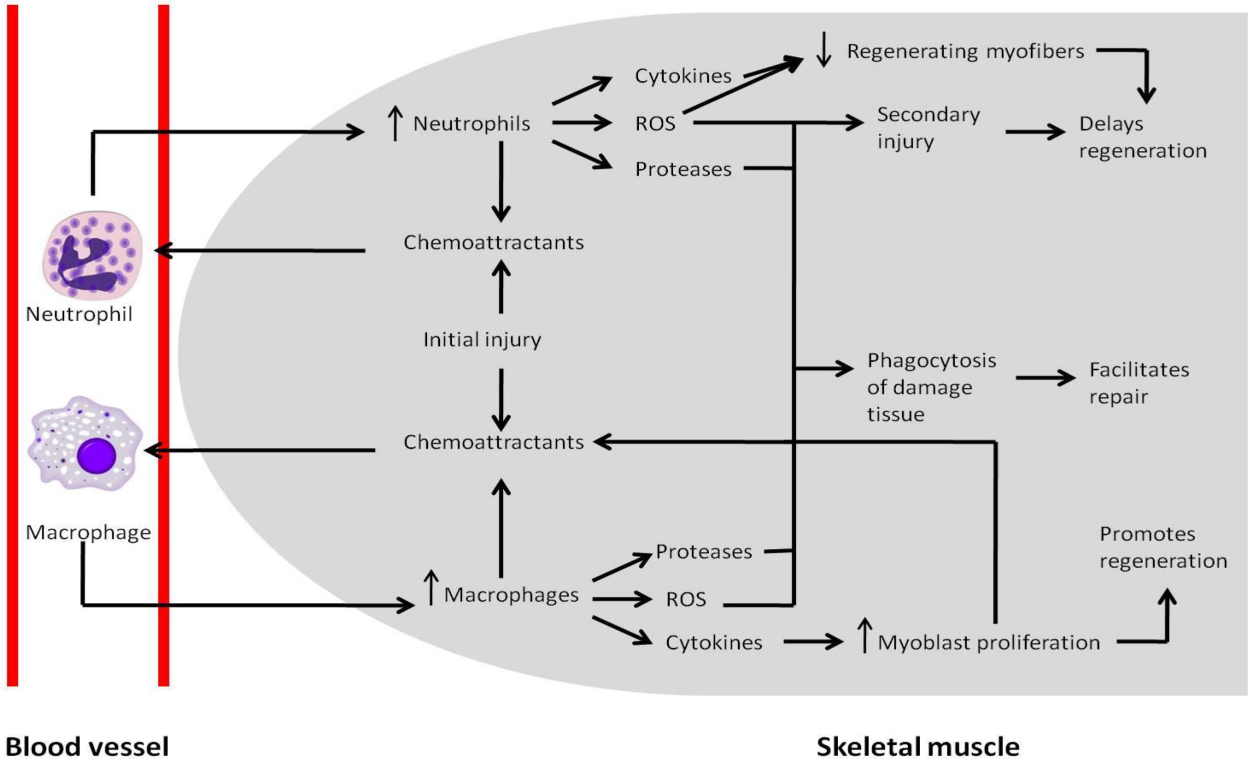


Figure 2.7: Neutrophil and macrophage accumulation and their contribution to the events of muscle injury and repair.

2.6.2.3 Role of cytokines in damage and repair

Muscle-derived cytokines, which are both pro- and anti-inflammatory regulators, are released after injury and they can either be beneficial to the repair process or hinder this process. It is therefore crucial that there is a time-balance between the secretion of pro-inflammatory and anti-inflammatory cytokines to prevent a prolonged inflammatory response, thereby preventing the development of fibrosis and promoting muscle regeneration.

Cytokines are regulatory proteins that are secreted mainly by cells of the immune system. Neutrophils, macrophages, fibroblasts, endothelial cells and even the damaged muscle cells themselves, all secrete cytokines [Cannon and St Pierre, 1998; Smith *et al.,* 2008]. They generally function as intercellular messenger molecules that exert their effect by binding to specific receptors on the responsive target cell. This happens either in an autocrine mode, by binding to the cell of their origin, or in a paracrine mode, by binding to a neighboring target cell expressing the appropriate receptor [Philippou *et al.,* 2012]. Therefore, in addition to the local muscle inflammation, there is also a systemic response, known as the acute phase response [Philippou *et al.,* 2012]. Circulating cytokines can therefore exert their effects at locations distant from the initial site of injury [Philippou *et al.,* 2012]. This makes it very difficult to study the exact contribution that each cytokine makes, since they are secreted by various cell types and they have interrelated functions. However, it is clear that cytokines play an essential role in muscle damage and repair. To date, there are more than 150 structurally distinct cytokines identified [Figarella-Branger *et al.,* 2003], which can be grouped into either pro-inflammatory or anti-inflammatory cytokines, based on their predominant action. Due to the vast amount of cytokines, only the main cytokines important in muscle regeneration will be addressed in the next few paragraphs.

After muscle injury, neutrophils, macrophages and damaged muscle cells secrete TNF-α [Tidball and Villalta, 2010; Warren *et al.,* 2002; Nguyen and Tidball, 2003a]. The production of TNF-α is generally controlled by cytokines such as IL-2, interferon (IFN)-γ, TNF-β and IL-6 [Figarella-Branger *et al.,* 2003]. TNF-α is seen as a pleiotropic cytokine, as it mediates inflammatory and apoptotic responses as well as modulating growth and differentiation of many cell types. TNF-α mediates inflammation, by its involvement in chemotaxis of leukocytes, the expression of adhesion molecules and the regulation and secretion of other pro-inflammatory cytokines [Tidball and Villalta, 2010; Warren *et al.*, 2002]. According to the literature, TNF-α participates in muscle protein loss during the degenerative phase of muscle regeneration, thought to be via activating NF-κB in skeletal muscle cells. If NF-κB, which is a transcription factor, is activated, it alters gene expression and causes proteolysis. Both *in vitro* and *in vivo* studies indicate that TNF-α promotes the expression of atrogin-1, which leads to the catabolism of muscle proteins. It is proposed that this occurs through the activation of the ubiquitin/proteasome pathway in muscle fibers, thought to be mediated via the p38 MAPK signaling pathway [Li, 2003]. Equally important to the role of TNF-α during the degenerative phase is its role during the regeneration phase. There is evidence that the regeneration and functional recovery of the damaged skeletal muscle are affected by TNF-α, since inhibiting its activity during the healing process results in defective muscle strength [Tidball and Villalta, 2005]. TNF-α is thought to have a double role during muscle regeneration. It activates satellite cells to enter the cell cycle and it induces satellite cell migration and proliferation [Tidball, 2010; Li, 2003]. Overall, it seems that during the early inflammatory response, the activation of M1 macrophages can promote their actions to lyse muscle cells, while during later stages post-damage, TNF-α can influence the regeneration process [Tidball and Villalta, 2010; Warren *et al.,* 2002].

IL-6 is another pleiotropic cytokine, as it has both pro-inflammatory as well as (indirect) anti-inflammatory properties. It is mainly secreted by skeletal muscle fibers, fibroblasts, endothelial cells, keratinocytes and peripheral blood mononuclear cells (PBMCs), specifically known as T_H2 cells and macrophages [Biffl *et al.,* 1996]. Its main immune-related function is to increase lymphocyte proliferation and differentiation and to activate the release of other pro-inflammatory cytokines [Heinrich *et al.,* 1990]. In addition, its anti-inflammatory effects include stimulating cortisol release, inducing hepatic synthesis of antioxidants and protease inhibitors [Steensberg *et al.,* 2003].

IL-10 is an anti-inflammatory cytokine and a key regulator of immune responses. It is produced primarily by monocytes, but lymphocytes, T_H2, B- cells and mast cells also produce it, however to a much lesser extent [Asadullah *et al.,* 2003]. Previous studies have shown that IL-10 can directly inhibit pro-inflammatory cytokine (IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-8, IL-12, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF)) production by T_H1 and T_H2 cells by acting on the antigen-presenting cells [De Waal Malefyt *et al.,*1991; Fiorentino *et al.,* 1991; Giannoudis *et al.,* 2000]. In addition, IL-10 has also been found to activate and attract M2 macrophages to the injured area, which aids muscle regeneration by facilitating the synthesis of more anti-inflammatory cytokines from the macrophages [De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991; Giannoudis *et al.,* 2000].

To summarize, the outcome of the muscle repair process depends greatly on the balance between the pro-inflammatory cytokines and the anti-inflammatory cytokines. By limiting the inflammatory response one can theoretically limit muscle degeneration and scar formation; however inflammation is a key response to muscle injury and is essential for muscle regeneration [Ostrowski *et al.,* 1999].

2.6.3 Other factors involved in muscle repair

2.6.3.1 Satellite cells

Satellite cells acquired their name from their location, which is on the periphery of adult muscle myofibers, beneath the basal lamina and outside the myofiber plasma membrane. They were first described in 1961 by Alexander Mauro who observed these mononucleated cells by electron microscopy [Mauro, 1961]. Satellite cells of adult skeletal muscle, express amongst others, Pax7, M-cadherin, c-Met and CD56 [Yin *et al.,* 2013; Seale *et al.*, 2000]. Even though all of these markers can be used to identify satellite cells, satellite cells do not represent a unique cell type, but a rather heterogeneous population of muscle precursor cells in various stages of activation, proliferation and differentiation. For example, the satellite cell markers Pax7, CD56 and CD34 are expressed at various stages during injury, but the expression pattern differs, with expression being higher during quiescence and significantly decreasing during activation and proliferation.

Under normal conditions, these cells are mitotically quiescent cells; however, after injury they are activated, after which they proliferate and differentiate, to give rise to myoblasts. In short, during the process of myogenic differentiation, proliferating myoblasts pause in the G_1 phase and withdraw from the cell cycle and then start to differentiate and fuse into multinucleated myotubes. These myotubes begin to produce muscle specific proteins and finally fuse with damaged fibers, where they mature into muscle fibers with peripherally located nuclei, or re-enter G_0, remain undifferentiated and replenish the satellite cell "stores" [Arnold and Winter, 2007; Bornemann *et al.,* 2000; Galliano *et al.,* 2000; Perry and Rudnicki, 2000; Yagami-Hiromasa *et al.,* 1995]. The evidence supporting the importance of satellite cells during muscle regeneration comes from studies where the total pool of satellite cells was abolished [Lepper *et al.,* 2011; Sambasivan *et al.,* 2011]. In these studies the injured muscles were unable to regenerate after all $Pax7^{+}$ (biomarker for satellite cells) cells were eliminated [Lepper *et al.,* 2011; Sambasivan *et al.,* 2011]. Cardiotoxin injected into the hindlimb of animals are frequently used as a reproducible model of severe muscle injury [Doroshow *et al.,* 1985; Nicolas *et al.,* 1996; Garry *et al.,* 2000]. In the study by Garry *et al.* (2000), they found that the intramuscular injection of 100 µl of 10 µM cardiotoxin into the gastrocnemius muscle of a mouse resulted in 80–90% muscle degeneration, followed by the activation of satellite cells within 6 hr after injury.

Satellite cell activation and differentiation is regulated by muscle-specific transcription factors called myogenic regulatory factors (MRF's). During the repair phase, quiescent $Pax7^{+}$ satellite cells migrate to the site of injury, up-regulate the MRFs, MyoD and Myf5 and start to proliferate [Smith *et al.*, 1994; Beauchamp *et al.,* 2000] and differentiate. The differentiation phase is marked by the down-regulation of Pax7 [Zammit *et al.,* 2004] and up-regulation of Mrf4 and myogenin [Smith *et al.,* 1994; Cornelison and Wold, 1997].

2.6.3.2 Growth factors

Growth factors are very important in the regulation of satellite cells. They are predominantly secreted by active immune cells post-injury. However, satellite cells themselves as well as the ECM, also contain growth factors that become active after muscle injury [Hawke and Garry, 2001]. The combination of these growth factors is responsible to attract, activate and induce differentiation of the satellite cells [Huard *et al.,* 2002].

Some of these **stimulatory growth factors**, such as fibroblast growth factor (FGF)-2 and -6, insulin-like growth factor (IGF)-1 and -2, hepatocyte growth factor (HGF), VEGF, platelet-derived growth factor (PDGF)-AA and –BB and stromal derived factor (SDF)-1 play a prominent role during myogenic proliferation and differentiation [Ten Broek *et al.*, 2010; Charge´ and Rudnicki, 2004; Hawke and Garry, 2001]. IGF-1 is particularly important as it has been found to be crucial for skeletal muscle growth [Menetrey *et al.,* 2000]. In *in vitro* studies, it has been found that both IGF-1 and later IGF-2 are able to alter the expression of myogenic regulatory factors and promote proliferation and differentiation in myoblasts [Charge´ and Rudnicki, 2004]. By over-expressing IGF-1 in transgenic mice, hypertrophy occurred [Adams and McCue, 1998] and direct injection of IGF-1 lead to improved muscle regeneration [Menetrey *et al.,* 2000], confirming the function of IGF-1. The assumption is that IGF-1 exerts its effect via the PI3K pathway and subsequent anti-apoptotic PKB/Akt activation [Watt and Hogan, 2000]. Another growth factor of importance is HGF [Huard *et al.,* 2002], which is bound to the ECM in muscle tissue and is released in response to injury. It is thought to induce proliferation of satellite cells by binding to c-met [Allen *et al.*, 1995]. HGF seems to be important during the early phase of muscle regeneration as the expression of HGF is high during the early phases [Tatsumi *et al.,* 2001; Suzuki *et al.,* 2002], however when HGF is introduced at a later stage during regeneration it does not promote skeletal muscle repair [Tatsumi *et al.*, 1998]. HGF has pleiotropic effects as it can play a role in satellite cell migration to the site of injury (stimulatory effect) [Suzuki *et al.,* 2000] as well as inhibit the formation of multinuclear myotubes (inhibitory effect) [Hayashi *et al.,* 2004]. In addition to the above-mentioned functions, HGF also seems to be secreted by muscle when it is stretched, through a NO-dependent manner [Tatsumi *et al.*, 2006], which might point to its possible role in satellite cell activation. As previously mentioned, there are numerous growth factors that have been found to play some role in the muscle repair process. For example, VEGF and PDGF have been found to be involved in satellite cell regulation. FGF-6 belongs to a family of cytokines that control cell proliferation, cell differentiation and morphogenic events and it has been found to be up-regulated during muscle regeneration [deLapeyriere *et al.,* 1993], however the specific role is still unknown. SDF-1 has been found to function as a chemoattractant [Ratajczak *et al.,* 2003]. However, by reviewing the literature, it seems as if IGF-1 is the main growth factor in the context of muscle regeneration.

The main **inhibitory growth factors** in skeletal muscle repair are myostatin, transforming growth factor (TGF)-α and -β1 and bone morphogenetic proteins (BMP) [Ten Broek *et al.*, 2010].

Myostatin forms part of the TGF-β superfamily where it plays an important role in regulating skeletal muscle growth. It is expressed in satellite cells as well as myoblasts, where it down-regulates Pax3 and Myf5 and prevents the expression of MyoD [Ten Broek *et al.*, 2010; Amthor *et al.*, 2002]. This function is adequately depicted in research done on knock-out mice. When myostatin lacks, these mice have extensive muscle hypertrophy [Shi and Garry, 2006]. Myostatin represses satellite cell self-renewal by inhibiting the cell-cycle [Shi and Garry, 2006]. Alongside TGF-β1, myostatin also reduces myoblast recruitment and differentiation [Massagua *et al.,* 1986; Ten Broek *et al.*, 2010]. TGF-β1 belongs to a small family of multifunctional growth factors, consisting of TGF-β1, β2 and β3 and it is responsible for inducing remodeling and repair of the ECM by stimulating fibroblasts. This process results in the production of collagen and fibronectin [Massagua *et al.,* 1986] resulting in the formation of scar tissue. It has been shown that decorin (TGF-β1 inhibitor) can prevent fibrosis and so-doing, enhance muscle regeneration [Sato *et al.,* 2003]. In conclusion, numerous other growth factors might be involved in skeletal muscle regeneration; however more research is needed to define the mechanism of action. TGF-β1 has the most obvious effect on proliferation and differentiation of satellite cells, which points to it being the major inhibitor of skeletal muscle regeneration.

2.6.3.3 Markers of regeneration

$ADAM_{12}$

Myogenic precursor cells express a number of proteins, which could be used to identify them. Several factors have been found to be involved in the control of the regenerative processes and the protein $ADAM_{12}$, belonging to the transmembrane metalloprotease ADAM (a disintegrin and metalloprotease) family, has been implicated in myogenesis and skeletal muscle repair [Engvall and Wewer, 2003; Kurisaki *et al.,* 2003; Moghadaszadeh *et al.,* 2003; Gilpin *et al.,* 1998], especially during muscle cell differentiation and fusion [Galliano *et al.,* 2000]. Galliano *et al.* (2000) conducted a study on C2C12 cells, in which they determined the expression levels of $ADAM_{12}$ *in vivo.* In this study they demonstrated that $ADAM_{12}$ is expressed at low levels in undifferentiated myoblasts and is dramatically up-regulated at the onset of differentiation when myoblasts fuse into multinucleated myotubes, i.e. during regeneration. Consequently, in skeletal muscle, $ADAM_{12}$ is expressed in the developing myofibers during the embryonic stage and during the early postnatal period [Borneman *et al.,* 2002; Kronqvist *et al.*, 2002]. In adult skeletal muscle, the expression level of $ADAM_{12}$ is very low in both differentiated muscle fibers and quiescent satellite cells [Bornemann *et al.,* 2000; Gilpin *et al.,* 1998; Yagami-Hiromasa *et al.,* 1995]. During regeneration, the amount of $ADAM_{12}$ protein increases dramatically [Galliano *et al.* (2000)], and its mRNA is readily detected in satellite cells following their activation [Bornemann *et al.,* 2000]. In spite of recent studies aimed at the biochemical characterization of $ADAM_{12}$, its role in development and/or regeneration of skeletal muscle is still unclear.

Desmin

Another protein implicated in tissue repair and regeneration is desmin [Paulin and Li, 2004]. Desmin (53-kDa cytoskeletal class III) is one of several intermediate filament proteins, including vimentin, nestin, lamins and cytokeratins [Paulin and Li, 2004], which together form an intracellular network that provides a three-dimensional scaffold in regenerating cells [Chourbagi *et al.,* 2011]. It is found mainly in the Z-disk of striated muscles and in the dense bodies of smooth muscle cells [Paulin and Li, 2004] and it is one of the earliest muscle-specific proteins to appear during myogenesis. The expression levels of desmin are low in proliferating myoblasts, but increase in differentiated myotubes [Paulin and Li, 2004]. Unlike many other myogenic markers, which are only expressed during myogenesis, desmin continues to be expressed in normal adult skeletal muscle. Inhibition of desmin mRNA in C2C12 cells, resulted in the complete inhibition of differentiation and fusion of the myoblasts, indicating its role in the formation of myofibers [Li *et al.,* 1994]. Conversely, desmin knockout mice showed irregular organization of myofibers with misaligned myofibrils, Z-disk and mitochondrial degeneration and disorganization. As a result of lack of anchorage of myofibrils to the sarcolemma, transmission of muscle force was impaired, which resulted in mice visibly lacking strength and fatigued significantly sooner compared to their controls [Paulin and Li, 2004].

After muscle damage, such as after myotoxin injury, desmin expression is rapidly lost, thought to be due to membrane disruption [Vater *et al.,* 1992]. In the study by Vater *et al.,* 1992 it was demonstrated that myotoxin injury in rats resulted in the total loss of desmin expression, validated by Western blot analysis, confirming that proteolysis of the intermediate filament network occurs in severe muscle damage. In addition, they observed that regenerating myotubes displayed intense desmin expression two days after toxin injection [Vater *et al.,* 1992]. Similarly Creuzet *et al.* 1998 found that desmin expression disappeared in necrotic mouse myofibers after freeze lesions to the *pectoralis major* muscle, but satellite cells and newly formed myofibers in the injured area showed increased staining intensity two to four days after injury.

Since desmin is expressed during skeletal muscle development, i.e., myotube formation, it is widely used as marker for distinguishing individual cell types within a tissue, such as myoblasts from fibroblasts in the regenerating and central zone of muscle injury [Stratos *et al.,* 2007].

2.6.4 Models used to study muscle injury and their limitations

It is very difficult to investigate muscle injury due to numerous technical issues. These issues include inter-individual variation in severity of injury if human subjects are used, since your sample will be consisting of individuals that have previously been injured. It is for this reason that animal models are the better option for studying muscle injury, especially when investigating a potential remedy. The other confounding issue is the invasiveness of some injury models. However, the use of animal models also has its limitations and it therefore depends on the end-point of your research as to which model will suit the best. In the next section I will be discussing the different animal models used for investigating muscle injury, highlighting the benefits and limitations of these methods.

2.6.4.1 Myotoxins

If the end-point of the experiment is to examine regeneration of damaged muscle, the use of myotoxins such as bupivacaine (marcaine), cardiotoxins (CTX) and notexin (NTX) is perhaps the easiest and most reproducible way to damage muscle [D'Albis *et al.,* 1988; Hall-Craggs, 1974; Harris and Johnson, 1978]. These toxins exert their effects on different pathways, for example, NTX and CTX are both peptides isolated from snake venom, where NTX exerts its effects by inhibiting neuromuscular transmission by blocking acetylcholine release and CTX exerting its effects by inducing the depolarization and contraction of muscular cells, disrupting the membrane organization and lysing various cell types. Injecting these myotoxins seems to generate highly reproducible muscle regeneration and may serve to elucidate molecular pathways or mechanisms. However, since it is likely to exert its toxic effects also on other cell types less prone to direct damage during physiological or accidental muscle damage, such as satellite cells, this model is probably not physiologically realistic.

2.6.4.2 Crush- and freeze-injury model

An alternative method to myotoxin injection is by direct infliction of a wound by crushing or freezing the muscle. Crush- and freeze-injury are invasive methods of examining both muscle regeneration and contusion injury. Before injuring, the muscle is first exposed surgically. The muscle is then bruised by manually pinching the exposed muscle with a pair of forceps or by dropping a weight on the forceps or by freezing the muscle in the case of the freeze-injury method. Manual injury is difficult to deliver in a standardized manner, however this technique has been used with success to investigate secondary damage [Merrick *et al.,* 1999] and processes of muscle regeneration [Vignaud *et al.,* 2005; Squarzoni *et al.,* 2005] after injury. Given the invasiveness of the technique, it is of course unsuitable for investigations focused on the inflammatory response to injury. Often, these injuries are also of extreme severity, again limiting extrapolation of results to physiological conditions.

Another variation of this model is a no impact version. This is where a heavy weight is placed on the muscle of the animal (not dropping the weight on the muscle) for a prolonged period of time [Akimau *et al.,* 2005]. This version leads to more severe muscle damage, since the weight is placed on the muscle for long periods, therefore leading to long-term occlusion of the blood vessels. This type of injury model simulates where an individual is e.g. in an accident and trapped for a period of time. It is deemed not a good model to examine muscle regeneration and inflammation.

2.6.4.3 Drop-mass model

The drop-mass method is a proven method of inducing mechanical injury in skeletal muscle of rodents [Kruger and Smith, 2012; Minamoto *et al.,* 1999; Beiner *et al.,* 1999]. The most commonly described model in the literature involves the dropping of a solid weight with a flat impact surface, varying in diameter and mass, from various heights onto the hind-limb of an anaesthetized animal. The mass is dropped on a surgically exposed muscle or on a non-exposed muscle. This model was first described by Kvist and colleagues (1974) and later elaborated by Stratton *et al.* (1984) (Fig. 2.8).

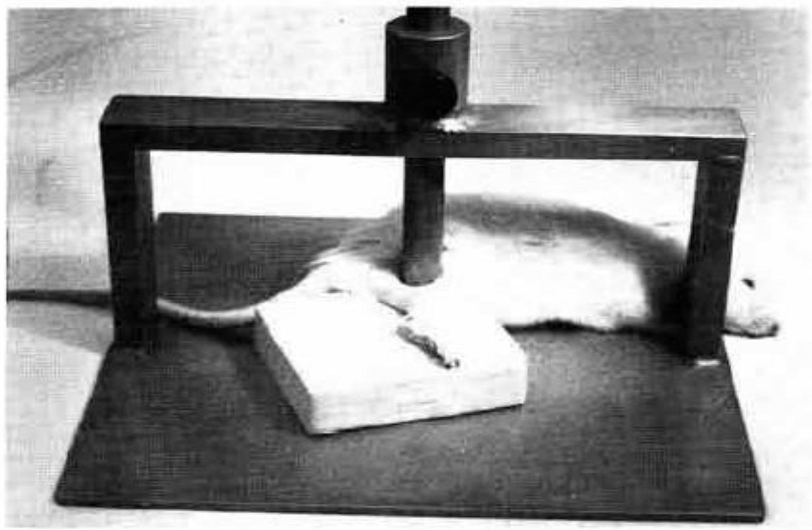


Figure 2.8: The drop-mass model as described by Stratton *et al.*, 1984

There are numerous variations of the drop-mass model described in studies using rats and mice found in the literature. Two variations previously used in rats to induce contusion injury are using either relatively heavy weights, dropped from a relatively short distance (640 and 700 g from 27 and 25 cm, respectively) [Stratton *et al.,* 1984; Fischer *et al.,* 1990] or a smaller weight, dropped from a larger distance (171 g from 102 cm) [Crisco *et al.,* 1994;

Markert *et al.,* 2005; Wilkin *et al.,* 2004]. Along with the weight of the mass, variations in the shape of the mass is also found. When the weight is flat the impact area is uniform in severity, however when the weight is spherical the center part of the impact area is more severe compared to the periphery [Beiner *et al.,* 1999; Wright-Carpenter *et al.,* 2004].

The drop-mass model has been used to examine outcome measures such as inflammation as a result of injury and both localized and systemic inflammation has been previously observed [Bunn *et al.,* 2004]. However, a limitation of the invasive version of this model is that if, your aim is to examine the inflammatory process, you will have to control for the effects that the surgery itself will have, since it has been found that by surgically exposing the muscle for the invasive injury, cytokines are released. It is known that TNF-α and IL-6 are released within the first few hours after injury [Nossuli *et al.,* 2000]. This time-frame of cytokine release is similar to that of cytokine release after injury and results would therefore not be a true reflection of the inflammatory response observed during injury. Therefore, if you wish to study inflammation, it would be advisable to use a non-invasive version of the drop-mass model.

The Department of Physiology at the Stellenbosch University has standardized a moderately severe, non-invasive drop-mass injury model, similar to that of Stratton's, in which contusion injury to the non-exposed gastrocnemius muscle of rats can be delivered by dropping a mass of 200 g from a height of 50 cm through a plastic tube fastened perpendicularly and directly above the muscle impact zone. This model produced reproducible contusions that were moderately severe and took about 14 days for full recovery [Kruger and Smith, 2012]. A further benefit of this model is that the blunt area of the mass is small enough so that the contusion may be delivered to the muscle without the risk of breaking any bones. This also adds to the ethical soundness of the technique, which results in an injury sufficient for studying recovery, whilst not severe enough to cause significant discomfort or loss of function (no limping).

2.6.5 Treatment modalities for skeletal muscle injury

Biological approaches to increase muscle regeneration and prevent the formation of scar tissue are currently being investigated in an effort to improve the muscle healing process after injury. The primary goals of these treatments are to minimize secondary damage, relieve pain, reduce bleeding and promote healing. The main treatment options to achieve these goals rely mainly on compression therapy, which limits bleeding, elevating the injured limb, local cooling and NSAID's [Järvinen *et al.*, 2005]. Alternative therapies include the use of antioxidants and natural anti-inflammatory agents. More recently, administration of growth promoting agents has received attention.

2.6.5.1 Non-steroidal anti-inflammatory drugs (NSAID)

NSAID's are frequently used by athletes after a soft-tissue injury, such as a contusion injury [Buckwalter, 1995; McCarberg and Argoff, 2010]. This class of drugs provides analgesic, fever-reducing and anti-inflammatory effects [Almekinders and Gilbert, 1986; Chen and Dragoo, 2013]. The ability of NSAID's to reduce inflammation and pain after injury is believed to be based on its ability to inhibit prostaglandin synthesis, from arachidonic acid, by COX [Bondesen *et al.,* 2004]. By inhibiting prostaglandin, the cascading inflammatory response will be decreased [Paoloni *et al.,* 2009]. COX is present in 3 isoforms, namely COX-1, COX-2 and COX-3. COX-1 and COX-2 are expressed in skeletal muscle [Weinheimer *et al.,* 2007] and will therefore be the only two isoforms discussed further. COX-1, which is constitutively expressed, has primarily homeostatic functions, but also appear to be involved in acute inflammation [Langenbach *et al.,* 1999]. COX-2 is normally present in low levels and is considered to be pro-inflammatory. It is induced by inflammatory mediators and cytokines to up-regulate the inflammatory process [Bondesen *et al.,* 2004]. The NSAID's that inhibit the COX-2 enzyme have been reported to have fewer gastrointestinal side-effects and renal side-effects but an increased risk of cardiovascular side-effects [McCarberg and Argoff, 2010; Paoloni *et al.,* 2009]. Similar to many of the treatment options available to treat muscle injury, the use of NSAID's are rather contradictory. Studies aimed at determining their ability to stunt the inflammatory response to injury and facilitating faster recovery are conflicting, since on one hand, NSAIDs may decrease the inflammatory response, decreasing pain and swelling [Almekinders, 1999]; on the other hand, it has been shown that the inflammatory response is a crucial phase during tissue healing [Järvinen *et al.,* 1992]. Therefore complete inhibition of this phase can lead to poor healing [Beiner *et al.,* 1999]. The intensity of the inflammatory response elicited by a particular insult differs as the severity of the insult differs. Therefore the response to a particular dose of NSAID would differ too, as it might alleviate, potentiate or have no effect at all on the symptoms associated with the injury. In addition, the type of NSAID used, the mode of administration (orally, intramuscular etc.), the duration of the treatment, as well as the onset of first dosage all seem to affect the outcome of the regeneration process [Smith *et al.,* 2008], as will be discussed in the section below.

Previous studies have reported that NSAID therapy delayed muscle regeneration [Almekinders and Gilbert, 1986]. For skeletal muscle to successfully regenerate after injury, dormant satellite cells are required to be activated, proliferate and fuse to damaged muscle. Therefore one of the concerns regarding NSAID usage in muscle injuries is the potential effect on satellite cells. In an early *in vitro* study by Santini *et al.* 1988, they found that differentiation of myoblasts into myotubes was impaired when myoblasts were kept in the presence of the non-specific COX-inhibitor indomethacin, which is a commonly used NSAID. This evidence is supported by another *in vitro* study in which both COX-1 and COX-2 enzymes were inhibited [Mendias *et al.*, 2004]. In this study they cultured skeletal muscle satellite cells from 9-month-old Sprague-Dawley rats and exposed them to naproxen sodium, which is a nonselective COX inhibitor, NS-398 (a selective COX-2 inhibitor) and SC-560 (a selective COX-1 inhibitor) for 96 h. They found that by inhibition of COX-2 alone, satellite cell proliferation was decreased. COX-1 and COX-2 inhibition resulted in decreased satellite cell differentiation and fusion. Interesting, inhibition of COX-1 alone had no effect on satellite cells. This study suggests that the COX enzymes play an important role in satellite cell proliferation, differentiation and fusion and that NSAID medication may have an adverse effect on muscle regeneration following injury. Similar results of *in vivo* studies suggest that COX-2-dependent prostaglandin synthesis is required during early stages of muscle regeneration. These studies have demonstrated that selective COX-2 suppression reduces satellite cell activation, proliferation and differentiation, as well as inhibiting myonuclear incorporation into muscle [Bondesen *et al.,* 2004; Bondesen *et al.* 2006]. Bondesen *et al.,* (2004) made use of localized freeze injury to the *tibialis anterior* muscles of mice. These mice were chronically treated with either a COX-1 (SC-560) or COX-2-selective inhibitor (SC-236), starting before injury. They analyzed the size of regenerating myofibers at time points up to 5 weeks after injury and found the size of regenerating myofibers to be decreased in the animals that were treated with the COX-2-selective inhibitor (SC-236) and in muscle samples of COX-$2^{-/-}$ mice. The mice that received the COX-1-selective inhibitor (SC-560) were unaffected. In contrast, COX-2-selective inhibitor (SC-236) had no effect on myofiber growth when it was administered 7 days after injury. The researchers ascribe the decrease in myofiber growth by COX-2-selective inhibitor (SC-236) treatment and in COX-$2^{-/-}$ muscles to the decrease in the number of myoblasts and intramuscular inflammatory cells at early times after injury. Similar to the study by Bondesen *et al.,* 2004, Mishra *et al.,* 1995 found that NSAID treatment resulted in the total inhibition of the inflammatory phase, decreasing the capacity for regeneration and thereby delaying muscle regeneration. The total inhibition of the inflammatory phase is therefore not necessarily beneficial.

In contrast to the studies above, a study that examined the effect of NSAID's on satellite cells could find no direct effect of NSAID's on satellite cell proliferation [Thorsson *et al.* 1998]. In this study, neither early nor late NSAID supplementation had any significant effect on muscle regeneration of the rat gastrocnemius muscles, as indicated by similar satellite cell and fibroblast activation/proliferation cycles, production of myotubes and capillaries in both NSAID and control groups. Late (3 days after injury) and early (6 hours after injury) treated groups both received daily intramuscular injections of naproxen at 10 mg/kg. The control animals received no injection, which is scientifically not advisable. The ideal situation would be the injection of a placebo. The early-treatment group was sacrificed on day 1, 3, 6 and 9, and the late-treatment group was sacrificed on day 5, 8 and 11. The above-mentioned studies mainly looked at the effects of COX inhibitors on satellite cell activity. The study by Vignaud *et al.* (2005) focused more on the accumulation of inflammatory cells. In this study, it was proposed that long-term NSAID and antioxidant drugs could significantly reduce the speed of muscle recovery after severe injury. They made use of two models of injury. The first model was by means of myotoxin injection. For this model the *tibialis anterior* muscle was injected with 20 µl of 0.9% saline containing a myotoxic agent (2 µg/kg per muscle of snake venom from *Notechis scutatus*). The second model was a crush-injury model. For this model the muscle of the animal was mildly crushed twice for 5 seconds with forceps placed from the distal tendon to the proximal extremity. Different NSAID and antioxidant drugs were administered at low doses via injection, namely, diclofenac, diferuloylmethane (DFM), dimethylthiourea (DMTU), dimethyl sulphoxide (DMSO), indomethacin and pyrrolidine dithiocarbamate (PDTC). The drugs either had known antioxidant and anti-inflammatory action or one of the two actions. Drug administration commenced on the day of injury and continued for 10 to 14 days. Analysis was conducted 10-42 days after injury by investigating the recovery of *in situ* muscle force production, size of regenerating muscle cells and expression of myosin heavy chain. Their results show that diclofenac, diferuloylmethane (curcumin), dimethylthiourea or pyrrolidine dithiocarbamate treatment did not significantly affect muscle recovery after myotoxic injury. Similarly, diferuloylmethane, dimethyl sulphoxide and indomethacin administration did not change muscle repair after crush injury. They also found that treatment used resulted in a decreased accumulation of inflammatory cells in the damaged muscle, as well as a limited production of free radicals/oxidants, prostaglandins, cytokines and chemokines in the first few days after injury. In addition, they found none of the drugs to have detrimental effects due to long-term (42 days) treatment, except high doses (> 2 mg kg^{-1}) of diferuloylmethane and indomethacin, which led to lethality and reduced muscle repair after crush injury. From this study it is not clear whether the inhibitory effects of NSAID's and antioxidants are due to the antioxidant or anti-inflammatory properties of these drugs, since some of the drugs displayed both beneficial properties. The above study by Vignaud *et al.* (2005), reports that low doses of NSAIDs might be beneficial to the muscle repair process while other studies indicate that high doses might have detrimental effects.

Human clinical trials examining the effects of NSAID's are also contradictory. For example, Paulsen *et al.* (2010) conducted a double-blind, placebo-controlled experiment, in which they evaluated the effects of a selective COX-2 inhibitor on muscle recovery following damaging exercise. This study consisted of 22 males and 11 female volunteers, all young and physically active. They were asked to perform 2 bouts of maximal lengthening actions of the elbow flexors. These exercises were done 3 weeks apart and only one arm was trained per session, the other arm serving as a non-exercise control. The volunteers were randomly divided into either an NSAID group or a placebo group. The NSAID groups received a daily dose of 400 mg of celecoxib orally for a total of 9 days, with the first dose administered ± 45 minutes prior to each exercise bout. The placebo group received lactose pills over the same time periods. Their results show no significant differences in the number of satellite cells/myoblasts per myofiber between groups. In addition, no significant differences were found in the number of macrophages between the different groups. This finding is in contrast to the findings of Mackey *et al.* (2007) and Mikkelsen *et al.* (2009), who both reported that NSAID administration had detrimental effects on satellite cell activity in their human clinical trials. In clinical practice, NSAIDs are often not prescribed for 24 to 48 hours after the injury [Rahusen *et al.*, 2004]; however, 1 to 2 days post-injury might be too late, due to the fact that neutrophils infiltrating the injured area may already have caused secondary damage, exacerbating the injury.

If the injury is severe and causing oedema, low dosage of NSAID's have been found to prevent the chain reaction oedema causes, namely anoxia and further cell death [Paoloni *et al.,* 2009]. In a study by Gierer *et al.* (2005), they intravenously infused a relatively low dose of NSAID (10 mg/kg parecoxib sodium) into the muscle of the experimental animal immediately before or 2 hours after contusion injury. They found that, by inhibiting COX-2, they were able to reduce leukocyte rolling and adhesion to the vascular endothelium and so-doing, reduce muscle tissue secondary damage [Gierer *et al.*, 2005]. When investigating the relationship between leukocytes, endothelium and muscle secondary damage Menth-Chiari *et al.* (1998) found that injured muscle had significantly higher numbers of rolling and adhering neutrophils compared to baseline pre-contusion, 5 hours post-injury. Gierer *et al.*, (2005) concluded that NSAID infusion both prior to and after contusion injury, resulted in a marked decrease in the inflammatory response and almost completely restored microcirculation to normal by 18 hours after trauma. These results indicate that NSAIDs might be a good treatment for contusion injuries. These studies seem to indicate that by limiting leukocyte infiltration into the damaged area, muscle regeneration may be accelerated. There are, however, also studies that suggest early administration (24 hr prior to injury) of NSAID does not have any effect on muscle recovery [Rahusen *et al.*, 2004]. This is unexpected, as one would assume that administration of NSAIDs would have some effect in blunting the inflammatory response as seen in the study by Gierer *et al.* (2005), rather than no effect at all. Since the effect seen by Gierer *et al.* (2005) is at a lower dose for a shorter time period before injury that that of Rahusen *et al.* (2004). NSAID's are also not recommended for long-term use (more than 7 days continually) as they might delay muscle regeneration by inactivating the proliferation and differentiation of satellite cells and inhibiting the production of growth factors [Rahusen *et al.,* 2004]. However, 7 days is already well into the macrophage stage, which is the beneficial stage with regard to regeneration, therefore I would suggest that this treatment phase be even shorter. These discrepancies indicate that research on the timing and dosage of NSAID administration are still of importance.

To conclude, possible reasons explaining the discrepancies found in the above-mentioned studies might be the differences in methodologies between studies, physiological differences between species and/or differences in the mechanisms of the various drugs used (i.e. selective vs. nonselective COX inhibitors). In injury-prone populations, such as athletes, it is a problem that NSAID's can be bought and used without prescription by a professional. Therefore, I am of the opinion that the main problem with NSAID's is their chronic, uncontrolled use. With this in mind, NSAID's should be used with caution.

2.6.5.2 RICE approach

The most used treatment, immediately after a skeletal muscle injury (or any soft-tissue injury), is the "RICE approach". RICE refers to rest, ice, compression and elevation. This technique is applied to try and minimize the haematoma formed due to the injury. This is believed to decrease the size of the connective tissue scar. However the RICE method, as a whole, has not been scientifically proven in any clinical trial [Järvinen *et al.*, 2005]. There is, nevertheless, scientific evidence to support the appropriateness of the distinct components of the concept, the evidence being derived largely from experimental studies.

The proof of "rest" as an effective method has been obtained largely from muscle immobilization studies [Järvinen *et al.,* 2005; Järvinen and Lehto, 1993]. Even though it is known that early mobilization of injured muscle assists in restoring muscle function to its pre-injury levels, it should not occur immediately after injury [Järvinen *et al.,* 2005; Järvinen, 1975]. Experimental studies have shown that if the injured muscle is active immediately after injury, a larger connective tissue scar ensues in comparison to immobilized muscle [Järvinen, 1975]. Furthermore, re-ruptures at the site of the original muscle injury are more common if injured muscle is mobilized immediately after the injury [Järvinen, 1975; Lehto *et al.,* 1985]. Conversely, inactivity for extended periods of time post-injury has been shown to be associated with significant atrophy of the healthy muscle fibers, excessive connective tissue formation within the muscle tissue and delayed recovery of the strength of the injured skeletal muscle [Järvinen *et al.,* 2005]. Therefore, a short period of immobilization (3 – 7 days) after muscle injury is beneficial [Järvinen and Lehto, 1993]; however it should be limited only to the first few days after the injury. Physical activity should therefore be gradual and performed without pain [Jarvinen *et al.*, 2005].

Regarding the application of ice to the injured area, it has been shown that the early use of cryotherapy is associated with a significantly smaller haematoma between the ruptured myofibers, less inflammation and accelerated regeneration [Deal *et al.*, 2002; Hurme *et al.*, 1993]. The prescribed application of cryotherapy is rather intricate, as it should be applied intermittently for 15 to 20 minutes with a rest period of 30 to 60 minutes, 2 to 4 times per day for the first 2 to 3 days post-injury [Kellet, 1986]. It has been found that lowering of tissue temperature decreases cellular metabolism and thereby diminishes oxygen and nutrient needs. However, cryotherapy treatment can also have detrimental consequences as it has been found that long periods of cold application (decreasing the temperature of muscle below 25 °C) leads to blood vessel dilation, resulting in increased haemorrhage and inflammatory response [Kellet 1986; Lehto and Jarvinen, 1991].

Even though the notion has always been that compression of the injured area reduces intramuscular blood flow [Kalimo *et al.,* 1997; Thorsson *et al.,* 1987], it is still not clear whether compression applied immediately after the injury accelerates the healing of the injured skeletal muscle [Thorsson *et al.,* 1997]. It is currently recommended that ice (cryotherapy) and compression be applied in combination for 15 to 20 minutes duration, repeated at every 30 to 60 minutes. This type of protocol has previously been shown to result in a 3°C to 7° C decrease in the intramuscular temperature and a 50% reduction in the intramuscular blood flow [Thorsson *et al.,* 1987; Thorsson *et al.,* 1985].

Finally, the rationale for the use of elevation after injury is based on the principle that elevation of an injured extremity above the level of the heart results in a decrease in hydrostatic pressure and reduces the accumulation of interstitial fluid [Järvinen *et al.,* 2005].

2.6.5.3 Growth factors

In recent years there have been various studies conducted on the potential impact of growth factors on muscle regeneration [Fu *et al.*, 2005; Kaariainen *et al.,* 2000]. The rationale behind this seems to be that it may increase muscle regeneration capacity by increasing the size and number of existing and newly regenerating muscle fibers and thereby improving muscle function [Kasemkijwattana *et al.,* 1998]. Researchers investigated several exogenous growth factors, which promote healing of injured fibers and inhibition of TGF-β1, to block muscle fibrosis.

Regeneration of an injured muscle consists of 2 elements. Firstly, proliferation and differentiation of myoblasts need to occur. This is promoted by growth factors such as FGF, IGF, nerve growth factor (NGF), TGF-β1 and PDGF, which are all capable of promoting muscle regeneration [Chargè and Rudnicki, 2004; Sartorelli and Fulco, 2004]. Mitchell and colleagues (1996) reported that basic FGF had limited stimulatory effects on satellite cells in three different injury models (crush-injured, denervated, and dystrophic (*mdx*) muscles) if administered exogenously. In this study basic fibroblast growth factor (bFGF) was administered at various doses and different time schedules, sometimes in combination with heparin, into injured tibialis anterior muscles of mice. It was delivered by either direct intramuscular injection or by the sustained release from 888polymers (Hydron or Elvax) implanted into the muscles. On the contrary, Armand *et al.* (2003) found that if bFGF-6 is directly delivered to the site of injury, it could accelerate regeneration by stimulating differentiation of myotubes in soleus muscle of mice. Takahashi *et al.* (2003) conducted a study in which they found that gene delivery of IGF-1 via electroporation resulted in (i) an increased number of regenerating myofibers 2 weeks post-injury and (ii) an increased regenerating myofiber size by 4 weeks after injury. In another study IGF was injected into healthy elderly men and it was found that the loss of muscle mass due to age was prevented [Huard *et al.,* 2002]. However positive this finding is, it is also known that IGF injections can have side-effects, amongst them being fibrosis development, by stimulating components such as collagen and decreasing the expression of collagenase [Huard *et al.,* 2002]. A study by Miller *et al.* (2000) showed that, when HGF was injected directly into the injured muscle, the number of myoblasts increased in a dose-dependent manner. However, they do report that this increased myoblast formation did not lead to better regeneration of the injected muscle. According to their study it seems as if injecting HGF is time-dependent, because when HGF was injected the first 4 days post-injury, muscle regeneration was inhibited, however when it was administered later it had no effect. The *in vivo* studies done by Kasemkijwattana *et al.* (1998; 2000) and Menetrey and Kasemkijwattana (2000) indicate that bFGF, IGF-1 and NGF are stimulators of proliferation and fusion of myoblasts after strain injury. In these studies the growth factors were injected into the injured gastrocnemius muscle of mice. It was found that the number of regenerating myofibers was 3.5 times higher for bFGF and IGF-1 and 1.5 times for NGF, in the treated group versus the untreated. This data points to the fact that these growth factors were able to improve regeneration of injured muscle.

Secondly, the amount of scar tissue formed needs to be decreased. According to the literature it seems as if the over-production of TGF-β1 leads to the excessive formation of fibrosis in animals and humans and it is therefore a target in drug development. In the study by Chan *et al.* (2005) they decreased the activity of TGF-β1 by injecting a TGF-β1-antagonist, suramin, immediately after injury, 7 days after injury or 14 days after injury. The mechanism of actions of suramin is based on competitive binding to TGF-β1 receptors. Suramin is used as an antiparasitic and anti-tumor drug with side-effects that include adrenocortical insufficiency, malaise, neuropathy and corneal deposits and to a lesser extent neutropenia, thrombocytopenia and renal failure [Chan *et al.* (2005)]. When suramin was administered immediately or after 7 days there was only a minor effect on fibrosis, yet when a high dose was administered 14 days after injury, fibrosis of the muscle was prevented. Chan *et al.* (2005) also reported more regenerating myofibers in all suramin-treated groups compared to controls. The study by Chan *et al.* (2005) coincides with the study by Nozaki *et al.* (2008) where they injected 2.5 mg of suramin 2 weeks after contusion injury and found less fibrosis and better healing of the muscle. Decorin has also been used to inactivate TGF-β1. Fukushima *et al.* (2001) could significantly reduce the amount of fibrosis after injury by injecting decorin 10 and 15 days post-injury. There seemed to be a dose-response effect as well.

2.6.5.4 Antioxidants as natural anti-inflammatory agents

The process of inflammation, as a result of injury, contributes to fibrosis and causes pain, which may impair skeletal muscle function [Abdelmagid *et al.,* 2012; Stauber, 2004]. It is for that reason that the mechanism of many treatments for muscle injury is to reduce inflammation by inhibiting it with drugs. The biggest problem with this mode of intervention is that even though inflammation causes secondary damage [Ebbeling and Clarkson, 1989; Tidball, 1995], preventing inflammation completely may hinder muscle recovery [Mackey *et al.,* 2007; Mikkelsen *et al.,* 2009].

There are different types of antioxidants: (i) the antioxidants that are produced by the body (enzymatic antioxidants), such as the SOD, glutathione peroxidase (GPx) and catalase, and (ii) the type of antioxidants that can be ingested from dietary sources (non-enzymatic antioxidants), such as lipid-soluble vitamin E, β-carotene, co-enzyme Q10 (CoQ) and the water-soluble vitamin C [Packer and Cadenas, 2007]. Antioxidants work by protecting the cell against ROS-induced damage [Packer and Cadenas, 2007], by either converting ROS into less reactive molecules (known as scavenging) or by preventing the transformation of less reactive ROS into the more highly reactive forms [Powers and Sen, 2000]. Under normal physiological conditions, mammalian cells do seem to have adequate antioxidant capacity to cope with ROS production, however, when ROS production is elevated above normal, such as during oxidative stress conditions, these antioxidant reserves may be inadequate. Therefore, antioxidant supplementation might prove beneficial to scavenge the extra ROS generated. In the publication by Basu (1999) he highlighted that deficiencies in some of these antioxidants were associated with oxidative stress. Similar to the observations of Basu (1999), the study by Brown *et al.,* 1994 linked acute dietary supplementation to a decrease in lipid peroxidation. The most common antioxidants used to scavenge free radicals are vitamins C and E. Vitamin C (ascorbic acid) is the most important water-soluble antioxidant vitamin [Frei *et al.,* 1989]. It functions as an antioxidant by directly scavenging specific ROS, as well as lipid hydroperoxides and it helps recycle vitamin E from its radical form [Carr and Frei, 1999; Powers and Sen, 2000]. Conversely, vitamin E is the most important lipid-soluble antioxidant vitamin [Packer, 1997]. It exists in eight different natural forms of which the most biologically active form is α-tocopherol [Traber, 2000]. In the literature there are large discrepancies on the effects of these antioxidants in animal studies. There are studies that report significant effects, moderate effects to no benefit at all of these common antioxidants [Ostman *et al.,* 2012; Strobel *et al.,* 2011; Theodorou *et al.,* 2011]. A fact to note when interpreting findings obtained from these types of animal studies and relating them to humans, is that it is done cautiously. The reason for this is, for example, the species differences observed in the animal (especially rodents) versus humans. Firstly, rodents have a different muscle fiber type population and different muscle architectures to humans. As a result thereof, animal muscle may respond differently to the same type of injury, than human muscle would. Secondly, humans acquire vitamin C through their diet, since it is not synthesized; however, many animals can synthesize vitamin C [Sen and Goldfarb, 2000]. In the following sections I will be discussing, in more detail, studies related to the popular antioxidants used as well as other antioxidants eliciting favorable effects on oxidative stress.

Vitamin C is water-soluble and therefore it is not stored in the human body in great amounts, the majority of it is transported in the plasma [McGinley *et al.,* 2009]. One would therefore assume that if vitamin C had any significant effect in protecting muscle against damage, that a single dose provided at the appropriate time would offer protection. I could however not find any conclusive evidence to support this assumption, since there are researchers reporting both positive effects after vitamin C treatment and no effect at all. For example, Thompson *et al.* (2001) conducted a placebo-controlled study in which they tested the effect of an acute dose (1g) of ascorbic acid 2 hours prior to 90 minutes of intermittent shuttle running. This type of exercise was designed to simulate the multiple-sprint sports. In this study they found that vitamin C supplementation increased plasma concentrations of vitamin C before exercise and that the plasma concentrations continued to increase during the shuttle-run and peaked at approximately 200 µmol × L^{-1} immediately after exercise. Supplementation did not affect the moderate increases in serum creatine kinase, serum aspartate aminotransferase or delayed onset muscle soreness. The authors suggested that the failure of vitamin C to attenuate indicators of oxidative stress might have been due to ineffective timing of supplement administration. However, in an earlier study by Ashton *et al.* (1999) they found a protective effect against ROS production using an identical dosing strategy. This study did not measure any indices of muscle damage [Ashton *et al.,* 1999]. They measured lipid hydroperoxides and malondialdehyde and found that vitamin C supplementation prevented a significant increase in lipid hydroperoxides and malondialdehyde after maximal aerobic exercise [Ashton *et al.,* 1999]. Several studies found in the literature make use of pre-supplementation strategies. In one such study vitamin C was administered for 2 weeks prior to different modes of exercise [Thompson *et al.,* 2001]. In this study the participants in the treatment group received 200 mg ascorbic acid twice a day and the placebo group received identical capsules containing 200 mg of lactose. They found that plasma malondialdehyde, which is a secondary marker of lipid peroxidation, was increased significantly after a 90-minute intermittent shuttle running test, and that vitamin C supplementation significantly reduced this elevated malondialdehyde level 2 and 24 hours after exercise. In addition, they found that inflammation demonstrated by a ~ 8-fold increase in serum IL-6 level, was reduced to baseline levels in the vitamin C-supplemented group. Conversely, another study provided evidence that vitamin C did not prevent the exercise-induced increase in IL-6 after 2.5 hours' cycling exercise [Davison and Gleeson, 2006]. The reason for this discrepancy is not completely clear, but use of exercise protocols differ, which might mean that they have differing levels of metabolic demand and thus different levels of ROS production.

In the literature there are also studies in which authors made use of a combination of pre- and post-supplementation strategies. One such study is the placebo-controlled cross-over design study by Kaminski and Boal (1992). This is one of the first studies to examine the effect of vitamin C supplementation on delayed-onset muscle soreness. In their study, ascorbic acid (1g three times a day) was provided to subjects for 3 days prior to and 4 days after eccentric exercise of the *plantar flexors*. This study reported less delayed-onset muscle soreness in the vitamin C-supplemented group. However, several subjects demonstrated no difference in soreness between treatment and placebo [Kaminski and Boal, 1992]. Importantly, the training status of subjects was not established and the dose of vitamin C was greater than the recommended upper tolerable levels [Hathcock *et al.,* 2005], which makes it difficult to draw inference from this study. A recent single-trial study in which a similar large daily dose of vitamin C (3 g per day) was used for 2 weeks before and 4 days after eccentric contractions of the elbow extensors, also reported lower delayed-onset muscle soreness in the vitamin C-supplemented group [Bryer and Goldfarb, 2006]. In this study they reported no significant difference between treatment and placebo groups with regard to range-of-motion, force loss or plasma creatine kinase response after exercise. The authors could find no evidence of a pro-oxidant effect of vitamin C in this study, with a reduction in the ratio of oxidized glutathione to total glutathione, evident in the blood at 4 and 24 hours post-exercise in the treatment group [Bryer and Goldfarb, 2006]. Close *et al.* (2006) also investigated the effects of supplementing with ascorbic acid for 2 hours before and 14 days after 30 minutes of downhill running. They found no effect of treatment on delayed-onset muscle soreness and torque loss was more prolonged with vitamin C supplementation. They found serum malondialdehyde levels were elevated at 72 and 96 hours post-exercise and the increase was reduced by vitamin C supplementation. Similarly, the study by Connolly *et al.,* 2006 reported that vitamin C treatment had no significant effect on delayed-onset muscle soreness in subjects receiving either vitamin C or placebo for 3 days prior to and 5 days after a bout of eccentric contractions of the elbow flexors [Connolly *et al.,* 2006]. Another study reported no effect on any indices of muscle damage after providing vitamin C supplementation to subjects for 14 days before and 3 days after eccentric downhill running [Thompson *et al.,* 2004]. Contradictory to previous findings from the same group [Thompson *et al.,* 2001]; vitamin C did not affect the time-course or extent of IL-6 response to exercise. These results are supported by similar findings after 90 minutes downhill running [Petersen *et al.,* 2001] but are in contrast to the findings from other studies [Childs *et al.,* 2001; Fischer *et al.,* 2004]. The exact reason for the differences is not known, however, it may be due to the use of exercise protocols with differing levels of metabolic demand. It is difficult to compare the studies mentioned above with regard to whether vitamin C supplementation has protective effects on exercise-induced oxidative stress, because they utilized a variety of supplementation strategies, exercise protocols or subject cohorts. Additionally, little direct measurement of muscle damage has been reported in these studies, thus it is unclear to what extent muscle damage was induced in many of these studies.

Unlike vitamin C, which is not stored in the body, vitamin E is stored. The likely assumption is therefore that it is necessary to build up in tissue stores, in order to optimize the potential protective effects. There are numerous studies found in the literature where researchers made use of a pre-exercise supplementation strategy or a combination of pre- and post-exercise supplementation, when investigating the effects of vitamin E on muscle damage. In a double-blind, crossover design study Cannon *et al.* (1990; 1991) set out to determine the effects of 48 days of vitamin E supplementation (800 IU/day) on eccentric muscle damage, the subjects received either α-tocopherol or a placebo until the day prior to the 45 minutes downhill running. Supplementation was also given 3 days after exercise. Additionally, subjects were grouped into either young (< 30 years old) or old (> 55 years old). From this study they found that the older subjects had higher plasma levels of vitamin E after supplementation, despite receiving the same dose. Plasma creatine kinase levels peaked in all subjects 1 day after exercise, never reaching more than 400 IU/L/g creatinine. The authors reported that vitamin E resulted in reduced creatine kinase in the younger participants and increased creatine kinase levels in the older participants; concentrations were not greatly elevated above baseline. This suggests that if damage was present, it was minimal. In addition, this study showed that vitamin E treatment attenuated the increased IL-1β but not TNF-α 24 hours after exercise. They also found that IL-6 release was not affected by exercise, but was found to be lower in the vitamin E group compared with placebo throughout the measurement period. Collectively, these data indicate that vitamin E supplementation had no effect on markers of muscle damage, but did moderate exercise-induced inflammation. In the study by Beaton *et al.* (2002), subjects received either vitamin E or placebo for 30 days prior to eccentric contractions of the quadriceps. Muscle damage in both treatment and placebo groups was determined by biopsies from which Z-band disruption could be visualized, taken 24 hours after exercise. From this study, no evidence of disruption to the structural proteins, desmin and dystrophin, were observed. They also found infiltration of the neutrophils and macrophages 24 hours after exercise, with no effect of treatment. They did no biopsies later than 24 hours after exercise, therefore evidence for secondary inflammation and damage may have been missed. There was no effect of vitamin E on serum creatine kinase, torque loss or delayed-onset muscle soreness. In another study the effect vitamin E supplementation 2 weeks prior to heavy resistance exercise, on exercise-induced muscle damage, was investigated using resistance-trained subjects [McBride *et al.,* 1998]. In this study, vitamin E supplementation significantly reduced the post-exercise increase in creatine kinase, but had no significant effect on delayed-onset muscle soreness or the increase in plasma malondialdehyde post-exercise. The creatine kinase response in this study was quite modest, indicative of minimal damage. In a separate study, where a similar dose of vitamin E supplementation was used, they found a greater increase in creatine kinase after resistance exercise; however, this study was done with untrained men [Avery *et al.,* 2003]. In a study by Sacheck *et al.* (2003) the oxidative stress response to downhill running in subjects receiving α-tocopherol for 12 weeks before exercise was assessed in young (26 ± 3 years) and old (71 ± 4 years) volunteers. In this study they found increased plasma lipid peroxidation, which was confirmed by an increase in malondialdehyde, immediately after exercise as well as a peak increase in F2-isoprostanes (which is a prostaglandin-like substances produced by ROS-induced oxidation of arachidonic acid) 72 hours after exercise. Vitamin E treatment elicited moderate effects with regard to the malondialdehyde response. In the younger vitamin E group the malondialdehyde levels were reduced at 72 hours, but elevated in the older group, whilst plasma F2-isoprostanes concentration was lower in the older vitamin E subjects. Moderate increases in serum creatine kinase were the only indicator of muscle damage, with a peak (maximum of ~500 IU/L) measured at 24 hours post-exercise in all groups. Mixed results of treatment were seen, with elevated baseline values in both treatment groups, but reduced creatine kinase levels in the young subjects vs. increased creatine kinase levels in the older subjects, at 24 hours post-exercise. Overall, there appeared to be a moderate protective effect of vitamin E against oxidative stress, but an interesting point is that, similar to the studies by Cannon *et al.* (1990; 1991) there were some contrasting responses between the young and old subjects. In the sections discussed above, I highlighted studies in which either vitamin C or vitamin E was used before or after or in a combination of before and after injury. The results are fairly contradictory. In the following section, I will discuss studies which examined the effects of vitamin C and vitamin E and occasionally other antioxidants, in combination, on indicators of oxidative stress and/or muscle damage.

In the study by Fischer *et al* (2004), supplementation with ascorbic acid (500 mg/day) and *RRR*-*α*-tocopherol (400 IU/day) was given for 28 days prior to a two-legged knee extensor exercise, at 50% of their individual power output. In this study, treatment was found to attenuate the exercise-induced IL-6 increase [Fischer *et al.,* 2004]. They reported that plasma IL-6 levels started to increase immediately after exercise, peaking after 4 hours. They also found that IL-6 protein and IL-6 gene expression increased after exercise, as seen by the ~6-fold higher levels in the placebo group, however treatment had no effect. The plasma IL-1ra, which is a receptor antagonist, increased with placebo treatment but not vitamin C and E treatment after 3 and 6 hours. Additionally, the C-reactive protein level increased at 23 hours post-exercise in the placebo group only. The antioxidant supplementation seemed to reduce exercise-induced inflammation, however, no data regarding the creatine kinase levels are available and therefore it is difficult to say to what extent the muscle was damaged. They also found that lipid peroxidation increased significantly 3 hours after exercise in the placebo and treatment with the antioxidants prevented this response. Lipid peroxidation was indicated by a ~2.4-fold increase in 8-epiprostaglandin F2a (the most commonly measured F2-isoprostane [McCall and Frei, 1999]).

In another study, a mixture of antioxidants (400 mg alpha-lipoic acid, 200 mg co-enzyme Q10, 12 mg manganese, 600 mg vitamin C, 800 mg NAC, 400 µg selenium, and 400 IU alpha-tocopherol per day) were given to participants for 7 days prior to a treadmill run-to-exhaustion exercise [Davison *et al.,* 2005]. When comparing the placebo group with the treatment group, they found that antioxidant treatment had no effect on deoxyribonucleic acid (DNA) damage. DNA damage was determined by measuring peripheral blood mononuclear cells, using the comet assay. In addition, treatment also had no effect with regard to the post-exercise increase in plasma total antioxidant capacity, or the increase in blood concentration of lactate dehydrogenase (LDH). According to the authors, their study demonstrated that exhaustive aerobic exercise induces DNA damage, however, antioxidant supplementation does not protect against this damage. The reason for the contradictory evidence of this study is unknown; however, one should keep in mind that the mixture of antioxidants that the participants received contained 800 mg of NAC. NAC has previously been found to have pro-oxidant activity in a similar dose of NAC combined with vitamin C in a post-exercise scenario [Childs *et al.,* 2001], which might have influenced the outcome of the study by Davison *et al.* (2005). In another study, where antioxidant treatment (500 mg of vitamin C and 400 mg of vitamin E) was given before and after exercise, the investigators could find no evidence that vitamins C en E had any protective effects with regard to muscle damage after downhill running [Petersen *et al.,* 2001]. Treatment started 2 weeks prior to exercise and continued for 1 week after exercise. With this treatment regime they found that the antioxidants had no effect on plasma creatine kinase, plasma IL-6, plasma IL-1ra or on a variety of inflammatory cells ($CD4^+$ memory T cells, $CD8^+$ memory cells, naïve T-cells and natural killer cells). The extent of muscle damage was not measured directly, but levels of plasma creatine kinase suggest that membrane damage was not too severe. In this article they did not report on the ROS levels, making it difficult to elucidate the potential effect of vitamin C and vitamin E in combination. However, Shafat *et al.* (2004) investigated the effects of vitamin C and vitamin E supplementation on functional measures of muscle damage after an eccentric exercise bout, in a single-blind, single-trial design. In this study, participants received vitamin C (500 mg) plus α-tocopherol (1,200 IU) or placebo daily for 30 days before and 7 days after eccentric contractions of the knee extensors. In this study they found that treatment could attenuate the reduction in maximal voluntary contraction post-exercise, but they could find no evidence that treatment had any effect on muscle soreness. No indicators of oxidative stress were measured, nor were there blood or muscle markers of muscle damage determined.

In conclusion, there appears to be some benefit in the usage of vitamins C and E by systematically manipulating the inflammatory response. A mild reduction of circulating oxidants and pro-inflammatory molecules may result in less secondary damage incurred after muscle injury, reduced soreness and faster time to recovery. Long term studies are warranted to verify that these acute changes post-injury do not affect skeletal muscle adaptation and repair.

In the following section, I will elaborate as to my reason for conducting the current study.

2.7 MOTIVATION AND HYPOTHESIS FOR CURRENT RESEARCH

By reviewing the literature, it is clear that the ideal treatments for skeletal muscle insulin resistance, skeletal muscle fatigue after exercise and muscle injury have yet to be found. This is mainly due to the complexity of these processes. The focus of this study therefore consisted of three main aims, centering on the ability of skeletal muscle to adapt in response to the above-mentioned altered demands.

Our first aim was to evaluate the effect of a plant-based substance, *P. glandulosa,* on skeletal muscle insulin sensitivity, in a hyperphagia-induced obese rat model. In previous studies conducted in our laboratory [George *et al.,* 2011], it was found that obese Zucker (*fa/fa*) rats displayed significantly lower fasting plasma glucose levels after *P. glandulosa* treatment, compared to their control counterparts (5.34 ± 0.17 mmol/l vs. 5.98 ± 0.20 mmol/l; $p < 0.05$). We also observed an improved glucose handling after glucose load, as indicated in an IPGTT, in the same model. Since skeletal muscle is responsible for up to 80% of the glucose disposal from the peripheral circulation, we hypothesized that *P. glandulosa* treatment would result in increased insulin sensitivity of skeletal muscle and therefore lead to the decreased plasma glucose levels observed.

Our second aim was to evaluate the effects of *P. glandulosa* on force generated by a slow-twitch skeletal muscle and the ability of this muscle to recover, after it had been electrically stimulated to fatigue. Our third aim was to determine the effects of *P. glandulosa* on the inflammatory mediators and the regenerative capacity of skeletal muscle, after a contusion injury to the hind-limb. The motivation for our second and third aim came from anecdotal claims that race horses that consumed large amounts of *P. glandulosa* seemed to recover faster after a muscle injury and seem to display delayed muscle fatigue. However, these were based on casual observations or indications rather than rigorous scientific analysis. We therefore hypothesized that treatment with *P. glandulosa* would reduce the influx of neutrophils at the sight of injury, therefore inhibiting the negative effects associated with the inflammatory response after skeletal muscle injury and so-doing enhance muscle regeneration. *P. glandulosa* treatment would also delay muscle fatigue after extensive stimulation.

To my knowledge, only Samoylenko *et al.*, 2009 and Rahman *et al.,* 2011 have characterized chemical compounds of *P. glandulosa*. Samoylenko and colleagues (2009) isolated four indolizidine (three new and one known) and one anti-infective and anti-parasitic compound (2,3-dihydro-1H-indolizinium chloride) from *P. glandulosa* and in 2011 Rahman and colleagues isolated a new indolizidine alkaloid, named $\Delta^{1,6}$-juliprosopine, together with previously known indolizidine analogs from the leaves of *P. glandulosa* collected from Nevada, USA - while two other known indolizidines, juliprosopine and juliprosine were isolated from *P. glandulosa* leaves collected in Texas, USA. In addition there are only two articles regarding its potential clinical benefit in the literature (publications from our laboratory) [George *et al.,* 2011; Huisamen *et al.*, 2013]. However, neither of the papers addressed either skeletal muscle fatigue or injury, so that the anecdotal observations on skeletal muscle function and recuperation remain unsubstantiated by science.

CHAPTER 3: THE EFFECT OF CHRONIC *PROSOPIS GLANDULOSA* TREATMENT ON OBESITY AND SKELETAL MUSCLE INSULIN SENSITIVITY

3.1 GENERAL INTRODUCTION

In light of the growing epidemic of obesity, large amounts of research are currently focused on this condition, due to the strong association between obesity and the risk of developing metabolic abnormalities [Poirier *et al.,* 2006]. This growing epidemic we are currently facing is as a result of a sedentary lifestyle and high-caloric and high-fat food intake. These unhealthy diets cause numerous pathological conditions including increased glucose and insulin levels, which lead to insulin resistance and later diabetes mellitus [Baur *et al.,* 2006]. Insulin resistance is defined as a reduced responsiveness of a target cell or a whole organism to the insulin concentration to which it is exposed, leading to hyperglycaemia [Shanik *et al.*, 2008]. The influence of body fat on insulin action is very important and the relation between obesity, especially when it is centrally located [Kissebah *et al.*, 1989], insulin resistance and the risk for developing T2D is well recognized. It is known that the effects of obesity and its related complications can be reversed. There is strong scientific evidence that shows that even a modest weight loss (5 – 10%) can lead to a significant reduction in the risk of obesity's co-morbidities [National Institutes of Health, 1998]. Overweight and obese individuals have a number of options for weight loss, of which lifestyle modification (healthy diet and exercise) is the most favourable and has the least risk. There are other options in which there are more risks involved, such as pharmacotherapy and surgery [National Institutes of Health, 1998]. When lifestyle changes are not effective in the weight loss process, pharmacotherapy is prescribed. If medication is not successful either, surgery is recommended. One should keep in mind that surgery is the last option and it is only prescribed to a limited number of morbidly obese patients (BMI > 40 kg/m^2 or >35 kg/m^2

with co-morbid conditions). This is selective therapy, which is reserved for patients who are suffering from the complications associated with extreme obesity or are unresponsive to non-surgical treatment [Fisher and Schauer, 2002].

It is known that plants produce chemicals that they use for self-preservation. From accumulating scientific evidence it is apparent that these extracted chemicals may have beneficial effects on human health. A wide variety of herbal preparations, especially those rich in phenolic compounds, alkaloids, flavonoids, terpenoids, coumarins and glycosides, have been shown to have anti-obesity and anti-diabetic activities [Tabatabaei-Malazy *et al.,* 2012]. They have been shown to, amongst others, effectively prevent diet-induced-obesity (by preventing weight gain), significantly reduce body weight (in already overweight individuals) and have glucose-lowering abilities [Astell *et al.*, 2013; Hasani-Ranjbar *et al.,* 2009; Hui *et al.,* 2009]. The use of over-the-counter herbal products, nutritional supplements and meal replacements in the management of obesity, insulin resistance and T2D mellitus has gained increasing attention in consumer arenas, due to their natural origin and perceived fewer side effects [Aggarwal, 2010]. According to an American publication, the sale of over-the-counter weight loss products is a billion dollar industry that is still fast growing [Saper *et al.,* 2004]. This is not at all surprising, since the main stream pharmacotherapy has been found to have adverse effects [Padwall and Majumdar, 2007; Filippatos *et al.,* 2008]; surgery comes with potential complications and maintaining a healthy lifestyle is perceived as being difficult.

During the last few decades, numerous researchers have investigated the potential clinical benefit of herbal substances on obesity and its associated complications. Ginseng is an example of an herbal substance that has been reported to have anti-diabetic effects. These anti-diabetic effects have been investigated with both aqueous and ethanol ginseng extracts [Hui *et al.*, 2009]. In a study where ginseng was administered orally (100 mg/kg body weight) for 20 days, a decreased serum level of glucose and HbA_1c in STZ-induced diabetic rats was reported [Kim *et al.*, 2007]. Decreased serum level of glucose in KKAy mice was also reported in the study by Chung *et al.* (2001). In the latter study the researchers propose the mechanism of action to be via ginseng possibly blocking intestinal glucose absorption and inhibiting hepatic glucose-6-phosphatase. Ginseng berry extracts have also been found to elicit anti-obesity effects in obese *ob/ob* and *db/db* mice, after a daily i.p injection of extract at 150 mg/kg for 12 days, by reducing weight gain in these obese animals [Xie *et al.,* 2002]. Additional studies found the same anti-obesity effect in *ob/ob* mice as well as an anti-hyperglycaemic effect of ginseng berry juice (0.6 ml/kg) [Xie *et al.,* 2007]. Lee *et al.*, 2012 also conducted a study in which they examined the anti-diabetic and anti-obesity effects of *Panax ginseng*, as well as the possible mechanism of action in an obese insulin resistant animal model. In their study, Sprague-Dawley rats were placed on an 18-week high-fat diet and half the experimental group received Korean red ginseng (200 mg/kg, oral) additionally. They found that the 18-week administration of Korean red ginseng was able to significantly reduce weight gain and it reduced fat mass. The proposed mechanism was related to increased energy expenditure, as there was no significant difference in food intake found between treated and non-treated groups. In addition they observed increased insulin sensitivity, as demonstrated by an insulin tolerance and hyperinsulinaemic-euglycaemic clamp test, in the Korean red ginseng-treated group. Finally, by means of Western blotting assays, they observed increased phosphorylation of the IRβ, IRS-1, PKB/Akt as well as increased membranous glucose transporter, GLUT4, in the muscle of the Korean red ginseng-treated group. From this data, they concluded that treatment with Korean red ginseng may have anti-diabetic effects and anti-obesity effects, due to partly increased insulin sensitivity, through increasing phosphorylation of IRβ, IRS-1, PKB/Akt and GSK3α/β and increasing GLUT4 translocation in skeletal muscle [Lee *et al.*, 2012]. In a similar study conducted by Tan *et al.,* 2011, they found that a Chinese herbal extract (denoted as SK0506), composed of *Gynostemma pentaphyllum*, *Coptis chinensis* and *Salvia miltiorrhiza* prevented weight gain and significantly reduced visceral fat mass during high-fat feeding. Similar to Lee *et al.* (2012), Tan *et al.* (2011) made use of Sprague–Dawley rats that were fed a high-fat diet, but they were only fed this diet for 4 weeks. They found that SK0506 significantly enhanced glucose uptake and glycogen synthesis during hyperinsulinaemic–euglycaemic clamp procedure and they propose that this occurs, in part, by the enhancement of GLUT4 expression and translocation in skeletal muscle tissue. Since glucose transport is the rate-limiting step of glucose uptake and metabolism in insulin-sensitive tissues, altered GLUT4 activity is one of the major factors responsible for decreased glucose utilization in skeletal muscle tissue in diseases such as T2D and its precursor, insulin resistance [Liu *et al.*, 2010]. Muller *et al.,* 2012 also found that aspalathin (a component of green rooibos tea), dose-dependently increased glucose uptake (5 × 10^{-5} to 5 μg/ml) in C2C12 myotubules in their *in vitro* study. Likewise, in the *in vivo* portion of the study, the extract sustained the blood glucose lowering effect in STZ-induced diabetic rats, validated by the decreased blood glucose levels. Mazibuko *et al.* (2013) too conducted a study on aspalathin in palmitate-induced insulin-resistance in C2C12 skeletal muscle cells and found that treatment increased glucose uptake and ATP production, down-regulated PKC θ activation, increased activation of AMPK and PKB/Akt and increased expression of GLUT4. They did not determine the translocation of GLUT4. Their proposed mechanism of action in which aspalathin ameliorates palmitate-induced insulin resistance is via PKC θ inhibition and increased activation of key regulatory proteins involved in insulin-dependent and non-insulin regulated signaling pathways, AMPK and PKB/Akt. Kawano *et al.* (2009) conducted an *in vivo* and *in vitro* study on aspalathin, which showed that aspalathin significantly increased glucose uptake by L6 myotubes, in a dose-dependent manner, at concentrations of 1–100 mM [Kawano *et al.,* 2009]. They also found aspalathin treatment significantly increased insulin secretion from cultured RIN-5F cells at 100 mM. The *in vitro* study showed that dietary aspalathin (0.1 – 0.2%) suppressed increased fasting blood glucose levels and the IPGTT showed an improvement in the impaired glucose tolerance of *db/db* mice. These results suggest that aspalathin has beneficial effects on glucose homeostasis through stimulating glucose uptake in muscle tissues and insulin secretion from pancreatic β-cells. Kim and Kim (2008) reported that the *in vivo* treatment with Korean red ginseng (0.1 – 1.0 g/ml) resulted in the significant release of insulin from isolated rat pancreatic islets at 3.3 mM glucose concentration. These results were in accordance with a previous study conducted by Kimura *et al.* (1981). In this 1981 study they reported that some fractions extracted from Korean white ginseng, stimulated glucose-induced insulin release from pancreatic islet. These positive results are thought to be as a result of its anti-oxidative capacity. It has been reported that oxidative stress impairs insulin action, in particular reducing glucose uptake in response to the insulin [Blair *et al.,* 1999]. It has also been observed that by suppressing oxidative stress, by means of antioxidant treatment, insulin sensitivity and glucose homeostasis can be improved [Houstis *et al.,* 2006].

From the above-mentioned studies, it seems as if herbal substances with anti-obesity and anti-diabetic properties elicit their effects by (i) decreasing or preventing weight gain, (ii) augmenting insulin sensitivity, by altering the key regulatory proteins involved in insulin action, (iii) increasing glucose uptake, by increasing glycogen synthesis and increasing the expression and translocation of GLUT4, (iii) increasing the release of insulin by the pancreas into the bloodstream and (iiii) suppressing oxidative stress, via its antioxidant properties.

In 2006 our laboratory conducted a study aimed at uncovering whether an herbal substance consisting solely of the plant species, *P. glandulosa,* possess any possible health benefits. In the initial pilot study [George *et al.,* 2011] obese Zucker (*fa/fa*) rats displayed significantly lower fasting plasma glucose levels after *P. glandulosa* treatment, compared to their control counterparts (5.34 ± 0.17 mmol/l vs. 5.98 ± 0.20 mmol/l; $p < 0.05$). We also observed an improved glucose handling after glucose load, as indicated in the IPGTT (Fig. 3.1).

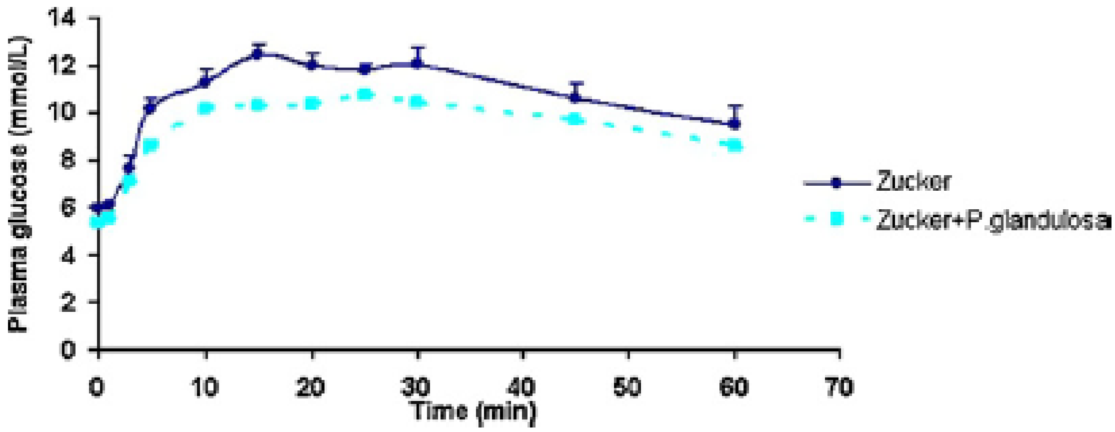


Figure 3.1: The response of plasma glucose to IPGTT of Zucker (*fa/fa*) rats. Rats were subjected to IPGTT by administering 1g/kg sucrose by i.p injection and measuring the glucose levels over a 60 min period. The data are expressed as mean ± SEM. n = 5. Data obtained from George *et al.,* 2011.

In addition, we examined whether the beneficial effects observed in the obese Zucker (*fa/fa*) rats were as a result of possible antioxidant capacity, since numerous herbal substances contain natural antioxidants. From this study it was evident that the beneficial effects observed were not due to an antioxidant mechanism. This was verified by the lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS) assays that both showed no significant difference between the serum from treated vs. untreated obese Zucker (*fa/fa*) rats (Fig. 3.2 (A) and (B)). These assays are done because ROS have extremely short half-lives and are therefore difficult to measure directly [Pryor, 1991].

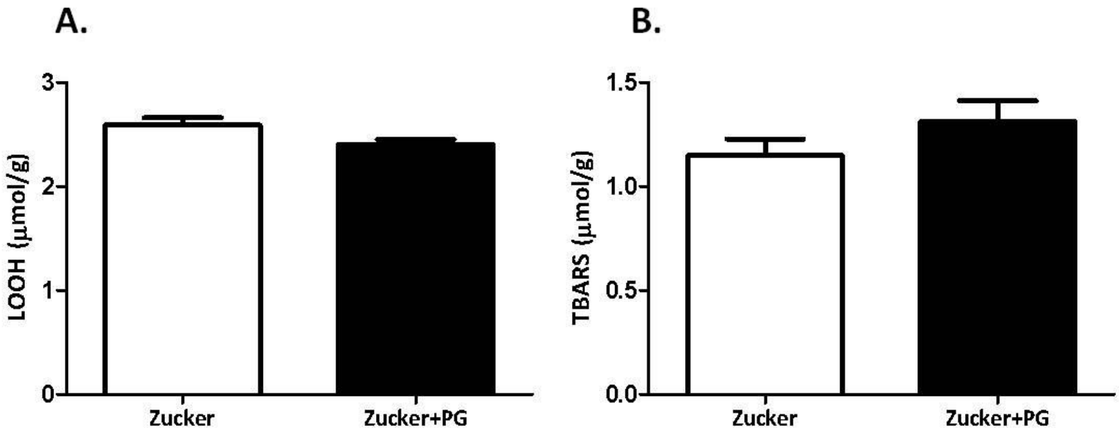


Figure 3.2: (A) LOOH and (B) TBARS assays of treated vs. untreated obese Zucker (fa/fa) rats. The data are expressed as mean ± SEM. n = 7. Unpublished data.

Since *P. glandulosa* does not act as an antioxidant, validated by data obtained through the LOOH and TBARS assays (Fig. 3.2 (A) and (B)), the results from this study could not completely explain the improved glucose tolerance. To investigate possibilities for this observation, we turned our attention to the effects that treatment with *P. glandulosa* has on skeletal muscle glucose uptake. Due to the enormous potential for the medicinal use of plant-based therapies, it is important that more research be done to scientifically validate these plant-based products.

3.2 METHODS

3.2.1 Research design and intervention

3.2.1.1 Animal care

Age- and weight-matched adult, male, Wistar rats were used. All animals were housed at the Stellenbosch University Central Research Facility, Tygerberg, in temperature controlled rooms (22 – 24 °C) and kept on a 12-hour light/dark cycle (lights on at 6:30 am). Rats were given *ad libitum* access to standard laboratory rat chow pellets and tap water for the duration of the experimentation. The animals received humane care in accordance with the principles of the South African National Standard for the care and use of animals for scientific purposes (South African Bureau of Standards, SANS 10386, 2008). The project was approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University (reference #10GK_HIL01).

3.2.1.2 Diet-induced obesity

A model of diet-induced obesity (DIO) [Pickavance *et al.*, 1999] with the concurrent development of insulin resistance was utilized. This model is one of hyperphagia-induced obesity and this 16-week diet has been characterized in our laboratory and shown to be physiologically relevant and comparable to the human equivalent of insulin resistance as a result of obesity [Du Toit *et al.*, 2005]. This high caloric diet consists of normal rat chow pellets, supplemented with sucrose and condensed milk, resulting in an elevated sugar and carbohydrate intake coupled to a low protein intake (refer to Table 3.1 for the nutritional composition of the control versus DIO diet). The animals in the control group received normal unsupplemented rat chow pellets.

Table 3.1: Macronutrient composition (% total energy value) of diet consumed by control versus diet-induced obese (DIO) animals

	Control	**DIO**
Fat (g/ 100g)	4.8	4.6
Cholesterol (mg/100g)	3	10
Sucrose (g/100g)	6.6	27.7
% Protein	17.1	9.4
% Carbohydrates	34.6	45.8
kJ/ 100g	1272	1173

3.2.1.3 *Prosopis glandulosa* treatment

The *P. glandulosa* powder used in the treatment protocol in the entire study is from herbal origin and it consists solely of the dry-milled pods of the *P. glandulosa* tree (commonly known as Honey mesquite) [George *et al.,* 2011]. Rats were treated with *P. glandulosa* at a dose of 100 mg/kg/day for a total period of 8 weeks (i. e. only the last 8 weeks of the 16 week high caloric diet). *P. glandulosa* was weighed daily for each animal in the treatment group and set in a mixture of commercially available gelatine/ jelly cubes of 1 ml volume. This jelly cubes were fed to each animal individually, to ensure absolute compliance and dose control. The dosage of 100 mg/kg/day *P. glandulosa* was calculated based on the daily dosage prescribed for human adults. We have previously shown this dose to elicit metabolic changes [George *et al.,* 2011; Huisamen *et al.,* 2012]. To accustom the animals to the researcher and the taste of the jelly cubes, all animals were fed placebo jelly cubes (jelly cubes without *P. glandulosa*) for 1 week prior to the start of the actual treatment program. During the 8 weeks experimental period, the control animals received placebo jelly cubes.

In the absence of published data on the medicinal value of *P. glandulosa*, there was not much known about its adverse effects. For the purpose of safety of our animals we had previously conducted a standard toxicology study, at the MRC, to determine the side effects of over-consumption of this product. *P. glandulosa* was fed to adult Vervet monkeys at dosages of 1x, 5x and 25x the therapeutic dose for a period of 3 months. The results showed no clinically relevant changes in any of the measured parameters and thus *P. glandulosa* consumption proved to be safe over this short-term period. In addition, the monkeys did not show signs of hypoglycaemia at any stage over the three-month experimental period. Data were compiled in a 66-page document [available on internet, George *et al.,* 2011].

3.2.1.4 Division into groups

Experimental rats were divided into 2 groups; a control group and a diet-induced obese group (DIO). The control group was sub-divided into a control placebo group (C-PLA), that received normal rat chow and jelly cubes without *P. glandulosa* and a control *P. glandulosa* group (C-PG) that received normal rat chow and *P. glandulosa* mixed into jelly cubes (n = 5). Rats in the DIO group were also sub-divided into two groups, a DIO placebo group (D-PLA), that received the special DIO diet with jelly cubes without *P. glandulosa* and a DIO *P. glandulosa* group (D-PG), that received the special DIO diet and *P. glandulosa* mixed in jelly cubes (n = 5). A total of 20 muscles were utilized, therefore 10 animals per experimental group (control vs. DIO) and 5 animals per sub-division (treatment vs. no treatment) (Fig. 3.3).

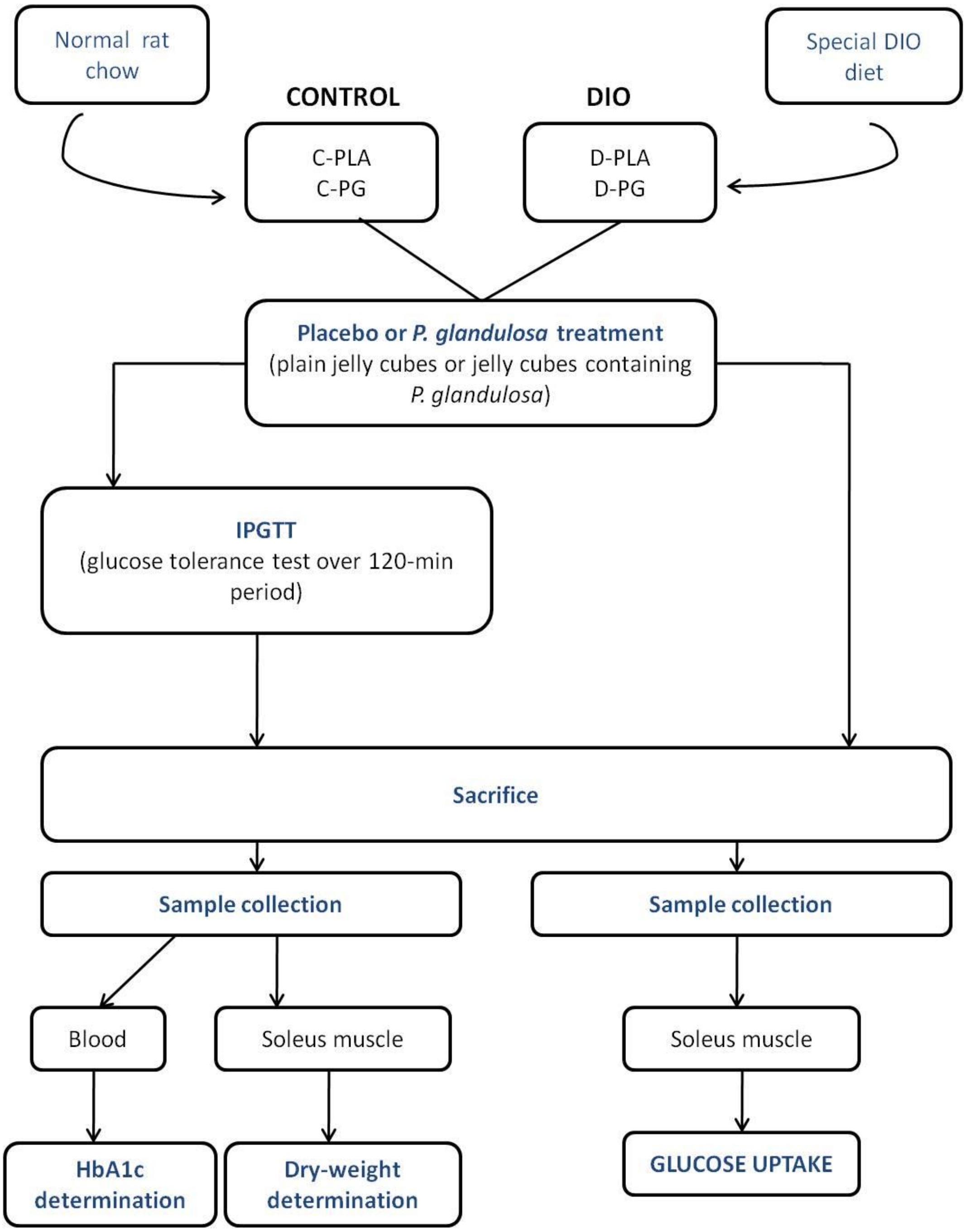


Figure 3.3: Schematic representation of the experimental design

3.2.2 Biochemical analysis

3.2.2.1 Sacrifice and sample collection

After an overnight fasting period, the animals were weighed (to determine body mass) and then received an overdose of sodium pentobarbital (200 mg/kg, intraperitoneal). The animals were continually monitored until total loss of consciousness was reached, as indicated by a total lack of response after a foot pinch. Blood samples were collected from the abdominal cavity, by means of a Pasteur pipette and allowed to clot on ice. After clotting the blood samples were subjected to centrifugation (microcentrifuge at 1000 g at 4°C for 10 min), where after aliquots were prepared for HbA_1c level determination. HbA_1c is used to monitor long-term glycaemic control, adjust therapy, assess the quality of diabetes care and predict the risk for the development of complications. The rest of the aliquots were stored at −80 °C for insulin level determination (refer to section 3.2.2.3). Abdominal fat was also removed and weighed (to determine intra-peritoneal fat mass). Serum insulin levels were determined via a Coat-A-Count® Radioimmunoassay (RIA) kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). In a separate non-fasted group of animals, both the soleus muscles were removed and placed in ice-cold Krebs Hensiliteit buffer (KHB) for further analysis (refer to section 3.2.3).

3.2.2.2 Intra-peritoneal Glucose Tolerance Test (IPGTT)

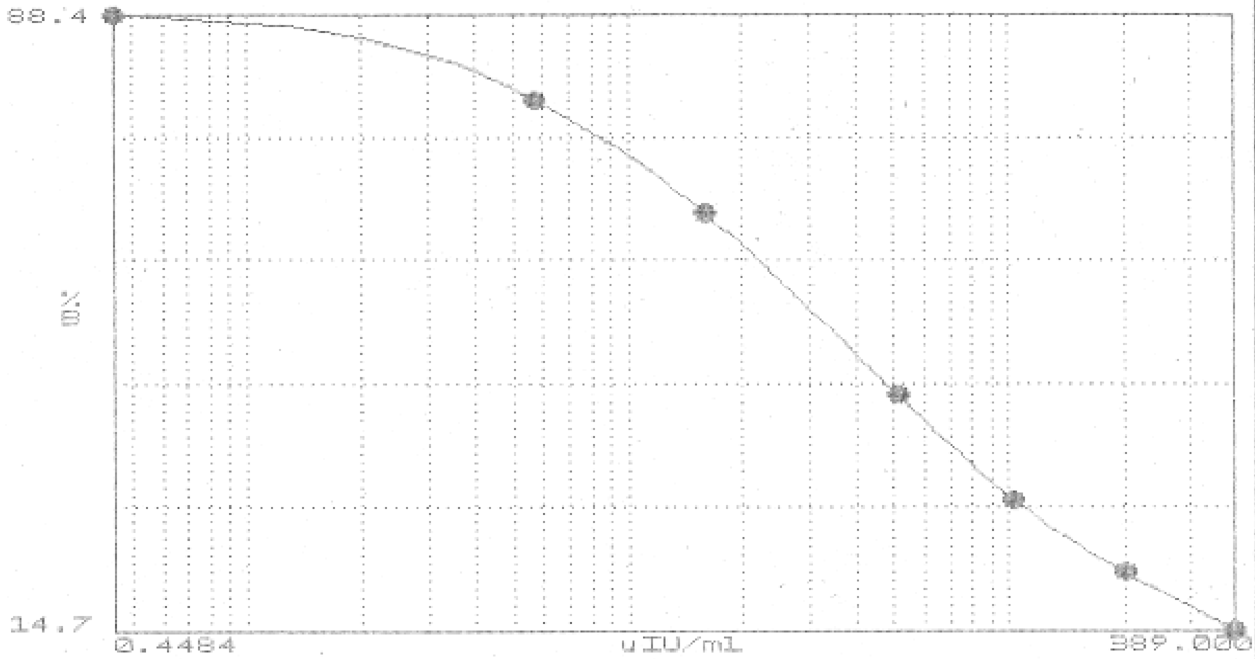
After an overnight fasting period, the intra-peritoneal glucose tolerance test was conducted on all the animals. At the start of the IPGTT, blood was collected via a once-off tail prick to determine baseline plasma glucose concentration (measured using a glucometer (GlucoPlus™, Montreal, Canada)). Animals were then injected intraperitoneally with 1 g/kg of a 50% sucrose solution, where after blood glucose levels were monitored at different time points over a 120-min period.

3.2.2.3 Serum insulin determination: Radioimmunoassay (RIA) (Coat-A-Count® Insulin, Diagnostic Products Corporation, LA, USA)

The fasting blood samples collected at the time of sacrifice (refer to section 3.2.2.1) were used for serum insulin determination. The Coat-A-Count Insulin procedure is a solid-phase RIA. ^{125}I-labeled insulin and the insulin in the blood sample, competes for binding to insulin-specific antibodies. These antibodies are immobilized to the wall of polypropylene tubes. Decanting the supernatant from the tubes terminates the competition and isolates the antibody bound fraction of the radiolabeled insulin. By counting the tubes in a gamma-counter, the presence of insulin in the blood sample can be measured. The calibration range of this assay is 5 – 350 µIU/ml (WHO 1st IRP 66/304). All samples were analyzed in duplicate. Prior to the commencement of the assay, all the components of the assay were brought to room temperature, as instructed by the manufacturers. Uncoated 12 x 74 mm polypropylene tubes were labeled for total count (T) and non-specific binding (NSB) respectively. Insulin-antibody coated tubes were labeled for standards and serum sample. 200 µl of the zero calibrator A was pipetted into the NSB and A tubes. 200 µL of the remaining calibrator and serum sample were pipetted in the tubes. 1.0 ml of ^{125}I insulin was added to each tube and subsequently vortexed. Samples were incubated for 18 to 24 hours at room temperature and decanted thoroughly. This was done by placing each tube (except the total count tube) in a foam decanting rack and allowing the tubes to drain for 2 to 3 minutes. Following this, each tube was struck on absorbent paper and excessive liquid dried from the tubes, to remove the excess moisture for enhanced precision of the assay. The radioactivity of each tube was then measured in a gamma counter (Cobra II Auto Gamma, A. D. P, South Africa) for 1 min per tube and the sample antibody binding affinities, calculated from an insulin standard curve, which was generated by the gamma counter. Figure 3.4 is an example of the standard curve generated by the gamma-counter.

Protocol #: 6 INSULIN2 User :

Assay Type: RIA %REF. BOUND



STD #	CPM	DEFINED DOSE	%B/F	CALC.DOSE	% DIFF
1	DET LIM	0.44837	88.43	0.44555	-0.63
2	12301	5.70000	78.15	5.70000	0.00
3	10211	16.00000	64.55	16.00000	0.00
4	6871	51.00000	42.82	51.00000	0.00
5	4959	102.00000	30.38	101.99997	0.00
6	3629	201.00000	21.72	200.99993	0.00
7	2557	389.00000	14.75	388.99883	0.00

Curve Fit : AUTO SPLINE	Transform X : LOG	Transform Y : NONE
Corr.Coef. : 1.0000		Slope :
Det.Limit : 0.4484		Para.Coef. : 1.000
%NSB/Total : 0.7568		%Ref/Total : 39.947
ED20 : 235.40	ED50 : 35.383	ED80 : 4.7403
%Sigma Std. : 2.4484		
RMS : .256802		Iterations : 1
Smoothing F.: 0.00000	%Smoothing : 0	Infl.Pt. : 41.3033
#Infl.Pts. : 1		#Extrema : 0

Figure 3.4: An example of the standard curve generated by the gamma-counter

3.2.3 2-Deoxy-D-^{3}[H] glucose uptake by isolated soleus muscle

The ability of the soleus muscle strip to accumulate 2-DG was measured as described previously by Sartori *et al.* (2009). After an overnight fast, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and both soleus muscles were rapidly isolated, separated into intact strips and placed in ice-cold oxygenated KHB (pH 7.3). The KHB was supplemented with 1% bovine serum albumin (BSA) and 2 mM sodium pyruvate. Glucose transport activity, in the presence or absence of insulin, was assessed as 2-DG uptake, as described by Sartori *et al.*, (2009). Muscle strips were left to equilibrate in KHB in a shaking waterbath (180 strokes/min) for 15 min at 37 °C. Muscle strips were then stimulated for 30 min with 1, 10 or 100 nM insulin while oxygenated (95% O_2 - 5% CO_2) continuously. After insulin treatment, the tissue was incubated with 1.5 µCi/ml 2-DG (PerkinElmer, Boston) for 30 min, to allow for glucose uptake. The reaction was stopped by the addition of 400 µM phloretin. After this treatment, the tissue was rinsed with KHB for 10 min and dissolved in 1 N sodium hydroxide (NaOH) at 70 °C. After complete solubilisation, 2 ml scintillation fluid was added to 200 µl aliquot of the samples and analysed for radioactivity in a scintillation counter (Beckman). An aliquot was used to determine protein levels by means of the Lowry method [Lowry *et al.*, 1951].

For protein content determination by the method of Lowry [Lowry *et al.*, 1951] three BSA protein standards of known concentration [0.238 mg/ml; 0.476 mg/ml and 0.952 mg/ml] were used and 0.5 N NaOH used as the blank. The reaction buffer, which contained 2% Na_2CO_3, 1% $CuSO_4.5H_2O$ and 2% NaK^+ tartrate, was freshly prepared prior to experimentation. The assay was done in duplicate and 50 µl of blank, standards and samples were used to perform the protein assay. 1 ml of the reaction buffer was added to the blank, standards and samples, rapidly vortexed and allowed to stand at room temperature for 10 min. Afterwards 0.1 ml Folin-Ciocalteu's phenol reagent (1:2 dilution with distilled water) was added, vortexed and permitted to stand for 30 min. This resulted in a colour development of which the absorbance was read at 750 nm against the blank. The standard curve was used to determine the unknown protein concentrations.

3.2.4 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance between groups was assessed via a 2 way-ANOVA, which was followed by a Bonferroni post-hoc test for multiple comparisons. $p < 0.05$ was considered as statistically significant. Statistical analysis of data was performed using GraphPad Prism version 5.

3.3 RESULTS

3.3.1 Biometric characteristics of experimental animals

As also seen in the publication by Huisamen *et al.* (2013), the animals on the high caloric diet (D-PLA) had a significantly higher body mass ($p < 0.0001$), compared to their control counterparts (C-PLA), after the 16-week feeding programme (Table 3.2). This obese state was also associated with significantly elevated intra-peritoneal fat mass ($p < 0.0001$), fasting blood glucose ($p < 0.001$) and fasting serum insulin levels ($p < 0.01$). This led to an increased homeostatic model assessment of insulin resistance index (HOMA-IR), which is an indicator of whole-body insulin resistance. These results show that the animals on the 16-week high caloric diet developed insulin resistance. *P. glandulosa* elicited no significant effect with regards to the body mass or intra-peritoneal fat mass, in neither control (C-PG) nor DIO (D-PG) groups, compared to their respective controls. The DIO animals had significantly lower blood glucose levels after *P. glandulosa* treatment (D-PG), compared to their control counter parts (D-PLA). In other words, the glucose levels of the D-PG group were no longer significantly elevated compared to the C-PLA; however the HOMA-IR of the D-PG was still significantly higher. No significant differences were observed in any of the groups with regards to the HbA_1c level. The HbA_1c level is used to identify the average plasma glucose concentration over prolonged periods of time. The HbA_1c levels are within the normal range, i.e. these rats were not yet type 2 diabetic. No significant differences were observed with regards to the dry-mass of the soleus muscles.

Table 3.2: Biometric characteristics of the animals after *P. glandulosa* treatment

	C-PLA	C-PG	D-PLA	D-PG	*p*-values
Body mass (g)	433.70 ± 9.30	438.60 ± 9.30	507.70 ± 22.90***	534.30 ± 11.70***	*** $p < 0.0001$ vs. respective control
Intraperitoneal fat mass (g)	18.10 ± 2.70	11.00 ± 1.80	28.00 ± 1.74***	34.00 ± 1.40***	*** $p < 0.0001$ vs. respective control
Soleus muscle mass (dry-weight) (mg)	33.00 ± 1.00	32.00 ± 3.00	40.00 ± 2.00	38.00 ± 2.00	No significance
Fasting glucose (mmol/L)	5.42 ± 0.17	5.43 ± 0.18	6.40 ± 0.17*	5.6 ± 0.19*	* $p < 0.05$ C-PLA vs. D-PLA; D-PLA vs. D-PG
Fasting insulin (µIU/ml)	17.12 ± 0.80	14.07 ± 1.50	34.33 ± 9.06*	35.93 ± 10.21*	* $p < 0.05$ vs. respective control
HOMA-IR	4.73 ± 0.71	3.40 ± 0.41	8.96 ± 2.65*	7.88 ± 3.30*	* $p < 0.05$ vs. respective control
HbA_1c (%)	2.58 ± 0.04	2.60 ± 0.03	2.80 ± 0.10	2.80 ± 0.10	No significance

The data are expressed as mean ± SEM; Analysis by two-way ANOVA; n = 6

3.3.2 Intraperitoneal glucose tolerance test (IPGTT)

Figure 3.5 shows that the blood glucose levels of the D-PLA group were significantly higher compared to the C-PLA group from 10 minutes through to 45 minutes post-glucose load (refer to Fig. 3.5 for p-values). Figure 3.5 also illustrates that, following oral glucose load, the blood glucose levels of PLA rats increased to 6.44 ± 0.42 mmol/L from a baseline value of 5.42 ± 0.17 mmol/L before slowly declining to 4.44 ± 0.15 mmol/L after 120 min. Similarly, in the treated control rats (C-PG), the blood glucose levels increased to 6.20 ± 0.43 mmol/L from a baseline value of 5.43 ± 0.18 mmol/L before slowly declining to 4.58 ± 0.07 mmol/L after 120 min. *P. glandulosa* treatment had no significant effect on the glucose handling of the rats in the control group. Conversely, the blood glucose levels of the animals on the high-caloric diet (D-PLA), increased from a basal level of 6.40 ± 0.17 mmol/L to peak at 7.12 ± 0.20 mmol/L and decrease to 5.22 ± 0.26 mmol/L by 120 min. The blood glucose levels of the D-PG group increased from a basal level of 5.6 ± 0.19 mmol/L to peak at 6.97 ± 0.13 mmol/L and decrease to 4.75 ± 0.21 mmol/L by 120 min. The slight effect on blood glucose handling that *P. glandulosa* treatment produces in animals on the high-caloric diet is emphasized by *P. glandulosa* treatment being able to slightly decrease the elevated glucose levels of the D-PG group when compared to the D-PLA group, however these groups do not differ statistically. Additionally, the glucose levels of the PLA rats peaked 5 minutes post-glucose load, where the glucose levels of the D-PLA rats only peaked after 10 minutes. *P. glandulosa* treatment was able to decrease the elevated glucose levels after only 3 minutes in both C-PG as well as D-PG groups. Furthermore, the area under the curve (AUC), which is a measure of impaired glucose tolerance, proved significantly larger in the D-PLA when compared to the C-PLA (697.22 ± 14.20 vs. 593.36 ± 12.02; $p < 0.001$). In addition, the high-caloric *P. glandulosa* treated rats displayed an AUC which was significantly smaller than the untreated rats on the high-caloric diet (649.98 ± 17.57 vs. 697.22 ± 14.20; $p < 0.05$).

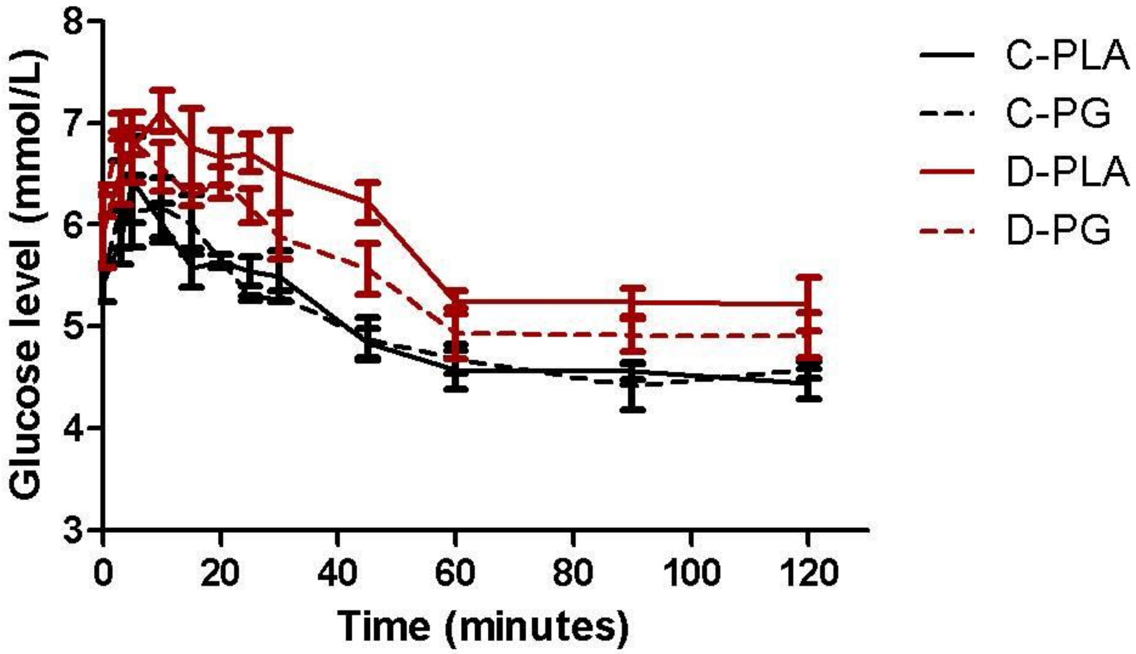


Figure 3.5: The response of plasma glucose to intra-peritoneal glucose tolerance test (IPGTT). Glucose handling was measured, by tail prick, over a 2-hour period after an i.p injection of a sucrose solution. Refer to section 3.2.2.2 of Methods. Data is expressed as mean ± SEM. *C-PLA vs. D-PLA:* 10min (*p < 0.05); 15min (**p < 0.01); 20min (*p < 0.05); 25min (**p < 0.01); 30min (*p < 0.05); 45min (***p < 0.001); *C-PLA vs. D-PG:* 3min (*p < 0.05); *C-PG vs. D-PLA:* 10min (*p < 0.05); 20min (*p < 0.05); 25min (***p < 0.001); 30min (**p < 0.01); 45min (**p < 0.01)

3.3.3 2-Deoxyglucose (2-DG) uptake by isolated soleus muscle

According to the results depicted in Figure 3.6, no significant differences were found between the control (C-PLA) and DIO (D-PLA) groups, neither under basal conditions nor after any concentration of insulin stimulation. *P. glandulosa* treatment significantly increased the uptake of glucose in the control group (C-PG) after 10 nM (0.143 ± 0.019 vs. 1.101 ± 0.017 pmol/mg/30 min; $p < 0.05$) and 100 nM (0.132 ± 0.017 vs. 0.099 ± 0.011 pmol/mg/30 min; $p < 0.01$) insulin stimulation, when compared to its control (C-PLA). No significant effect in die DIO (D-PG) group was observed.

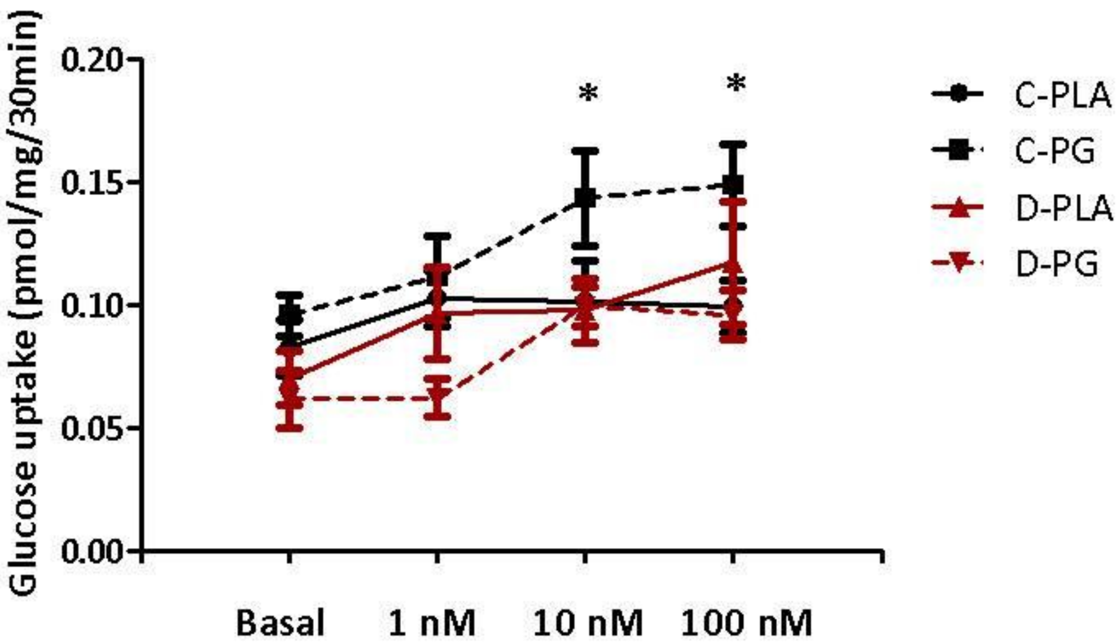


Figure 3.6: Glucose uptake by isolated soleus muscle strips from control and DIO rats at basal levels and after stimulation with 1 nM, 10 nM and 100 nM insulin. The insulin sensitivity of the muscle strips was determined by means of measuring the ability of the muscle to accumulate radio-labeled deoxyglucose before and after insulin stimulation. Refer to section 3.2.3 of Methods. Data is expressed as mean ± SEM. * $p < 0.05$ C-PLA vs. C-PG (10 nM); * $p < 0.01$ C-PLA vs. C-PG (100 nM)

3.4 DISCUSSION

According to the WHO, the prevalence of obesity has reached epidemic proportions, with more than 1.4 billion adults reported to have been overweight in 2008 [WHO, 2013]. Obesity has always been linked to insulin resistance or decreased insulin sensitivity and the consequent development of T2D [DeFronzo, 1997]. The general assumption has always been that insulin resistance is acquired as a result of elevated free fatty acids, which is accompanied by hyperglycaemia and hyperinsulinaemia [DeFronzo, 1997; DeFronzo and Ferrannini, 1991].

Drugs such as sulphonylureas and metformin are currently used as oral anti-diabetics to improve insulin sensitivity in peripheral tissues and reduce hyperglycaemia and hyperinsulinaemia [Aljada *et al.,* 2009]. However, many of these current diabetes drugs are often associated with serious side effects [Viner *et al.,* 2010], e.g. thiazolidinediones have been withdrawn from the market because of liver toxicity. Therefore, the use of complementary and alternative medicine has increased in recent years [Kennedy, 2005]. Numerous herbal substances, especially those rich in phenolic compounds, alkaloids, flavonoids, terpenoids, coumarins and glycosides, have previously been demonstrated to be potential treatment candidates for addressing the issues of obesity and diabetes [Tabatabaei-Malazy *et al.,* 2012]. These herbal substances have been shown to, amongst others, effectively prevent weight gain, significantly reduce body weight of individuals that were already overweight and have glucose-lowering abilities [Astell *et al.*, 2013; Hasani-Ranjbar *et al.,* 2009; Hui *et al.,* 2009].

P. glandulosa is a herbal product currently marketed as a food supplement with, amongst others, blood glucose stabilizing properties as well as having the ability to enhance glucose utilization. It is manufactured and distributed in South Africa and consists solely of the dry-milled pods of the *P. glandulosa* tree (Honey mesquite). In the current study, we evaluated the possible anti-obesity effects, which included glucose-lowering and insulin-sensitizing abilities of *P. glandulosa,* in a hyperphagia-induced obese rat model.

Following a 16-week high-caloric feeding program, we successfully generated a model of insulin resistance, as a result of obesity. Glucose tolerance was determined in these animals, before and after treatment with *P. glandulosa* as well as the skeletal muscle of these animals used to determine radioactive glucose uptake. An important observation was that the 16-week high-caloric diet was not sufficient to induce T2D in these animals, as the fasting blood glucose levels never rose above 6.5 mmol/L and the HbA_1c levels always falling below the diabetic range of 7.5% [Rodríguez-Mañas *et al.,* 1998] in all four the experimental groups. The rats in our study presented with HbA_1c levels lower that 3%.

The DIO insulin resistant model used in this study has previously been characterized in our laboratory and shown to be physiologically relevant and comparable to the human equivalent of insulin resistance as a result of obesity [Du Toit *et al.,* 2005, 2008]. Hyperphagia, increased thermogenesis, hyperleptinaemia and mild insulin resistance characterize this model [Pickavance *et al.*, 1999]. In this study it was established that the rats were obese and insulin resistant after 16 weeks on a high caloric diet. This was validated by the significant increased body weight, intraperitoneal fat weight, fasting blood glucose and -insulin levels as well as an increased HOMA-IR index of DIO (D-PLA) versus control (C-PLA) rats (Table 3.2). Chronic treatment with *P. glandulosa* could significantly decrease the elevated blood glucose levels, observed in the obese rats, to near control levels. This is depicted in Table 3.2, where the D-PLA rats displayed elevated fasting glucose levels of 6.40 ± 0.17 mmol/L compared to 5.42 ± 0.17 mmol/L in the PLA rats and after *P. glandulosa* treatment, the fasting glucose levels were significantly reduced to 5.6 ± 0.19 mmol/L. This same phenomenon was observed in the clinically important two-hour blood glucose tolerance test, where the values, after an i.p glucose load, were significantly higher in the D-PLA animals, compared to the PLA animals, yet these elevated levels were slightly reduced by *P. glandulosa* treatment (Fig. 3.5), however not significantly. As previously reported [George *et al.*, 2011], this underscores the slight effect on glucose handling that *P. glandulosa* elicits. As the results of a previous study conducted in our laboratory also demonstrated, when treating insulin resistant DIO rats with *P. glandulosa* for 16 weeks, a significant increase in cardiomyocyte insulin sensitivity was observed, though without any observed differences in the fasting plasma glucose and insulin levels [George *et al.,* 2011]. Conversely, in our current study we found a slight decrease in the elevated fasting glucose levels of the insulin resistant (D-PG) animals treated with *P. glandulosa*, but no significant differences was found with regards to the 2-DG uptake in skeletal muscle in the DIO animals. The only significant differences observed in the 2-DG uptake in skeletal muscle were between the treated and untreated control animals. The mechanism by which *P. glandulosa* effectively reduces high levels of glucose and increases insulin sensitivity of skeletal muscle of control rats is still unknown. Studies of herbal substances acting as anti-diabetic and anti-obesity treatments, suggest that the herbal substances might be act via increasing the animals energy expenditure, which would lead to reduced weight gain and fat mass [Lee *et al.,* 2012; Tan *et al.*, 2011], consequently reducing blood glucose levels and ultimately ameliorate insulin resistance. Studies have indicated that plant-based products may contribute to body weight loss or gain in animals [Xu *et al.,* 2013; Bwititi *et al.,* 2001]; however in our study *P. glandulosa* treatment did not significantly alter the body mass or intraperitoneal fat mass, suggesting that this was not the mode of action of *P. glandulosa*. Since insulin resistance is associated with abnormalities in insulin's signal transduction [Haring and Mehnert, 1993; Nolan *et al.*, 1994] and glucose transport [Lee *et al.*, 2012; Tan *et al.,* 2011], another possible mechanism of action, is by increasing the phosphorylation of proteins regulating glucose uptake. In the study by Lee *et al.* (2012) they found that IRβ, IRS-1, PKB/Akt, GSK3α/β and GLUT4 were upregulated after treating rats with 200 mg/kg *Panax ginseng* for 18 weeks. Glucose transport is the rate-limiting step of glucose uptake and metabolism in insulin-sensitive tissues, therefore altered GLUT4 activity is one of the major factors responsible for decreased glucose utilization in skeletal muscle tissue in diseases such as T2D and its precursor, insulin resistance [Liu *et al.*, 2010]. Many researchers therefore allude to the increased expression and translocation of GLUT4 as the mechanism of action to increase glucose uptake by muscle cells and so-doing decrease blood glucose levels [Tan *et al.,* 2011]. This is amplified in studies such as Stenbit *et al.* (1997), where they showed that GLUT4 heterozygous knockout mice exhibited increased serum glucose and insulin and reduced muscle glucose uptake. Conversely, Galuska *et al.* (1998) showed that genetic over-expression of GLUT4 in skeletal muscle ameliorated the development of insulin resistance. Lastly, herbal substances can act by means of inducing insulin release [Kim and Kim, 2008; Kawano *et al.,* 2009; Blair *et al.,* 1999], which was not evident in our study. With this in mind, it is however very difficult to determine the precise mechanism of action when researching herbal substances as herbal preparations may contain more than one active compound, each with a different therapeutic effect. Therefore more research is needed.

In summary, while it is difficult to point out the exact single compound which exerted the specific effects on glucose metabolism, since the active ingredient/s of *P. glandulosa* has not yet been identified, this natural herbal substance showed end-point effects of enhanced glucose tolerance in our obese rat model. A lot more research is needed to determine the active ingredient/s in *P. glandulosa* as well as the exact mode of action of that active ingredient/s.

CHAPTER 4: THE EFFECT OF CHRONIC *PROSOPIS GLANDULOSA* TREATMENT ON MUSCLE STRENGTH AND FATIGUE AFTER ELECTRICAL FIELD STIMULATION

4.1 GENERAL INTRODUCTION

Physical fatigue, also referred to as peripheral fatigue, is usually accompanied by deterioration in physical performance [Roots *et al.*, 2008; Fitts, 1994]. The two mechanisms thought to be responsible for this decrease in muscle performance are oxidative stress and exhaustion. It has been found that intense exercise leads to the production and accumulation of excessive amounts of reactive free radicals, resulting in oxidative stress injury to the body [Allen *et al.*, 2008]. The exhaustion theory proposes that fatigue is the result of energy source depletion and excess metabolite accumulation [You *et al.,* 2011; Allen *et al.*, 2008]. Numerous studies have reported on the anti-fatigue, ergogenic and adaptogenic effects of various medicinal plants. An ergogenic aid can be broadly defined as a substance used for the purpose of enhancing physical performance [Thein *et al.*, 1995; Calfee and Fadale, 2006], whereas an adaptogenic aid refers to any of various natural substances that combats stress and increase resistance to stress, usually without producing any side-effects [Rege *et al.,* 1999]. For centuries, athletes have used herbal substances to improve their physical performance. It has been reported that the Greek Olympians, as early as BC 776, used dried figs, mushrooms and strychnine to perform better at sporting events [Grivetti and Applegate, 1997]. Research into the use of performance-enhancers has gained enormous prominence since the days of the ancient Greeks. In 1889, Dr Brown-Sequard announced his landmark discovery at a scientific meeting in Paris, were he revealed that he had found a substance that reversed the ailments he experienced as a 72-year-old man. He had reportedly injected himself with the extract of dog and guinea pig testicles, under the assumption that these organs had "internal secretions that acted as physiologic regulators"

[Calfee and Fadale, 2006; Hoberman and Yesalis, 1995]. This claim was later substantiated by the discovery of hormones, in 1905, and the isolation of testosterone, in 1935 [Calfee and Fadale, 2006]. During the 1950's, Russian Olympian weightlifters began injecting themselves with performance-enhancers and later the Americans followed suit, by producing an anabolic steroid, known as Dianabol [McDevitt, 2003]. From then on, steroids and various stimulants spread throughout sport without reproach. It was not until the 1960's that the International Olympic Committee banned steroid use and began formal drug testing [Williams, 1994]. To highlight the "win at all costs" attitude of many athletes, Goldman conducted a survey in 1994. In this survey he asked aspiring Olympians 2 simple questions. The first question he asked them was, "If you were offered a banned performance-enhancing substance that guaranteed that you would win an Olympic medal and you could not be caught, would you take it?" Amazingly, 98% of the athletes replied, "yes". The second question he asked was, "Would you take a banned performance-enhancing drug with a guarantee that you will not be caught, you will win every competition for the next 5 years, but will then die from adverse effects of the substance?" More than 50% of the athletes said that they would take the banned substance [Calfee and Fadale, 2006]. Due to the "win at all costs" attitude of many athletes, they still make use of banned substances, in the hope of not being caught. A recent and very public incident was that of the seven consecutive winner of the Tour de France cycling race, Lance Armstrong, who after being charged in June 2012, subsequently admitted to having used illicit performance-enhancers.

The trend of utilizing herbal substances to improve performance, speed up recovery, maintain health and fitness during intense periods of training, increase muscle mass and reduce body fat has increased. Herbal substances can exert anti-fatigue effects by means of increasing glycogen storage, prolonging performance in exercise endurance, and decreasing metabolite accumulation. Numerous studies have shown that exogenous antioxidants can reduce exercise-induced oxidative stress [Zheng *et al.,* 2012; Chen *et al.,* 2012; Bucci, 2000; Song *et al.* 2009; Bae *et al.* 2002; Ni *et al.* 2009; Stavro *et al.* 2005]. Ginseng (especially Korean and Chinese ginseng) is among the most popular and most researched herbal substances consumed to enhance exercise and sport performance [Chen *et al.,* 2012; Bucci, 2000]. The term ginseng refers to different species, all of the Araliaceae plant family, each plant having its own specific physiological effects. Panax ginseng (Chinese or Korean ginseng) is one of the most commonly used and highly researched species of ginseng. A total of 705 components have been isolated from ginseng, such as ginsenosides, polysaccharides, peptides and polyacetylenic alcohols [Hui *et al.,* 2009]. Panax ginseng has traditionally been used as a “tonic”, performance-enhancer, anti-cancer agent and aphrodisiac [O’Hara *et al.,* 1998]. To date it has been found that Panax ginseng has multiple effects, amongst others, anti-inflammatory, antioxidative, anti-diabetic and anti-fatigue effects [Chae *et al.* 2009; Mochizuki *et al.* 1995; Song *et al.* 2009; Bae *et al.* 2002; Ni *et al.* 2009; Stavro *et al.* 2005; Lee *et al.* 2005; Liu *et al.* 2003]. In a study by Wang *et al.* 2010, they evaluated the anti-fatigue effects of water-soluble polysaccharides from ginseng, using FST, which is an animal model of fatigue. They also tested the effects of these water-soluble polysaccharides on biochemical markers for fatigue, such as glucose, triglycerides, lactic dehydrogenase, creatine phosphokinase, malondialdehyde, superoxide dismutase and glutathione peroxidase. In their article they reported that mice treated with the water-soluble polysaccharides displayed less time in an immobile state during the FST. In addition, the FST-induced reduction in glucose and glutathione peroxidise, and the increase in creatine phosphokinase, lactic dehydrogenase and malondialdehyde levels, all indicators of fatigue, were restored to baseline levels in the animals treated with the water-soluble polysaccharides. Their findings coincides with those of Yu *et al.* (2006), who have demonstrated similar effects of polysaccharides from another plant species, the *Euphorbia kansui* (Euphorbiaceae), on malondialdehyde and glutathione peroxidase levels. Wang *et al.* (2010) proposed two possible anti-fatigue mechanisms: (1) via prevention of lipid oxidation, by means of modifying several enzyme activities and (2) via triglyceride (or fat) mobilization during exercise, as indicated by the decrease in triglyceride levels and the simultaneous increase in glucose levels in the blood. However, further investigation is needed in order to identify the mechanism by which ginseng polysaccharides affect fat mobilization. If fat mobilization is indeed ginseng’s anti-fatigue mechanism, this would be advantageous during prolonged exercise since better utilization of triglycerides allows the sparing of glycogen and glucose and therefore delays fatigue [Jung *et al.,* 2004]. Therefore, more research into herbal substances is needed to find agents that have the ability to reduce metabolite production and/or improve energy utilization.

The motivation for the current part of this study came from anecdotal claims that race horses that consumed large amounts of *P. glandulosa* seemed to be able to endure longer periods of physical activity and thus seemed to be resistant to fatigue. However, these claims are based on casual observations or indications rather than rigorous scientific analysis. Therefore, I opted to scientifically examine this observation by electrically stimulating soleus muscle from rats to fatigue and determining the extent of recovery after the fatigue period. If a cheap, natural and readily available substance is proven to augment muscle fatigue; it could have enormous implications in the sporting arena.

4.2 METHODS

4.2.1 Experimental design and sample collection

4.2.1.1 Animal care and treatment regime

The animal care and *P. glandulosa* treatment was the same for all groups of animals in this study. Refer back to section 3.2.1.1 for "animal care" and section 3.2.1.3 for *P. glandulosa* treatment".

4.2.1.2 Division into groups

Experimental rats were divided into 2 groups: a control placebo group (PLA), that received normal rat chow pellets and jelly cubes without *P. glandulosa* and a *P. glandulosa* group (PG) that received normal rat chow and *P. glandulosa* mixed into jelly cubes (n = 10 each). A total of 20 isolated muscles were utilized, therefore 10 animals per experimental group (treatment vs. no treatment).

4.2.1.3 Sacrifice and sample collection

After 10-weeks of *P. glandulosa* treatment, the animals were weighed (to determine body mass) and then received an overdose of sodium pentobarbital (200 mg/kg, intraperitoneal). The animals were continually monitored until total loss of consciousness was reached (as explained in section 3.2.2.1). Both the soleus muscles were removed and placed in ice-cold KHB for further analysis (refer to section 4.2.2 below).

4.2.2 Muscle fatigue stimulation protocol

Skeletal muscle fatigue was determined by methods previously described by Gordon *et al.* (2010) and El-Khoury *et al.* (2012). After the animals were euthanized with an overdose of sodium pentobarbital (200 mg/kg, intraperitoneal), one of the soleus muscles, with tendinous insertions intact, was removed and placed in ice-cold KHB. The KHB solution contained in mM: NaCl 119, KCl 4.74, $CaCl_2.2H_2O$ 1.25, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 24.9, Na_2SO_4 0.6 and glucose 10. All these chemicals were purchased at Merck (Pty) Ltd – South Africa. The intact soleus muscle was then removed from the cold KHB buffer and vertically suspended between a pair of platinum electrodes in a water-jacketed organ bath of a PowerLab® apparatus (ADInstruments, Inc., Colorado Springs, CO), containing continuously gassed (95% O_2/5% CO_2) KHB solution at 25°C (pH 7.4) (Fig. 4.1). The physiological stability of rat skeletal muscle *in vitro* is temperature-dependent and stability for muscle strips of 1-2mm diameter is better at 25°C compared to the *in vivo* temperature of 37°C [Segal *et al.*, 1986]. The base of the muscle was fixed to an immobile hook and the other end tied to an isometric force transducer. The position of the force transducer could be adjusted by a micro-positioner, thus altering preload. The muscles were left to stabilize for 30 minutes before electrical stimulation commenced.

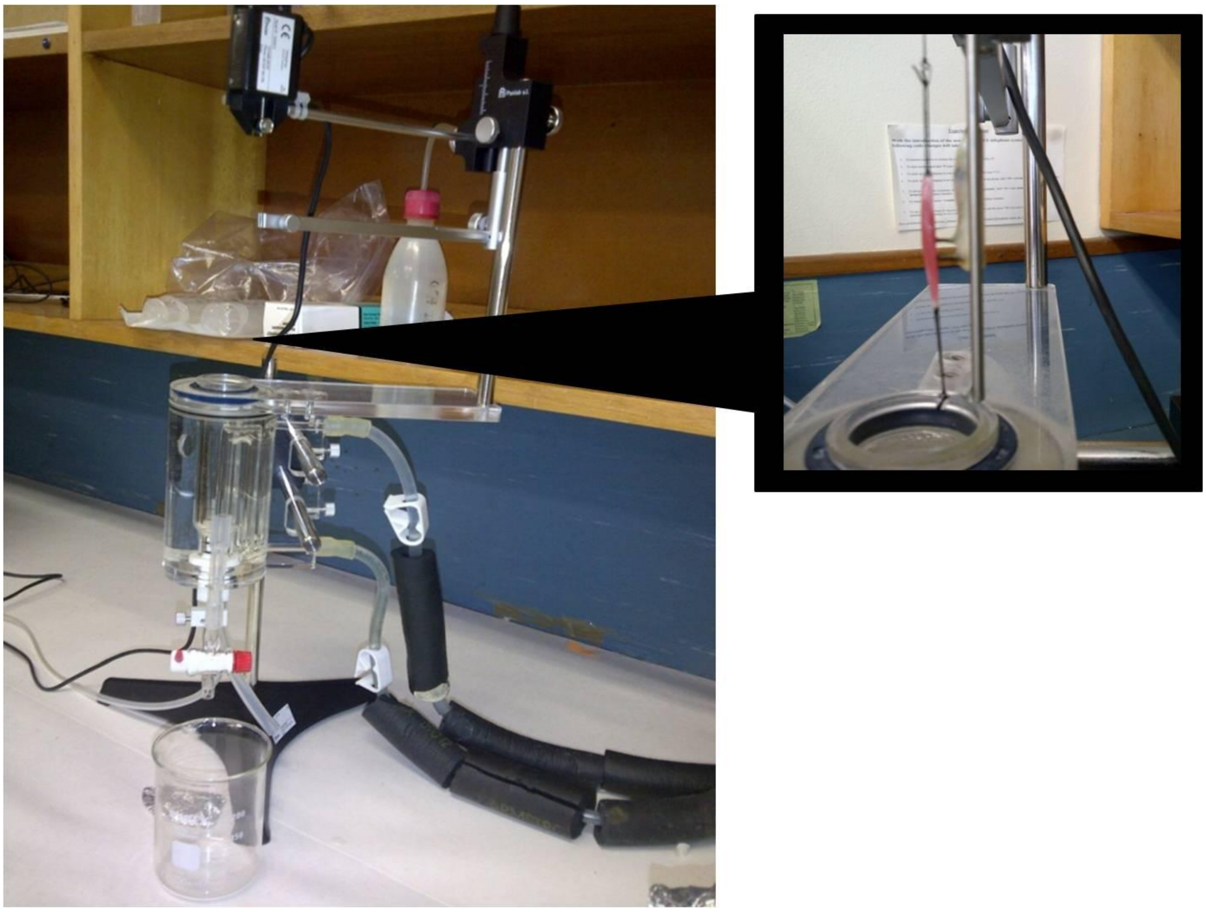


Figure 4.1: Soleus muscle mounted on the PowerLab® apparatus. The base of the muscle was fixed to an immobile hook and the other end tied to an isometric force transducer. The position of the force transducer could be adjusted by a micro-positioner, thus altering preload.

After an equilibration period of 30 min, the optimal length (*i.e.* muscle length producing maximal isometric twitch force) and optimal voltage was determined. Optimal muscle length and voltage was determined for each muscle by generating single twitch contractions at increasing muscle lengths and voltages, respectively, until no increase in single-twitch force production was observed. The muscle length and voltage that generated the highest single twitch amplitude was then used throughout the entire stimulation protocol. The pulse duration was set to 1 msec for all twitch and tetanic contractions. The stimulation protocol consisted of the generation of a single twitch, force frequency curve to determine F_{max}, tetanus, a 2 minute stimulation period to determine fatigue resistance and ended off with two sets of tetanus stimulations at 5 and 20 minutes after fatigue. F_{max} was determined using brief, repeated stimulations at increasing pulse frequencies (1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Hz for 3 sec allowing a 2 min recovery interval between each stimulus). The greatest force achieved for each animal using this protocol was considered the F_{max}. Following a 10 min resting period after F_{max} determination, muscle fatigue rate was determined over a 2 minute period of intermittent contractions, stimulating the muscle for 2 seconds on and 2 seconds off at a frequency of 40 Hz (predetermined to be F_{max}). Force was measured at 20 second intervals during fatigue (Fig. 4.2). Twitch amplitude (force), contraction time (time to peak tension) and half-relaxation time (time for peak force to decay by 50%) were determined before and after the fatigue protocol. Contraction time (time to peak tension) was defined as the time elapsed from the base to the peak of a single twitch. Half-relaxation time was defined as the time elapsed from the peak of a single twitch to the point of the twitch amplitude returning halfway to baseline. All muscle function data were collected through an AD Instruments Bridge Amp and Powerlab 4/30, and analyzed with Chart5 PowerLab software (ADInstruments, Inc., Colorado Springs, CO).

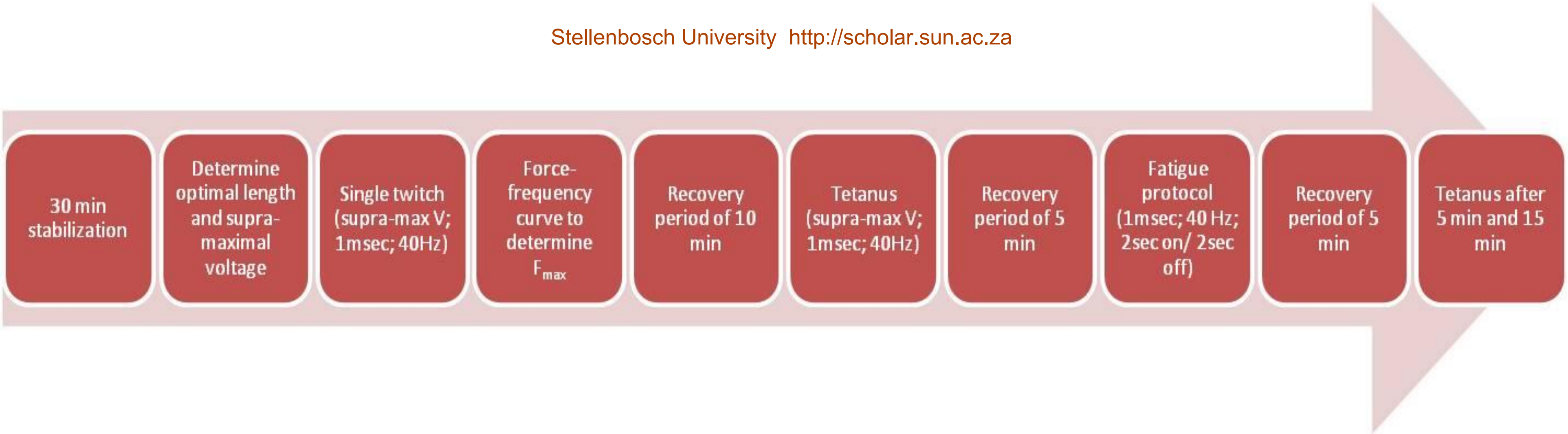


Figure 4.2: Graphical depiction of the muscle fatigue stimulation protocol. In short, the stimulation protocol consisted of the generation of a single twitch, force frequency curve to determine F_{max}, tetanus, a 2 minute stimulation period to determine fatigue resistance and ended off with two sets of tetanus stimulations at 5 and 20 minutes after fatigue.

Specific force was calculated in N/cm^2 of muscle cross-sectional area. The latter was approximated by dividing the dry-weight of the muscle by the product of optimal length and muscle density (assumed to be 1.056 g/cm^3). The force transducers were calibrated using known weights. The contraction time and half-relaxation time were measured as indices of isometric twitch kinetics. For the fatigue protocol, values were normalized by expressing the force generated at each 20 second time point, as a percentage of the initial force at the beginning of the fatigue trial.

4.2.3 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance between two groups was assessed via a Student t-test and between two or more groups; a two-way ANOVA was used, followed by a Bonferroni post-hoc test. $p < 0.05$ was considered as statistically significant. Statistical analysis of data was performed using GraphPad Prism version 5.

4.3 RESULTS

4.3.1 Biometric characteristics of experimental animals

Rats were matched for body mass at the onset of the 10 week *P. glandulosa* treatment and treatment was found to have no effect on weight gain. Skeletal muscle biometrics (mass, optimal length and width), which is a key determinant of the force output, displayed no significant differences between the treated and untreated groups (Table 4.1). In essence, the soleus muscles of the PLA and PG where biometrically similar.

Table 4.1: Biometric characteristics of the animals after *P. glandulosa* treatment

	PLA	PG	*p*-values
Body mass (g)	438.00 ± 14.97	426.43 ± 16.26	**Not significant**
Muscle mass (g)	0.20 ± 0.01	0.19 ± 0.012	**Not significant**
Muscle dry-mass (g)	0.03 ± 0.003	0.03 ± 0.003	**Not significant**
Optimal muscle length (mm)	31.20 ± 0.66	31.14 ± 0.99	**Not significant**
Muscle width (mm)	4.60 ± 0.24	4.43 ± 0.20	**Not significant**
Muscle mass/body mass ratio	0.04 ± 0.002	0.05 ± 0.002	**Not significant**

The data are expressed as mean ± SEM; Analysis by Student t-test; n = 10

4.3.2 Contractile properties of soleus muscle

The induction of muscle fatigue resulted in the significant reduction in both twitch- and peak tetanic force generated by the soleus muscle, when comparing PLA (BF) to PLA (AF) and PG (BF) to PG (AF). Therefore as a consequence the twitch/tetanus ratio was significantly reduced after fatigue compared to before fatigue. Despite fatigue ensuing, the contraction time was unaffected by *P. glandulosa* treatment, remaining constant throughout. Ten weeks of *P. glandulosa* treatment sufficiently increased force generated by the soleus muscle, as depicted by the significantly elevated twitch- and peak tetanic force production at baseline (PG (AF) vs. PLA (AF)). *P. glandulosa* treatment also resulted in a significantly increased half-relaxation time post-fatigue, compared to the untreated controls.

Table 4.2: Contractile properties of soleus muscle from control vs. *P. glandulosa*-treated rats before and after fatigue

	PLA (BF)	PG (BF)	PLA (AF)	PG (AF)	*p*-values
Twitch force (N/cm^2)	7.80 ± 0.65	11.95 ± 0.72*	3.09 ± 0.41***	4.22 ± 0.17***	* $p < 0.05$ PG (BF) vs. PLA (BF) *** $p < 0.0001$ PLA (AF) vs. PLA (BF); PG (AF) vs. PG (BF)
Contraction time (ms)	150.0 ± 10.0	114.29 ± 3.49	120.00 ± 5.48	128.57 ± 5.62	No significance
Half-relaxation time (ms)	447.5 ± 10.37	442.86 ± 11.88	387.5 ± 16.01	453.21 ± 9.83*	* $p < 0.05$ PG (AF) vs. PLA (AF)
Tetanic force (N/cm^2)	47.91 ± 2.60	62.20 ± 2.68*	26.39 ± 5.98***	28.45 ± 1.92***	* $p < 0.05$ PG (BF) vs. PLA (BF) *** $p < 0.0001$ PLA (AF) vs. PLA (BF); PG (AF) vs. PG (BF)
Twitch/Tetanus ratio	15.83 ± 1.49	19.16 ± 1.50	11.90 ± 2.40*	14.75 ± 1.04*	* $p < 0.05$ PLA (AF) vs. PLA (BF); PG (AF) vs. PG (BF)

The data are expressed as mean ± SEM; Analysis by two-way ANOVA; n = 10

4.3.3 Force-frequency relationship

The force-frequency relationship, which is the sigmoid relationship between a muscle's activation frequency and the consequent isometric force output, displayed a similar trend for both muscles in the treated and untreated groups (Fig. 4.3 B). This trend is displayed in Figure 4.3 (B), representing the force generated at each frequency, expressed as a % of the maximum force generated. In contrast, the absolute values of the force generated at different frequencies displays that the soleus muscles of the treated rats generated significantly more force, during electrical stimulation, compared to the untreated rats, at all the different frequencies ($p < 0.001$). As illustrated in Figure 4.3 (A), the force generated by the soleus muscle of the untreated rats incrementally increased from 15.34 ± 2.92 N/cm^2 at a frequency of 5 Hz to a maximum force of 47.77 ± 5.73 N/cm^2 at a frequency of 40 Hz, where after the generated force slowly decreased to a force equal to 38.55 ± 6.27 N/cm^2 at a frequency of 100 Hz. A similar trend is followed by the treated rats, however, at significantly higher levels. The force generated by the soleus muscle of the *P. glandulosa* treated rats incrementally increased from 24.37 ± 3.18 N/cm^2 at a frequency of 5 Hz to a maximum force of 61.65 ± 5.05 N/cm^2 at a frequency of 40 Hz, before slowly declining to a force of 53.48 ± 6.41 N/cm^2 at a frequency of 100 Hz. The level of force generated peaked at a frequency of 40 Hz in both groups.

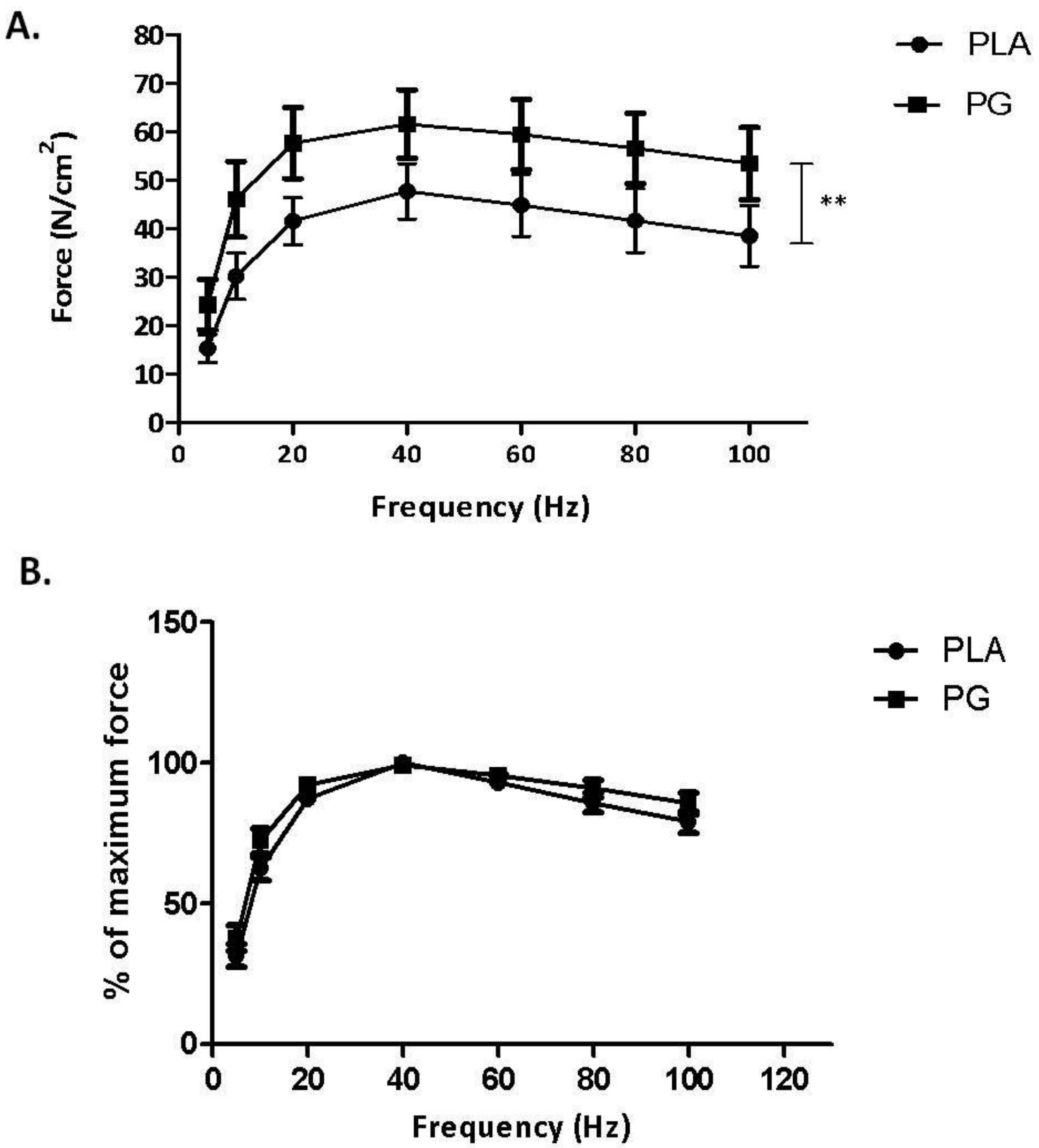


Figure 4.3: Force-frequency relationship characteristics of rat soleus muscle in control and *P. glandulosa*-treated rats. Figure (A) represents the specific force generated by the soleus muscles at the different frequencies and Figure (B) represents the force generated at each frequency when expressed relative to the maximum force generated. Force-frequency curve was generated by means of brief, repeated stimulations at increasing pulse frequencies. The greatest force achieved for each muscle using this protocol was considered the F_{max}. The data are expressed as mean ± SEM. Analysis were by two-way ANOVA. n = 7 ** $p < 0.001$ PLA vs. PG (10 Hz to 100 Hz)

4.3.4 Fatigue characteristics

The 2 minute intermitted stimulation (fatigue protocol) was sufficient to significantly decrease the force generated by both the treated and untreated group by at least 50%. In other words, the force measured after the 2 minute fatigue protocol was 50% lower than the force measured before the induction of fatigue (18.03 ± 3.36 vs. 42.62 ± 5.00 N/cm^2; $p < 0.0001$) (Fig. 4.4 B). *P. glandulosa* treatment was unable to reduce fatigue tolerance, as fatigue development was not significantly different between the treated versus untreated group at any point during the 2 minute fatigue protocol. However, the initial force generated, was significantly higher in the treated group, when compared to the untreated group (56.39 ± 4.21 vs. 42.62 ± 5.00 N/cm^2; $p < 0.001$) (Fig. 4.4 A).

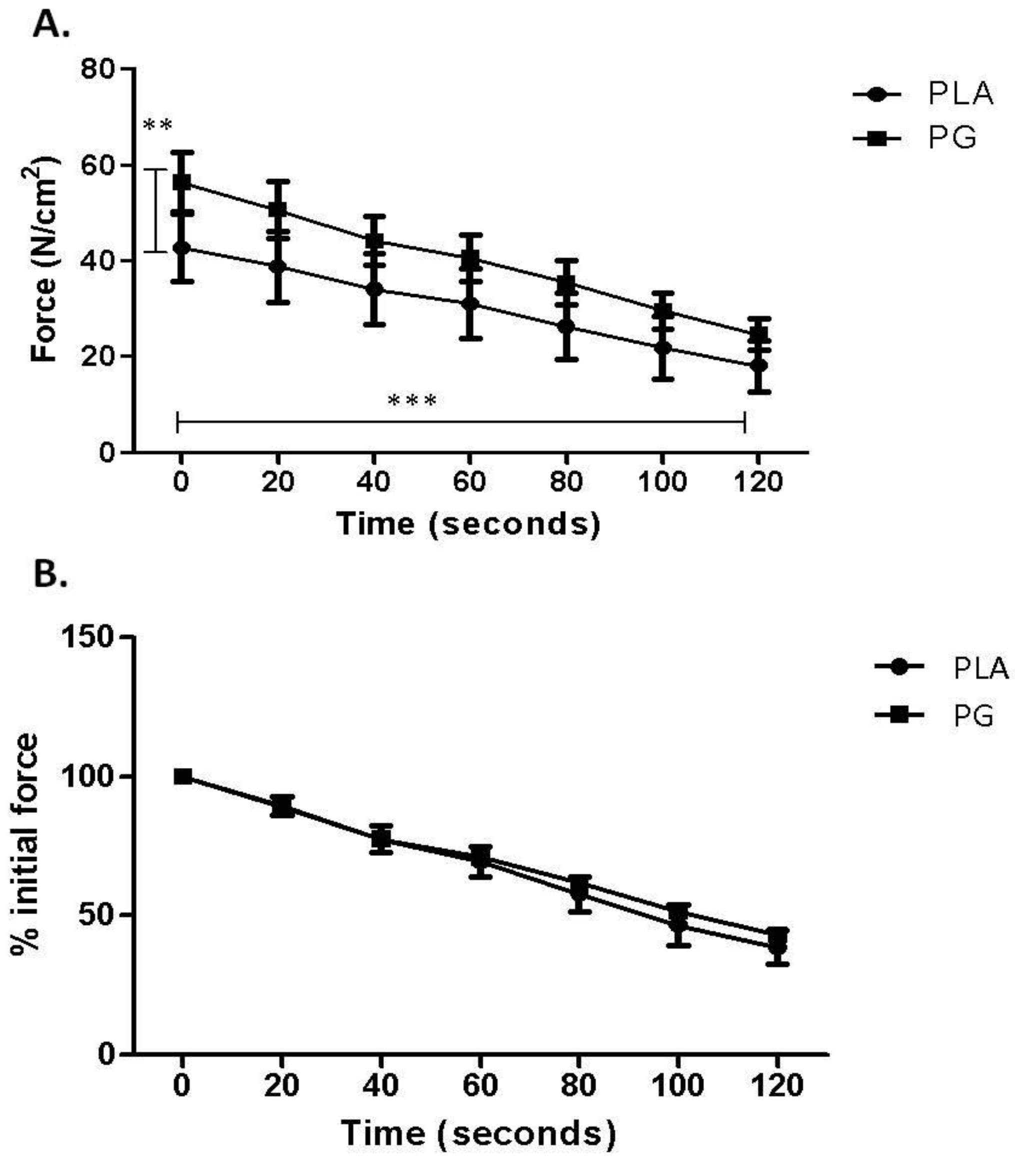


Figure 4.4: Fatigue characteristics for soleus muscle in control and *P. glandulosa*-treated rats. Figure (A) represents the specific force generated by the soleus muscles and Figure (B) represents the force generated, expressed as a % of the initial force generated. Muscle fatigue was determined over a 2 minute period of intermittent contractions, stimulating the muscle for 2 seconds on and 2 seconds off at a frequency of 40 Hz (predetermined to be F_{max}). Force was measured at 20 second intervals during the fatigue protocol. The data are expressed as mean ± SEM. Analysis were by two-way ANOVA. n = 10 ** $p < 0.001$ t = 0 min; PG vs. PLA; *** $p < 0.0001$ t = 0 min vs. t = 120 min PLA; PG

4.3.5 Tetanic force produced before and after fatigue

Figure 4.5 depicts the specific force generated before fatigue, 5 minutes after fatigue and 20 minutes after fatigue. As represented in Figure 4.5 the specific force generated by the soleus muscle was significantly lower in both untreated (26.39 ± 5.98 vs. 47.91 ± 2.60 N/cm^2; $p < 0.0001$) and treated (28.45 ± 5.07 vs. 62.20 ± 2.68 N/cm^2; $p < 0.0001$) groups compared to their respective baseline values, 5 minutes after the 2 minute fatigue protocol. When comparing the specific force generated 5 minutes after fatigue with the specific force generated 20 minutes after fatigue, there is a significant increase in force generated 20 minutes after fatigue in both untreated (42.12 ± 7.16 vs. 26.39 ± 5.97 N/cm^2; $p < 0.001$) and treated groups (52.37 ± 7.48 vs. 28.45 ± 5.07 N/cm^2; $p < 0.001$). In addition, *P. glandulosa* treatment only had a significant effect on the specific force generated before the fatigue protocol (62.20 ± 2.68 vs. 47.91 ± 2.60 N/cm^2; $p < 0.05$). No significant differences were observed between the treated and untreated groups 5 minutes and 20 minutes after fatigue.

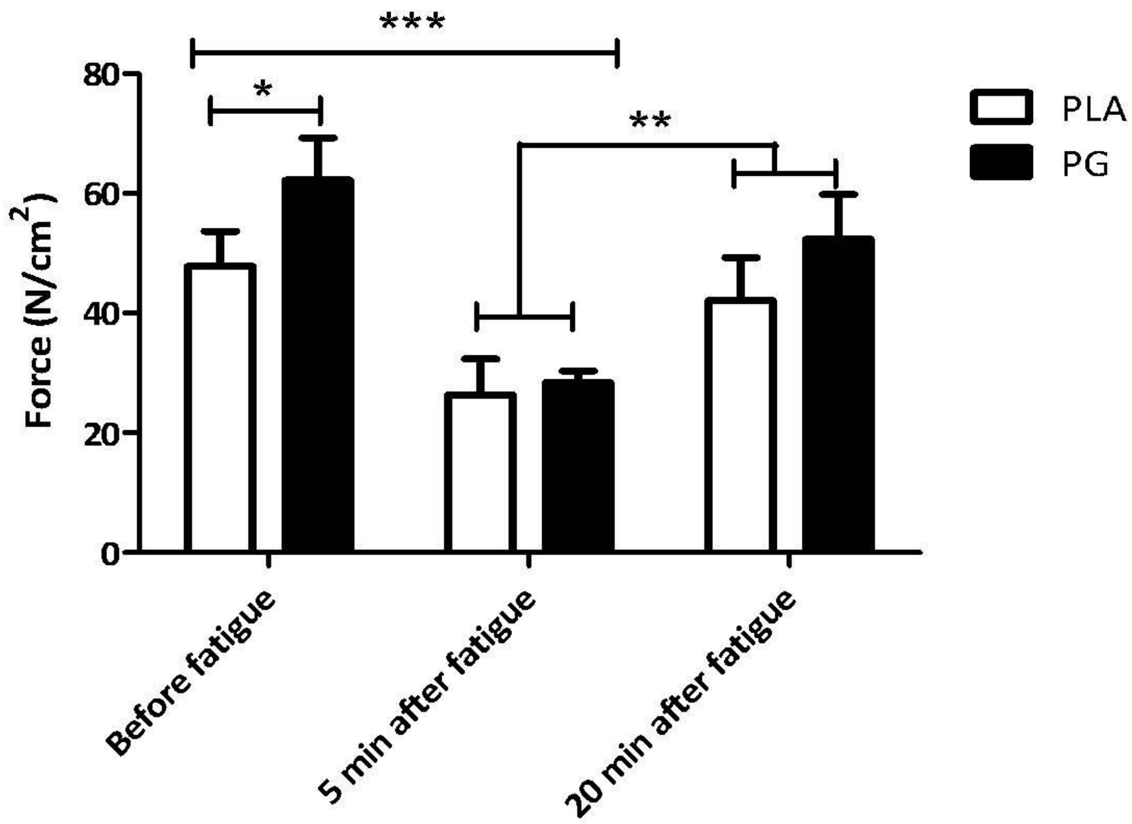


Figure 4.5: Tetanic force production before, 5- and 20 min after fatigue. Tetanic contractions were generated by stimulating the muscle at its supra-maximal voltage at a frequency of 40 Hz. The data are expressed as mean ± SEM; Analysis by two-way ANOVA; n = 10; * $p < 0.05$ PLA vs. PG (Before fatigue); ** $p < 0.001$ PLA vs. PLA (5 min vs. 20 min); PG vs. PG (5 min vs. 20 min); *** $p < 0.0001$ PLA vs. PLA (Before fatigue vs. 5 min); PG vs. PG (Before fatigue vs. 5 min)

4.4 DISCUSSION

Many herbal substances have been studied as possible supplements to improve muscle fatigue symptoms. Most of these studies focused mainly on plant extracts and emphasized the importance of compounds such as polysaccharides [Wang *et al.,* 2010], flavonoids [Yu *et al.,* 2010] and peptides [You *et al.,* 2011]. Not many studies made use of whole plant crude preparations as we did in our study. Since the anecdotal evidence obtained previously, was of animals consuming the pods of the *P. glandulosa* plant whole, we opted to use this mode of administration. In addition, the formulation used in this study is also how this plant product is currently marketed as an over-the-counter food supplement.

The energy metabolism during muscle activity determines the rate and intensity of physiological fatigue [Belluardo *et al.,* 2001], which makes exercise endurance an essential variable in evaluating anti-fatigue treatments. In this current study, we examined the possible strength-increasing and anti-fatigue effects of *P. glandulosa* on soleus muscle during electrical field stimulation of healthy rats. In this *ex vivo* study, intense exercise was mimicked by electrically stimulating a single muscle type (soleus muscle) to fatigue and determining the muscle's recovery after fatigue induction. As mentioned in section 2.5.4, there are numerous methods and at various temperatures in which to study muscle fatigue. The intact perfused muscle is under the control of the central nervous system [Allen *et al.,* 2008], and since we wanted to investigate peripheral fatigue we had to avoid central complications. Isolated whole muscle, which is isometrically stimulated with repeated tetani, in an organ bath, until force is reduced [Allen *et al.,* 2008] was the method selected for this study. This method does have its limitations, as the absence of circulation may lead to the muscle core becoming anoxic and K^+ accumulating extracellularly. Active muscle fibers release K^+ and it accumulates in the extracellular spaces until a diffusion gradient develops which is sufficient to allow K^+ to diffuse out of the preparation. As a result thereof, the concentration of K^+ will be much higher at the core of the muscle than it is in the perfusate. Additionally, CO_2, H^+ and lactate will also accumulate in the extracellular spaces in a similar fashion [Barclay, 2005]. All these factors complicate the analysis of the mechanisms of muscle fatigue. However, the aim of the study was not to determine the mechanism of fatigue, but rather if *P. glandulosa* had any effect on fatigue development, i. e. if *P. glandulosa* treatment could delay the onset of fatigue. In addition to trying and overcome the issue of the anoxic core and the K^+ gradient, we utilized a small muscle, the soleus muscle. One could however also use single fibers, which would completely eliminate the issue of the anoxic core, but our primary aim was to investigate the effect of *P. glandulosa* treatment on a particular muscle type. If any effect was observed in our study, further studies would have been conducted on the effects of *P. glandulosa* on the different fiber types. The temperature, which the preparation was to be stimulated at, was also a factor that needed assessment. In research done by Segal *et al.* (1986), they determined the performance of isolated muscle (soleus and EDL) at different temperatures, ranging from 20 °C to 40 °C. In this study they found that optimal muscle performance was obtained at between 25 °C and 30 °C, rather than at higher or lower temperatures. In addition to the study by Segal *et al.* (1986), we also conducted a pilot study and found that temperatures above 25 ˚C led to slower development of fatigue and temperatures below 25 ˚C led to fast development of fatigue.

In our study, muscle fatigue was established when the force generated after the fatigue protocol was 50% of the initial force generated. In a trial study, this was set at 2 minutes, as it took the soleus muscle 2 minutes to lose 50% of its initial force. As can be seen from Figure 4.4 (A), the force generated at time point 120 seconds (2 minutes) is 50% of the initial force generated. The main findings from this study showed that treatment with pulverized *P. glandulosa* pods had no significant effect on muscle fatigue tolerance, as both treated (PG) and untreated (PLA) groups fatigued at the same rate (Fig. 4.4 (B)). Even though the fatigue curves from the treated group diverged slightly from the curve of the untreated group, 80 seconds into the fatigue protocol, it was not significant. The magnitude of the decline in fatigue tolerance (Fig. 4.4 (A)) in the treated group could presumably have continued decreasing with extended time, until it reached the levels of the untreated group.

In the current study, we also measured the tetanic force generated before fatigue as well as 5 minutes and 20 minutes after fatigue (Fig. 4.5). From this data it is clear that the force generated 5 minutes after the fatigue protocol is significantly lower than the force generated before fatigue. This is a good indication that the muscles were indeed exhausted, post-fatigue protocol. It is known that exercise-induced fatigue is reversible and that after a modest resting period the muscle is able to generate the same force as it did before fatigue set in [Allen *et al.,* 2008]. This phenomenon was evident in our study. We found that the soleus muscle could regain its force after a 20 minute resting period (Fig. 4.5), since the force generated 20 minutes after fatigue is similar to the initial force (before fatigue). Biochemical variables, including lactate and creatine kinase are also important indicators of muscle fatigue after exercise [Brancaccio *et al.,* 2007], since the muscle produces a large quantity of lactate and creatine kinase during high-intensity exercise. The increased lactate level reduces intracellular pH, which is thought to partially contribute to muscle fatigue. To further evaluate the effects of *P. glandulosa* on muscle fatigue, the biochemical variables, such as lactate or creatine kinase needs to be measured. We did not measure these variables in the current study.

Another aspect also changing during exercise-induced fatigue includes the slowing of muscle relaxation [Allen *et al.,* 2008]. In our study we found that there was no significant difference in the initial phase of half-relaxation time in either the control group or the treated group before and after fatigue (Table 4.2), however it should be noted that muscle can fatigue without any major decrease in the rate of relaxation. In both the studies by Bruton *et al.* (2003) and Lunde *et al.* (2006) they demonstrated that isolated slow-twitch fibers of mouse soleus muscles displayed little to no slowing during fatiguing stimulation. However, when rats were treated with *P. glandulosa* for 16 weeks and electrically stimulated to fatigue (PG (AF)), their soleus muscles relaxed at a significantly slower rate than the soleus muscles of the untreated rats, 5 minutes after fatigue (PLA (AF)) (Table 4.2). It is difficult to draw inference from our current study as relaxation of skeletal muscle cells is a complex process that involves many major steps. Firstly, SR Ca^{2+} release stops, then Ca^{2+} is taken up by the SR via ATP-driven pumps, which results in the decline in $[Ca^{2+}]_{i,}$ leading to Ca^{2+} dissociating from troponin and inevitably the cross-bridge cycling ceases [Allen *et al.,* 2008]. Due to this intricate process, any of these steps could possibly have been influenced by *P. glandulosa* treatment. Therefore, more research is needed to determine the exact effect *P. glandulosa* has on any of the above mentioned steps. Researchers have developed techniques by which they can simultaneously measure force generated and $[Ca^{2+}]_i$ in single muscle fibers, allowing the assessment of the relative contribution of changes in SR Ca^{2+} handling and cross-bridge action [Westerblad and Allen, 1993; Westerblad *et al.,* 1997].

An important novel result was that despite *P. glandulosa* not acting as an ergogenic aid, the soleus muscles of the treated rats (PG) generated significantly higher force when the muscle was stimulated to generate a single twitch or tetanus (Table 4.2), prior to the induction of fatigue. This same phenomenon was observed after the force-frequency relationship was determined. As depicted in Figure 4.3 (A), the force generated by the soleus muscle of the untreated rats, incrementally increased to reach its maximum force at 40 Hz, where after the generated force slowly decreased again. A similar trend was observed in the treated group; however, the specific force generated by the soleus muscles of the treated rats was significantly higher at all the different frequencies (Fig. 4.3 (A)). Figure 4.3 (B) representing the force generated at each frequency when expressed relative to the maximum force generated, shows the similar trend in which both treated and untreated soleus muscles generated force after being stimulated at the respective frequencies. The augmented effect on force generation described above, disappeared after the fatigue protocol, as no significant differences were observed during either a single twitch or tetanic stimulation (Table 4.2) when measured 5 minutes after the 2 minute fatigue protocol. However, later time points after fatigue termination, need to be measured in order to determine whether *P. glandulosa* treatment will have a similar effect after an extended period of rest (> 20 minutes). Referring back to Figure 4.3 a 20 minute resting period allowed the muscle to recover most of its initial force in both the control (PLA) and treated (PG) groups, when compared to the force generated directly after fatigue (5 minutes). Therefore, one can assume that this augmented effect on force will persist if the muscle is left to completely recover.

An individual's strength is determined by mainly two factors, namely, the cross-sectional area of the muscle fibers recruited to generate the force needed and the intensity of the recruitment needed [Maughan *et al.,* 1983; Lee *et al.,* 2013]. In our study we found no significant difference in the biometrics of the muscles in the different experimental groups, i.e. the muscles seemed phenotypically similar (Table 4.1). However, the diameter of the individual muscle fibers was not measured, so we can not conclusively state that the cross-sectional area of the muscle did not contribute to the increase in muscle strength observed.

From previous studies conducted in our laboratory [unpublished data] we have shown that *P. glandulosa* does not seem to elicit its effects via an antioxidant mechanism, as numerous plant-based substances do. This was verified by the LOOH and TBARS assays that both showed no significant difference between the treated vs. untreated obese Zucker (*fa/fa*) rats (Fig. 3.2). In addition, it has also previously been shown that antioxidants may impair muscle force production *in situ* [Coombes *et al.*, 2001] and *in vitro* [Reid *et al.*, 1993], which is the opposite of our findings. In the study by Reid *et al.* (1993) they incubated isolated diaphragm fiber bundles with the antioxidant enzymes catalase and SOD and found that these fiber bundles displayed depressed sub-maximal tetanic contractile force generation. In similar studies, however with NAC incubation, the researchers found a depression of sub-maximal tetanic force production [Diaz *et al.,* 1994]. These studies collectively showed that high levels of antioxidants can negatively affect both twitch and sub-maximal tetanic contractions in unfatigued muscle.

A possible explanation for the increase in muscle strength might be that *P. glandulosa* treatment led to the transition of the fiber type, i.e. from a slow-twitch to a fast-twitch phenotype or activated the "transition fibers". Neunhäuserer *et al.* (2011) proposed that in addition to the different fiber types, there are also "transition fibers" in the different muscles. A muscle composed of a high proportion of slow-twitch fibers will be relatively weaker than a similar muscle with a high proportion of fast-twitch fibers. Soleus muscle has been found to consist of predominantly (84%) slow-twitch fibers [Ariano *et al.,* 1973]. It is known that fiber composition is regulated in response to changes in physical activity, environment and pathological conditions [Schiaffino *et al.*, 2007]. For example, endurance exercise training induces a fast-to-slow fiber type transition, transforming the myofibers to an increased oxidative metabolism [Demirel *et al.,* 1999; Pette and Staron, 2001; Yuan *et al.,* 2011]. Additional factors leading to fiber type transition include mechanical loading and unloading, hormones and aging [Pette and Staron, 2001]. Scant research could be found in which an herbal substance *per se* was responsible for such a transition. Wang *et al.* (2003), aimed to investigate the effects of Jiang-Tang-Ke-Li, a traditional Chinese medicine, on insulin resistance and hypertension as well as attempting to determine the mechanisms by which Jiang-Tang-Ke-Li improves insulin sensitivity in fructose-fed rats (FFR). In this study they found that the ratio of type I fibers (slow-twitch fibers) in soleus muscles decreased significantly in the FFR compared to that in the control group and treatment with Jiang-Tang-Ke-Li led to recovery of the composite ratio of type I fibers to the same level as that of the control group [Wang *et al.,* 2003]. They made use of the adenosine-triphosphatase method [Higashiura *et al.,* 1999]. It is therefore possible that an herbal substance can induce the transition of one fiber type to another; however more research on this topic is needed.

To summarize, the main findings of our current study was that no significant difference with regards to fatigue index of control vs. *P. glandulosa* treated groups was observed, suggesting that *P. glandulosa* did not increase the endurance capacity of isolated skeletal muscles. In addition we found a significant increase in specific force generated by the soleus muscle of the *P. glandulosa* treated rats compared to the untreated rats. The possible explanations for this phenomenon are merely speculative, since research into these particular areas has not yet been explored.

CHAPTER 5: THE EFFECT OF CHRONIC AND ACUTE *PROSOPIS GLANDULOSA* TREATMENT ON SKELETAL MUSCLE INJURY AND REPAIR AFTER A CONTUSION INJURY TO THE RAT HINDLIMB

5.1 GENERAL INTRODUCTION

Muscle injuries are frequently seen during sporting events, with contusion injuries being reported as 12.1% of all injuries [Fernandez *et al.*, 2007]. A contusion injury is an injury caused by a blunt non-penetrating object, resulting in the rupturing of muscle fibers at or adjacent to the injured area [Järvinen *et al.,* 2005]. Appropriate treatment of a contusion injury is very important as failure to properly treat these injuries can lead to prolonged disability and even incomplete recovery of the damaged muscle. There are various therapies for treating muscle injuries, all of which are directed at restoring skeletal muscle function, enhancing normal muscle repair and regeneration by limiting inflammation and muscle fibrosis and in so-doing, reduce scar formation. Currently, the most common clinically prescribed treatment for muscle injuries are anti-inflammatory drugs [Järvinen *et al.,* 1992; Vignaud *et al.,* 2005]. It has been estimated that ±70 million prescriptions for NSAID's are issued annually and 30 billion purchases are made for over-the-counter NSAID's [Elnachef *et al.,* 2008].

In response to injury, the COX enzymes (COX-1 and 2) produce prostaglandins which promote inflammation and pain. Therefore, the assumption is that by inhibiting these enzymes, inflammation and pain would be reduced. However, this mode of treatment may lead to delayed or even incomplete recovery, as a result of incorrect dosing or timing of administration of the anti-inflammatory drug. It has been found that prolonged use and therefore prolonged inhibition of the inflammatory response may inhibit the positive events associated with inflammation, which in-turn results is poor recovery [Järvinen *et al.,* 2005].

The general injury and repair mechanism is similar, regardless of the type of injury, with the different stages typically including degeneration, inflammation, regeneration and fibrosis [Teixeira *et al.,* 2009]. During the inflammatory phase, the neutrophils are the first immune cells to infiltrate the injured muscle area, closely followed by the infiltration of the macrophages. Neutrophils have been shown to be present in the injured area from approximately 1 hour after injury, at which time they have been found to be responsible for clearing the injured area of any debris [Fielding *et al.,* 1993; Tidball *et al.,* 2005], whilst macrophages infiltrate the damaged tissue roughly 24-48 hours after injury, being a major role player in both phagocytosis and muscle repair [Duffield, 2003]. The process of inflammation is regarded as a complex process, as it is a known contributor to both secondary damage as well as muscle recovery. Proof of its contribution to muscle recovery comes from studies in which the inflammatory process was prevented completely, an action that led to incomplete muscle recovery [Mackey *et al.,* 2007; Mikkelsen *et al.,* 2009]. The research conducted on the activity of the immune cells after contusion injuries, offers varying results. This discrepancy is partly due to the varying severity of the injury incurred as well as the model used to produce the experimental contusion [Kami *et al.,* 2000; St. Pierre Schneider *et al.,* 2002; Bunn *et al.,* 2004; Smith *et al.,* 2008; Farnebo *et al.,* 2009]. We are of the opinion that a suitable model of muscle injury for our study, in which the activity of the immune cells and factors involved during muscle regeneration needs to be evaluated, is the contusion injury. The drop-mass model used is a contusion injury model, which delivers an injury that is standardized with regards to size of injury and severity of injury.

Though it is known that the inflammatory response is required for removal of debris and promotion of cytokine-mediated processes involved in regeneration, oxidants which are released from neutrophils, macrophages and satellite cells can potentially cause secondary damage, known as oxidative stress. Oxidative stress occurs when the ROS and RNS produced, overpower the endogenous antioxidant enzymes (SOD, GPx and catalase) of the body [Packer and Cadenas, 2007]. ROS is thought to be involved in the initiation of the inflammatory response and the damage incurred during exercise-induced muscle damage [Tiidus, 1998]. In the study by Tiidus (1998), it was documented that the oxygen radicals generated through the neutrophil respiratory burst are crucial in removing the damaged muscle tissue; conversely the presence of excessive oxygen radicals may result in the spread of further tissue damage. It is also known that macrophages produce large amounts of NO, which is necessary to recruit satellite cells to the site of injury, where they proliferate and mature, as well as to recruit additional macrophages [Chazaud *et al.,* 2003]. Since NO production is time and concentration dependent, the more macrophages present at a given time, the more satellite cells will be recruited and activated [Anderson, 2000]. Therefore, a balance needs to be obtained with regards to free radical production. It seems logical that if one can limit oxidative stress, you can also limit the degree of inflammation and the associated damage in the injured area, therefore potentially accelerating the recovery process.

Several studies have been conducted on the properties and effectiveness of dietary antioxidant, of which vitamins C, E and A are the most common. Research has shown that a lack in antioxidant capacity leads to increased oxidative stress [Basu, 1999] and antioxidant supplementation is associated with a decrease in lipid peroxidation [Brown *et al.,* 1994]. Numerous studies have also shown that vitamin supplementation may have favourable effects with regards to muscle damage. However, many of these studies only investigated pro-inflammatory markers, such as IL-6 and TNF-α [Thompson *et al.,* 2004] or indirect indicators of damage, such as creatine kinase release [Petersen *et al.,* 2001] and not direct markers of recovery, such as recovery of force after injury or markers of regeneration, such as satellite cell response. Many plant-derived substances, especially those rich in triterpenoids and polyphenols, have also been found to have antioxidant capacity. For example, the grape-derived antioxidant, resveratrol, has been found to act as an anti-inflammatory agent. It has been proposed that resveratrol elicits its anti-inflammatory effects by either inhibiting the production of the pro-inflammatory cytokines (IL-8 and IL-6) or by partially inhibiting the activation of the immune cells [Donnelly *et al.,* 2004]. In another study conducted by Kruger *et al.* 2011, they found that the chronic and acute supplementation of a plant-derived antioxidant, proanthocyanidolic oligomer, in a rat hindlimb contusion injury model, resulted in a blunted neutrophil response and earlier macrophage infiltration, leading to earlier muscle recovery. Guabiju extract [Andrade *et al.,* 2011] and quercetin [Derlindati *et al.,* 2012] have, in separate studies, also been reported to have anti-inflammatory effects. Guabiju extract was found to inhibit neutrophil chemotaxis [Andrade *et al.,* 2011] and quercetin-3-O-glucuronide was found to reduce the transcription of genes involved in inflammation, such as pro-inflammatory interleukins and enzymes involved in oxidative stress responses [Derlindati *et al.,* 2012]. Another well-known herb, Chamomile (*Matricaria recutita*), has also been found to have anti-inflammatory abilities. In the study by Srivastava *et al.* (2009), they treated lipopolysaccharide-activated RAW 264.7 macrophages with an aqueous chamomile extract and found that this extract had the ability to inhibit the release of prostaglandin E2 from the LPS-activated macrophages. The authors speculated that the inhibitory activity of chamomile was due to a dose-dependent inhibition of COX-2 enzyme activity. They also found that chamomile treatment could reduce COX-2 mRNA and protein expression, without affecting the activity or expression of the constitutive form of cyclooxygenase, COX-1. Contrary to the positive outcomes of the studies mentioned above, one should keep in mind that it is also possible that by completely preventing free radical production, it may lead to insufficient phagocytosis and/or too little activation of repair.

It is well known that skeletal muscle has the capacity to regenerate after injury. This process is mainly dependent on skeletal muscle stem cells, known as satellite cells [Chargè and Rudnicki, 2004]. Under normal conditions, these cells are mitotically quiescent; however, after injury they are activated, after which they proliferate and differentiate, to give rise to myoblasts. In short, during the process of myogenic differentiation, proliferating myoblasts pause in the G_1 phase and withdraw from the cell cycle and then start to differentiate and fuse into multinucleated myotubes. These myotubes begin to produce muscle specific proteins and finally fuse with damaged fibers, where they mature into muscle fibers with peripherally located nuclei or re-enter G_0, remain undifferentiated and replenish the satellite cell "stores" [Arnold and Winter, 2007; Bornemann *et al.,* 2000; Galliano *et al.,* 2000; Perry and Rudnicki, 2000; Yagami-Hiromasa *et al.,* 1995].

Several factors have been found to be involved in the control of the regenerative processes. One such factor, adhesion protein $ADAM_{12}$ [Przewoźniak *et al.,* 2013], belonging to the transmembrane metalloprotease ADAM (a disintegrin and metalloprotease) family, has been implicated in myogenesis and skeletal muscle repair [Engvall and Wewer, 2003; Kurisaki *et al.,* 2003; Moghadaszadeh *et al.,* 2003; Gilpin *et al.,* 1998], especially during muscle cell differentiation and fusion processes [Galliano *et al.,* 2000]. Galliano *et al.* (2000) conducted a study on C2C12 cells, in which they determined the expression levels of $ADAM_{12}$ *in vivo.* In this study they demonstrated that $ADAM_{12}$ is expressed at low levels in undifferentiated myoblasts and is dramatically up-regulated at the onset of differentiation when myoblasts fuse into multinucleated myotubes. Consequently, in skeletal muscle, $ADAM_{12}$ is expressed in the developing myofibers during the embryonic stage and during the early postnatal period [Borneman *et al.,* 2002; Kronqvist *et al.*, 2002]. In adult skeletal muscle, the expression level of $ADAM_{12}$ is very low in both differentiated muscle fibers and quiescent satellite cells [Bornemann *et al.,* 2000; Gilpin *et al.,* 1998; Yagami-Hiromasa *et al.,* 1995]. During regeneration, the amount of $ADAM_{12}$ protein increases dramatically [Galliano *et al.,* 2000], and its mRNA is readily detected in satellite cells following their activation [Bornemann *et al.,* 2000]. In spite of recent studies aimed at the biochemical characterization of $ADAM_{12}$, its role in development and/or regeneration of skeletal muscle is still unclear.

Another protein implicated in tissue repair and regeneration is the 53-kDa cytoskeletal class III intermediate filament protein, desmin [Paulin and Li, 2004]. In skeletal muscle, desmin is the earliest muscle-specific protein to appear during myogenesis and it is found mainly in the Z-discs [Paulin and Li, 2004]. Desmin, along with the other intermediate filament proteins, vimentin, nestin, lamins and cytokeratins, forms an intracellular network that provides a three-dimensional scaffold in regenerating cells [Paulin and Li, 2004]. It has been found that when translation of desmin mRNA was inhibited in C2C12 cells, differentiation and fusion was blocked, indicating its vital role in the formation of myofibers [Li *et al.*, 1994]. The expression levels of desmin are low in proliferating myoblasts, but increase in differentiated myotubes [Chourbagi *et al.,* 2011; Paulin and Li, 2004], i.e. it is expressed at higher levels during skeletal muscle development (myotube formation). It is therefore widely used as a marker to distinguish between individual cell types within a tissue, such as myoblast from fibroblasts in the regenerating and central zone of muscle injury [Stratos *et al.,* 2007].

The motivation for the current part of this study came from two observations. The main motivation was the anecdotal information from the race horse industry, which stated that, in horses that consumed *P. glandulosa,* skeletal muscle injuries healed faster. Secondly, research that had previously been conducted in our laboratories documented that *P. glandulosa* treatment could significantly increase the formation of small pancreatic β-cells (0–2500 μm^2) in a STZ-induced diabetic animal model (Fig. 5.1) [George *et al.,* 2011].

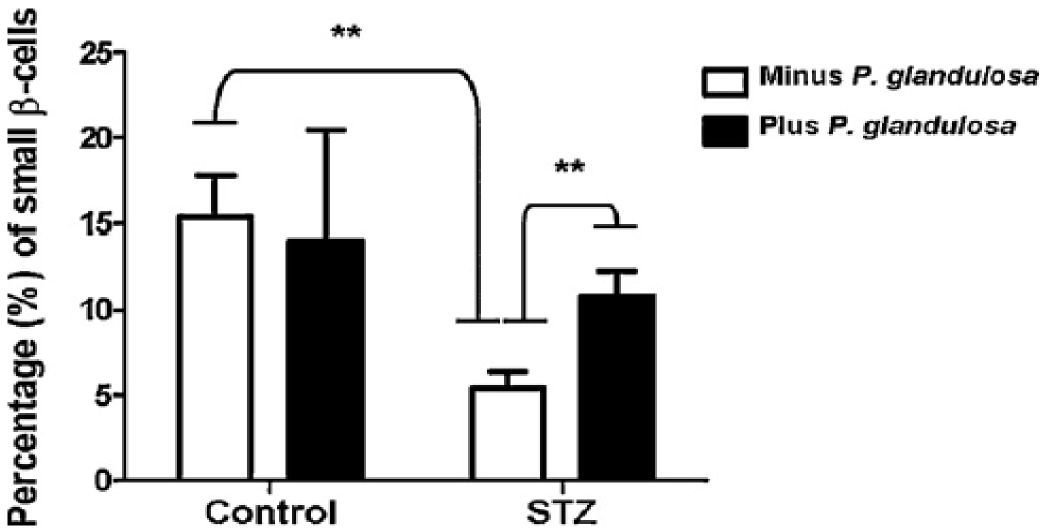


Figure 5.1: Percentage small β-cells (0–2500 µm2) per islet. Pancreatic β-cells were sized using different area parameters. The data are expressed as mean ± SEM. ***p < 0.001 control vs. STZ; **p < 0.01 STZ vs. STZ + *P. glandulosa*; n = 5–8. [George *et al.,* 2011]

The STZ-induced diabetic animal model is a model of type 1 diabetes. Type 1 diabetes is achieved in these animals by injecting them with a single, intraperitoneal injection of STZ. This model has previously been shown to result in a graded diabetic response, due to the partial ablation of the β-cell reserve [BrØndum *et al.,* 2005]. The data obtained from this study implied possible β-cell neogenesis [George *et al.,* 2011]. Findings from this study alluded to the fact that *P. glandulosa* treatment might activate the regenerative systems employed by the pancreas. We therefore argued that if *P. glandulosa* had the ability to regenerate ablated pancreatic cells after it had been partially destroyed by a toxin; it might also have the ability to regenerate skeletal muscle cells after it had been damaged through injury, which would explain the faster "healing" observed in the race-horses. Therefore, we hypothesized that treatment with *P. glandulosa* could enhance muscle regeneration after a contusion injury. Since we observed in previous studies conducted in our laboratory that *P. glandulosa* does not elicit its effects by antioxidant mechanisms (Fig. 3.2), we hypothesized that the mechanism of action might be through *P. glandulosa* treatment blunting the neutrophil response and/or advancing the infiltration of macrophages (alleviating the inflammatory response), into the injured muscle, or enhancing factors responsible for muscle regeneration.

5.2 METHODS

5.2.1 Research design and intervention

5.2.1.1 Animal care and treatment regime

The animal care and *P. glandulosa* treatment was the same for all groups of animals in this study. Refer back to section 3.2.1.1 for "animal care" and section 3.2.1.3 for "*P. glandulosa* treatment".

Diclofenac, a known NSAID, served as a positive control for the inflammatory effects. Diclofenac sodium*,* in the form of Voltaren Emulgel®, was applied to the injured area on the hindlimb of the rats after different time periods post-injury (section 5.2.3). The dosage of Voltaren Emulgel® was calculated at 57.14 mg/kg/day, which equals 0.57 mg/kg Diclofenac. The dosage of 57.14 mg/kg/day was calculated based on the daily dosage prescribed for human adults.

5.2.1.2 Division into groups

Experimental rats were divided into 4 groups. The four groups consisted of one control placebo group (PLA) and three treatment groups, namely (1) PG-CHR, animals pre-treated with *P. glandulosa* from the start of the experiment, (2) PG-AI, animals treated with *P. glandulosa* after contusion injury up to time of sacrifice and (3) NSAID, animals treated with Voltaren Emulgel® (Diclofenac) after contusion injury up to time of sacrifice. The latter group served as a positive control for inflammatory effects. The PLA, received jelly cubes without *P. glandulosa* for the entire duration of the experimental feeding programme, the PG-CHR group received *P. glandulosa* mixed into jelly cubes for the entire duration of the experimental feeding programme up to the time of sacrifice, the PG-AI group only received *P. glandulosa* jelly cubes after the contusion injury was induced up to the time of sacrifice and the NSAID group received placebo jelly cubes for the entire duration of the feeding protocol and in addition Voltaren Emulgel® (Diclofenac) was applied to the injured area after contusion injury up to time of sacrifice (Fig. 5.2).

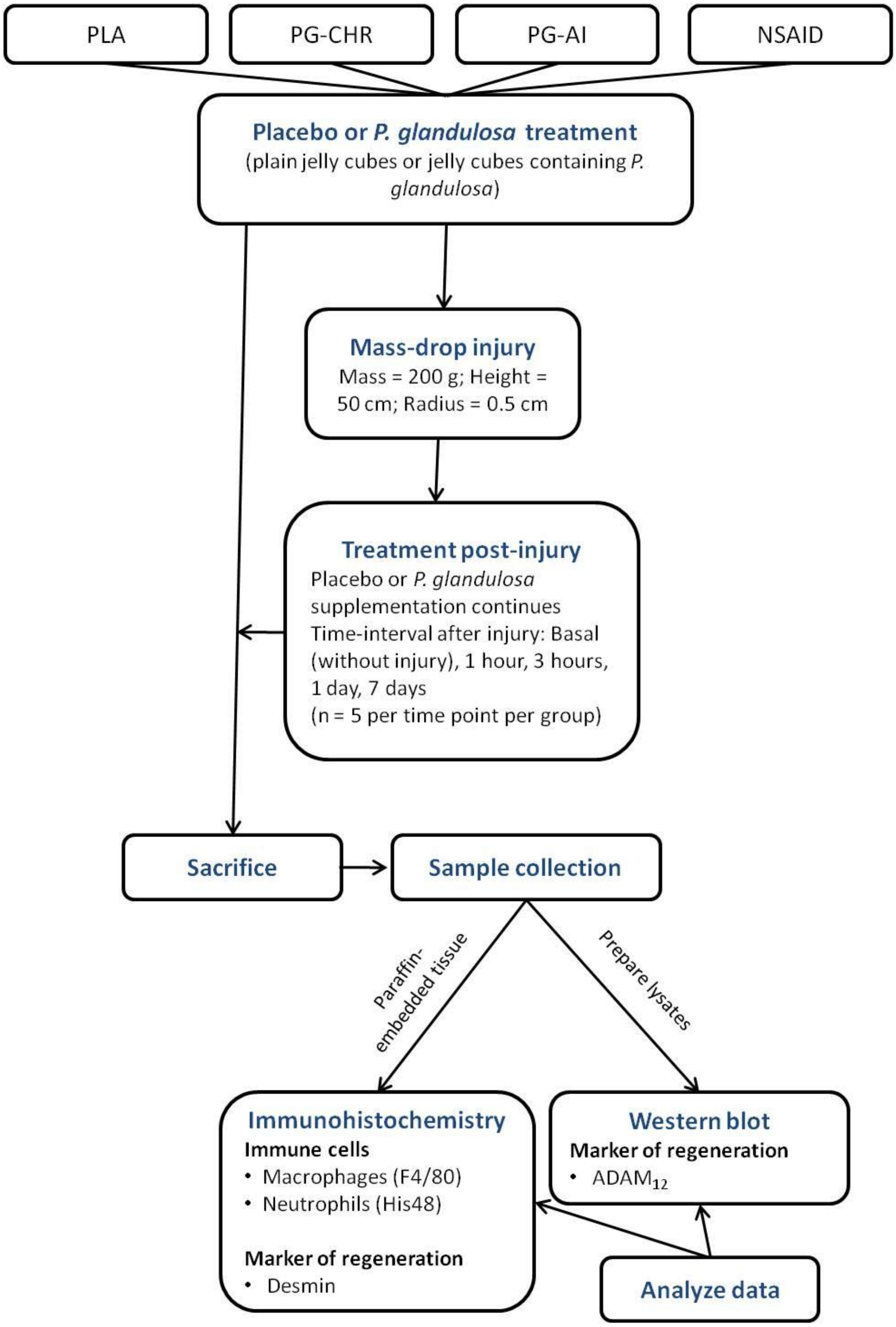


Figure 5.2: Schematic representation of the experimental design

5.2.2 Sacrifice and sample collection

All animals were sacrificed as previously described (refer to section 3.2.2.1). The only difference with this set of experiments was that the sacrifice procedure took place early morning (between 09h00 and 11h00) for the basal, 1-day and 7-day post-injury groups. The 1-hour post-injury group and the 3-hour post-injury group were sacrificed at their respective time point after injury.

After the injury procedures (refer to section 5.2.3), the gastrocnemius muscle of the right (injured) hindlimb was exposed by removing the skin and connective tissue surrounding the muscle. The central section of the damaged area of the gastrocnemius muscle was harvested and the harvested muscle randomly divided into two parts, so that one part could be processed for immunohistochemistry and the other part snap-frozen for Western blotting analysis, using Wollenberger tongs, pre-cooled in liquid nitrogen. After the muscles were snap-frozen, they were submerged into and stored in liquid nitrogen until later use (refer to section 5.2.4.4).

5.2.3 Induction of contusion injury

The contusion injury to the rat hind-limb was produced similarly to the drop-mass model first described by Stratton *et al.* (1984). Briefly, the rats were anaesthetised with an intra-peritoneal injection of sodium pentobarbital (40 mg/kg, intraperitoneal) and left until complete sedation was observed. There after the right lateral thigh (biceps femoris) of the rat was extended away from the hip joint and the muscle injured, being cautious not to injure the thigh bone. The anaesthetic left the rats unconscious for up to 2 hours post-injury. The Department of Physiology at Stellenbosch University has standardized and validated the moderately severe, non-invasive drop-mass injury model, in which contusion injury to the non-exposed gastrocnemius muscle of rats was delivered. The mass-drop contusion apparatus consists of a large round metal platform with a smaller round platform in its centre. The smaller platform is the part on which the hindlimb of the animal rests during injury. Directly above the smaller platform is a plastic tube which is mounted perpendicularly in such a way that it directs the passage of a 200 g circular-bottomed weight from a height of 50 cm onto the medial surface of the right gastrocnemius muscle of the rat (Fig. 5.3) [Kruger, 2011]. This contusion injury was moderately severe and did not leave the animals limping.

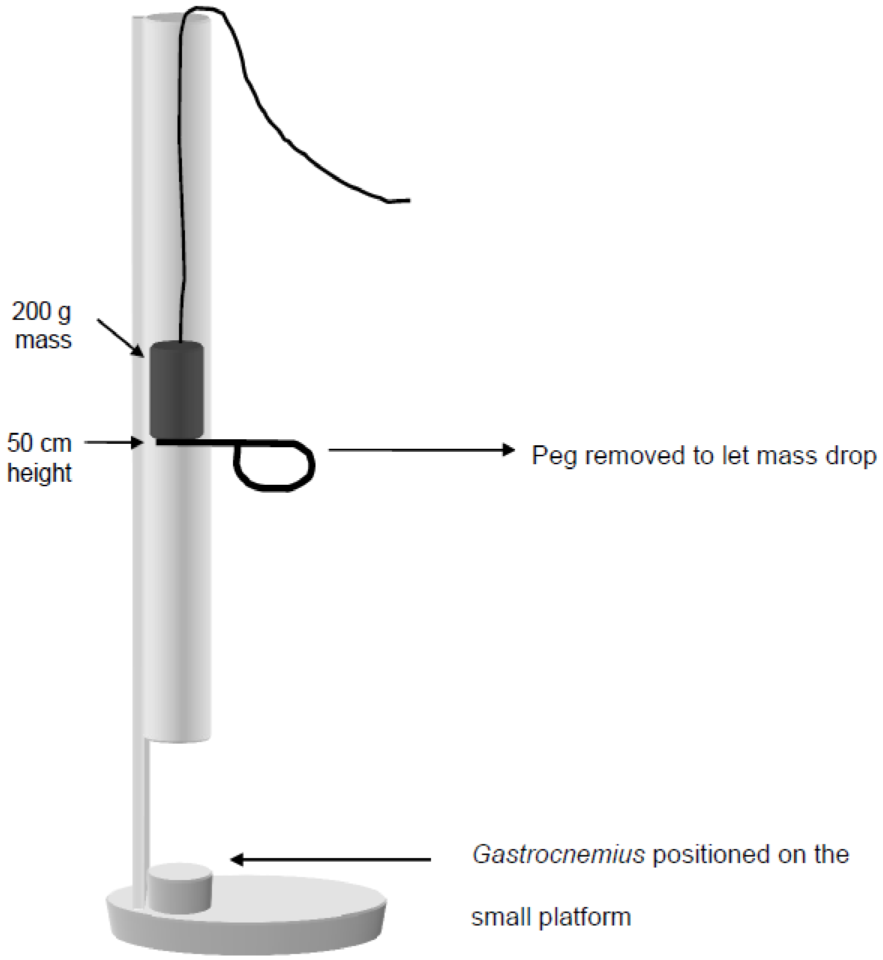


Figure 5.3: Schematical representation of muscle contusion injury jig. A mass of 200 g was dropped from a height of 50 cm onto a small platform on which the right gastrocnemius muscle of the anaesthetised rat was positioned. Engelbrecht, 2013.

5.2.4 Sample analysis

5.2.4.1 Processing and sectioning of paraffin-embedded tissue

Immediately after the careful excision of the gastrocnemius muscle, it was fixed in 10% formalin PBS solution. The tissue was kept in the fixation solution for at least 48 hours before further processing commenced. The processing protocol consisted of three steps, namely, (1) dehydration with a series of alcohol washes, (2) clearing with xylene and (3) infiltration with paraffin wax. The muscle specimens were processed using an automated processor (Duplex processor, Shandon Elliot, Optolabor (Pty) Ltd.). The processing protocol is shown in Table 5.1.

Table 5.1: Processing protocol

Steps	Solution	Time (min)	Temperature (˚C)
1	70 % Ethanol	30	40
2	80 % Ethanol	30	40
3	95 % Ethanol	45	40
4	95 % Ethanol	45	40
5	100 % Ethanol	45	40
6	100 % Ethanol	45	40
7	100 % Xylene	45	40
8	100 % Xylene	45	40
9	Paraffin wax	30	58
10	Paraffin wax	30	58
11	Paraffin wax	30	58
12	Paraffin wax	30	58

After processing, the tissue was embedded in paraffin wax at 60 ˚C. This was done by placing the tissue in a metal embedding mould and filling the mould with wax. A cassette was fixed to the mould and placed on an iced surface to allow the wax to set. Once the wax had set, it was removed from the mould to obtain the tissue wax block. Tissue blocks were kept at temperatures of between 20 – 25 ˚C until sectioning commenced. Two hours prior to sectioning, the tissue blocks were placed in a freezer too cool down. The tissue blocks were trimmed and sectioned using a Leica RM 2125 RT microtome to obtain uniform 5 µm sections. The sections were then placed in a water bath (± 40 ˚C), allowing the tissue to smooth out before being positioned onto glass slides and stained.

5.2.4.2 Haematoxylin and eosin (H&E) staining

Haematoxylin and eosin (H&E) stained sections were used to qualitatively assess the extent of the recovery process. This is a commonly used technique in animal histology and routine pathology. The basic dye, haematoxylin, stains basophilic structures a purplish blue. Cell nuclei, ribosomes and endoplasmic reticulum have strong affinity for this dye owing to their high content of DNA and RNA, respectively. In contrast, alcohol-based eosin stains eosinophilic structures bright red or pink. The eosinophilic structures are intra- or extracellular proteins, mostly cytoplasm and red blood cells. In general, when the H&E staining technique is applied to animal cells, nuclei stain blue and cytoplasm stains pink or red [Young *et al.*, 2006].

Prior to staining, slides were placed in an incubator in order for the wax to melt. Muscle tissue sections were stained with H&E with an autostainer (Leica Auto Stainer XL, SMM Instruments (Pty) Ltd.). The autostaining process included steps to de-wax, rehydrate and clear tissue, so that the slides may be permanently mounted. The H&E staining protocol is shown in Table 5.2.

Table 5.2: H&E staining protocol

Steps	Solution	Time	Repetitions
1	Xylene	10 min	X 2
2	99 % Ethanol	5 min	X 2
3	95 % Ethanol	2 min	X 1
4	70 % Ethanol	2 min	X 1
5	Distilled water	5 sec	X 1
6	Haematoxylin	8 min	X 1
7	Running water	5 min	X 1
8	1 % acid alcohol	30 sec	X 1
9	Running water	1 min	X 1
10	0.2 % Ammonia	45 sec	X 1
11	Running water	5 min	X 2
12	95 % Ethanol	10 dips	X 1
13	Eosin	45 sec	X 1
14	95 % Ethanol	5 min	X 2
15	Xylene	5 min	X 2

5.2.4.3 Immunohistochemistry

For immunohistochemistry, muscles were fixed in 4% formaldehyde for 7 days, where after they were cut to size, placed into embedding cassettes, processed and impregnated with paraffin wax (section 5.2.4.1 for details on tissue processing) using an automated tissue processor (Duplex processor, Shandon Elliot, Optolabor (Pty) Ltd.). Five µm thick cross sections were cut using a rotary microtome (Leica RM 2125 RT microtome). The immunohistochemistry staining (antibody information in Table 5.5) procedure was conducted using the automated Leica Bond Autostainer in combination with the Bond Polymer Refine detection kit (Leica Biosystems, SMM Instruments (Pty) Ltd). The staining protocol is shown in Table 5.3.

Table 5.3: Immunohistochemistry staining protocol

Steps	Type	Incubation time (min)	Temperature	Dispense type
1	Peroxide block	5	Ambient	Selected volume
2	Bond wash solution	0	Ambient	Selected volume
3	Bond wash solution	0	Ambient	Open
4	Bond wash solution	0	Ambient	Selected volume
5	Primary antibody	15	Ambient	Selected volume
6	Bond wash solution	0	Ambient	Selected volume
7	Bond wash solution	0	Ambient	Selected volume
8	Bond wash solution	0	Ambient	Selected volume
9	Post primary	8	Ambient	Selected volume
10	Bond wash solution	2	Ambient	Selected volume
11	Bond wash solution	2	Ambient	Selected volume
12	Bond wash solution	2	Ambient	Selected volume
13	Polymer	8	Ambient	Selected volume
14	Bond wash solution	2	Ambient	Selected volume
15	Bond wash solution	2	Ambient	Selected volume
16	Deionized water	0	Ambient	Selected volume
17	Deionized water	0	Ambient	Selected volume
18	Mixed DAB refine	10	Ambient	Selected volume
19	Deionized water	0	Ambient	Selected volume
20	Deionized water	0	Ambient	Selected volume
21	Deionized water	0	Ambient	Selected volume
22	Hematoxylin	5	Ambient	Selected volume
23	Deionized water	0	Ambient	Selected volume
24	Deionized water	0	Ambient	Selected volume
25	Deionized water	0	Ambient	Selected volume

After the automated staining protocol, the tissue samples were rehydrated and cleared manually. The steps for the rehydration process are shown in Table 5.4.

Table 5.4: Rehydration of tissue samples

Steps	Solution	Duration
1	10 % alcohol	5 dips
2	96 % alcohol	5 dips
3	96 % alcohol	5 dips
4	99 % alcohol	5 dips
5	99 % alcohol	5 dips
6	Xylene	Dip for 1 min
7	Xylene	Dip for 1 min

After the rehydration step, the slides were mounted and were ready for visualization by means of microscopy. All imaging data were obtained by analyzing one section from each muscle sample, at each time point for each antibody. In the injured area, five fields of view per section were imaged using a microscope (Nikon ECLIPSE E400; 40x objective used; actual enlargement thus 400x), equipped with a colour digital camera (Nikon 5.0 Mega Pixels Color Digital Camera head DS-Fi2).

Note that the images presented here are only partial images of those taken at 400x. All stained samples were assessed in sections. Desmin-stained cells and immune cells were counted manually and expressed as the number of positively labeled immune cells per field of view (350 μm^2) in the injured area, using the NIS-Elements BR imaging software package. To ensure accurate counting of neutrophils, multilobular nuclei had to be present.

Table 5.5: Antibodies used to identify neutrophils (His48), macrophages (F4/80), and Desmin.

Antibodies	Supplier	Catalogue no	[Stock]
Granulocytes (His48) mouse monoclonal IgG	Santa Cruz	sc- 19613	200 µg/ml
F4/80 (M-300) rabbit polyclonal IgG	Santa Cruz	sc- 25830	200 µg/ml
Desmin (Y-20) goat polyclonal IgG	Santa Cruz	sc- 7559	200 µg/ml

5.2.4.4 Western blotting

Protein levels were determined by standard Western blotting techniques. Briefly, 30 µg protein per 9 µl lysate was prepared. Samples were then analyzed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were immunoblotted for $ADAM_{12}$ (Biocom Biotech, Clubview, South Africa). β-tubulin was used to determine equal loading.

Protein extraction

The proteins of interest were extracted from the gastrocnemius muscle tissue by means of a lysis buffer that contained: 2 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1 mM β-glycerophosphate, 2.5 mM tetrasodiumpyrophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 50 µg/ml phenylmethyl sulfonyl fluoride (PMSF). Frozen gastrocnemius muscle tissue (± 200 mg) was pulverized and homogenized, using a Polytron PT-10 homogenizer (2 x 4 sec, setting 4) in 0.9 ml cold lysis buffer. The homogenate was left to stand on ice for 15 minutes to allow digestive processes to take place. After 15 minutes, the homogenate was transferred to Eppendorf tubes and samples were subjected to centrifugation at 1000 g for 10 min at 4 °C, where after the supernatant was collected in a separate set of Eppendorf tubes.

The protein content of each sample was measured by means of the Bradford protein method [Bradford 1976]. Bradford solution contained: 0.6 mM Coomassie Brilliant Blue G-250, 95% ethanol and 85% (w/v) phosphoric acid. Colour development (absorbance) was read at 595 nm against a blank and sample values were determined from a standard curve generated from bovine serum albumin (BSA) of known concentrations. This sensitive method is suitable for measuring microgram quantities of proteins. The supernatant was then diluted in lysis buffer and Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptothanol, 0.0004% bromphenol blue and 0.125 M Tris-HCl) to contain equal amounts of protein per volume unit [Laemmli, 1970]. The samples were boiled for 5 min and aliquots stored at -80 °C.

Protein separation

All stored aliquots were boiled for 5 min and subjected to centrifugation at 15000 rpm for 2 min. Of each sample, 30 µg of protein was loaded in a 4% stacking polyacrylamide gel and separated according to their molecular weights by subjection to a 7.5% SDS-PAGE in running buffer. The running buffer contained: 50 mM Tris, 384 mM glycine and 1% SDS. A standard Bio-RAD Mini-Protean III system was used. A protein ladder, obtained from Fermentas Life Sciences, was utilized as marker to identify the molecular weights of the proteins of interest.

The proteins separated within the SDS-gel were then transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon™ P, Millipore) with an applied electrical current of 200 V for 1 hour, in a tank filled with transfer buffer. The transfer buffer consisted of 25 mM Tris, 192 mM glycine and 20% methanol. At the end of the transfer period, the membranes were immersed in fresh methanol and left to air dry. This was done so that the membranes could be stained with 5% Ponçeau Red in acetic acid (reversible protein stain), for visualization of proteins and to confirm whether adequate transfer did occur.

Once the Ponçeau Red was rinsed off, the non-specific binding sites on the membranes were blocked by gently incubating them in fat-free milk, made up in a TBS-Tween solution (Tris-buffered saline (TBS) plus 0.1% Tween 20), for 2 hours, at room temperature on a shaker. At the end of the "blocking" period, the membranes were thoroughly washed in the TBS-Tween solution. These membranes were then probed with primary antibodies directed against $ADAM_{12}$ (ABCAM) and left to incubate overnight at 4 °C. $ADAM_{12}$ was diluted to a 1:5000 ratio (1 µl primary antibody in 5 ml TBS/Tween).

Immunodetection of protein

After the overnight primary antibody incubation, the membranes were thoroughly washed in TBS-Tween and thereafter incubated in secondary antibody for 1 hour at room temperature on a shaker. The secondary antibody was a horseradish-peroxidase conjugated donkey anti-rabbit immunoglobulin G (Amersham life Science, Sandton, Johannesburg). The secondary antibody was diluted to a 1:4000 dilution (5 µl secondary antibody per 20 ml of a 2.5% milk/TBS-Tween solution). This conjugated antibody now bound to the already bound primary antibody. To remove the excess secondary antibody, the membranes were washed extensively in TBS-Tween and kept moist.

Proteins were visualized by covering the membrane with enhanced chemiluminescence (ECL) detection reagent (from Amersham life Science, Sandton, Johannesburg) for 1 minute and then exposing it to an autoradiography film (Hyperfilm ECL, RPN 2103). The horseradish-peroxidase reacts with the detection reagent in a luminescence reaction and light emission that results, is captured on the radiography film. Band intensities were then densitometrically quantified using UN-SCAN-IT^{TM} (version 5.1, Silkscience) image analysis software.

For comparison purposes, samples from negative control gastrocnemius muscle were always included in each blot and used for normalization of the unknown samples (i.e. calculation of the ratio between the sample and negative control). Normalized data was expressed in arbitrary units (AU).

In all instances the membranes were stripped, by incubating for 5 min in 0.2 M NaOH and reblotted with antibody against β-tubulin (1:1000, Cell Signaling Technology, Beverly, MA) to verify the uniformity of protein load and the transfer efficiency across the test samples.

5.2.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance between two groups was assessed via a Student t-test and between two or more groups, a two-way ANOVA, followed by a Bonferroni-post hoc test was used. $p < 0.05$ was considered as statistically significant. Statistical analysis of data was performed using GraphPad Prism version 5.

5.3 RESULTS

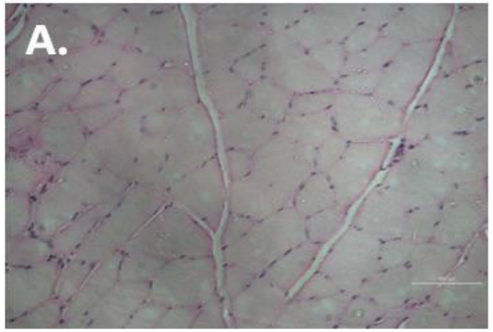
5.3.1 Body weight

Rats in the different experimental groups were matched for body mass at the start of the protocol. There were no significant differences in body mass found between the different groups at the time of sacrifice (PLA: 456.47 ± 9.74 g; PG-CHR: 445.98 ± 11.21 g; PG-AI 439.12 ± 14.84 g; NSAID: 442.25 ± 12.58 g).

5.3.2 Muscle injury: Immune cell infiltration

5.3.2.1 H&E

Qualitative microscopic analysis of the fiber architecture post-contusion injury, indicted that irrespective of treatment, the blunt force to the muscle belly significantly damaged and disrupted the skeletal muscle fibers, resulting in red blood cell accumulation in the interstitial spaces at 1 hour and 3 hours after injury (Figs. 5.4 B, E, H, K). Representative pictures for 1 hour after injury are not included here as 1 hour and 3 hours post-injury does not visually differ. In addition, edema was also present in both treated and untreated groups, confirmed by the widening of the interstitial spaces between the fibers at this early time point. Histological comparison between the PLA and the treatment groups illustrated a significant influx of immune cells 1 day after injury in all four groups. However, this influx was relatively limited in the group chronically treated with *P. glandulosa* (PG-CHR), compared to all other groups. The immune cells remained visible in the injured area of the PLA, PG-AI and NSAID groups for up to day 7 post-injury (Figs. 5.4 D, J, M), but were undetectable in the PG-CHR group at the same time point (Fig. 5.4 G). By day 7 only the chronically treated *P. glandulosa* group (Fig. 5.4 G) displayed near normal muscle architecture, indicative of successful progressing muscle regeneration.



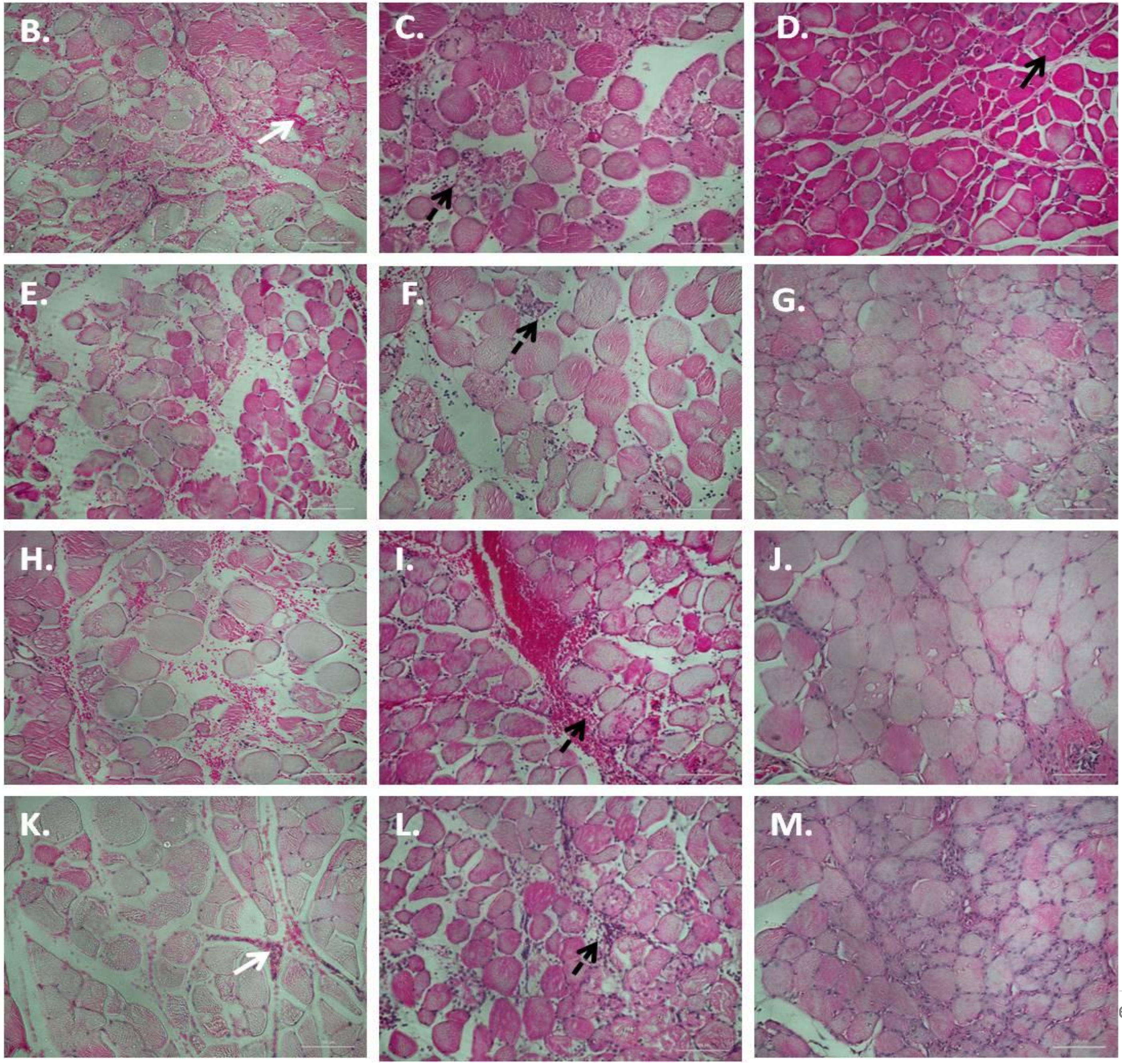


Figure 5.4: H&E stains, illustrating the clearing of inflammation after injury. E. Picture A represent uninjured samples. Pictures B–D represent samples taken from PLA animals at 3 hours (B), 1 day (C) and 7 days (D) post-injury. Pictures E-G, H-J and K-M represent similar time points in the PG-CHR, PG-AI and NSAID groups respectively. Scale bar represents 100 µm. Pictures B, E, H and K represent muscle fiber destruction and vascular disruption. Immune cells infiltration into the injured area is visible from 1 day post-injury (Pictures C, F, I and L). *Solid white arrows* indicate red blood cells, *solid black arrows* points to newly regenerated muscle fibers and *dashed arrows* indicate immune cells.

5.3.2.2 Neutrophils

Clear differences were evident between the various experimental groups with regards to neutrophil infiltration. No neutrophils were present in the any of the experimental groups before injury, whereas contusion injury resulted in a significant (between 30- and 40-fold) transient elevation in neutrophils on day 1 after injury, which had normalised by day 7 post-injury ($p < 0.0001$) (Fig. 5.6). On day 1 post-injury, the PG-CHR ($p < 0.0001$), PG-AI ($p < 0.001$) as well as the NSAID treatment groups ($p < 0.0001$) displayed a significantly lower number of neutrophils compared to the untreated group (PLA). Furthermore, the magnitude of the neutrophils response as assessed on day 1 post-injury was similar in these three treatments groups. Neutrophil infiltration on day 1 and day 7 after injury is represented by Figure 5.5.

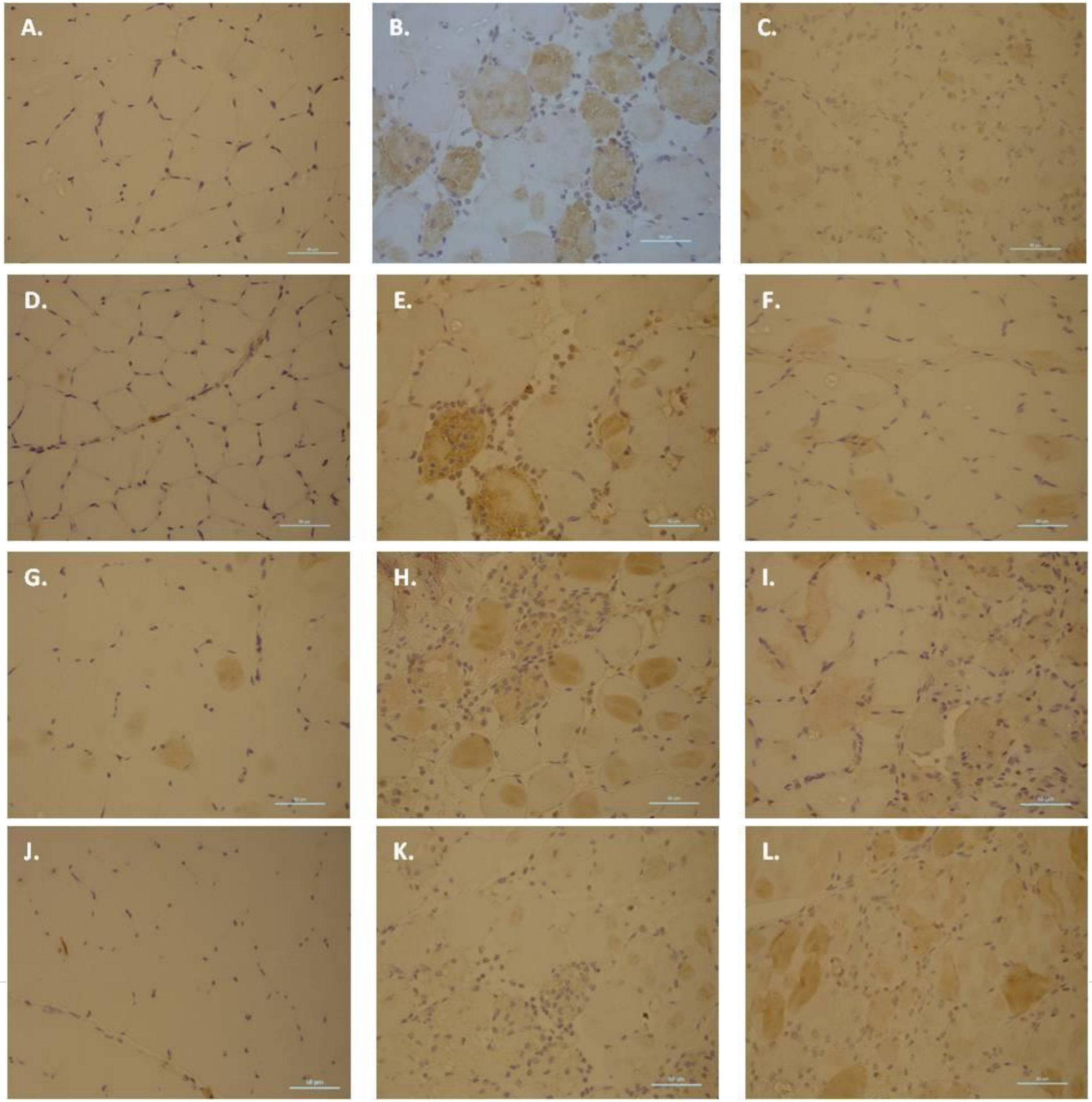


Figure 5.5: Neutrophil (His48) expression and infiltration into the injured area of muscle of PLA (**A-C**), PG-CHR (**D-F**), PG-AI (**G-I**) and NSAID (**J-L**). Figures A, D, G and J represent samples taken from uninjured rats, Figures B, E, H and K represent samples taken 1 day post-injury and Figures C, F, I and L represent samples taken 7 days post-injury. Scale bar represents 50 µm, with the original magnification of 400x used for the image acquisition. His48-positive cells with multi-lobed nuclei were counted as neutrophils.

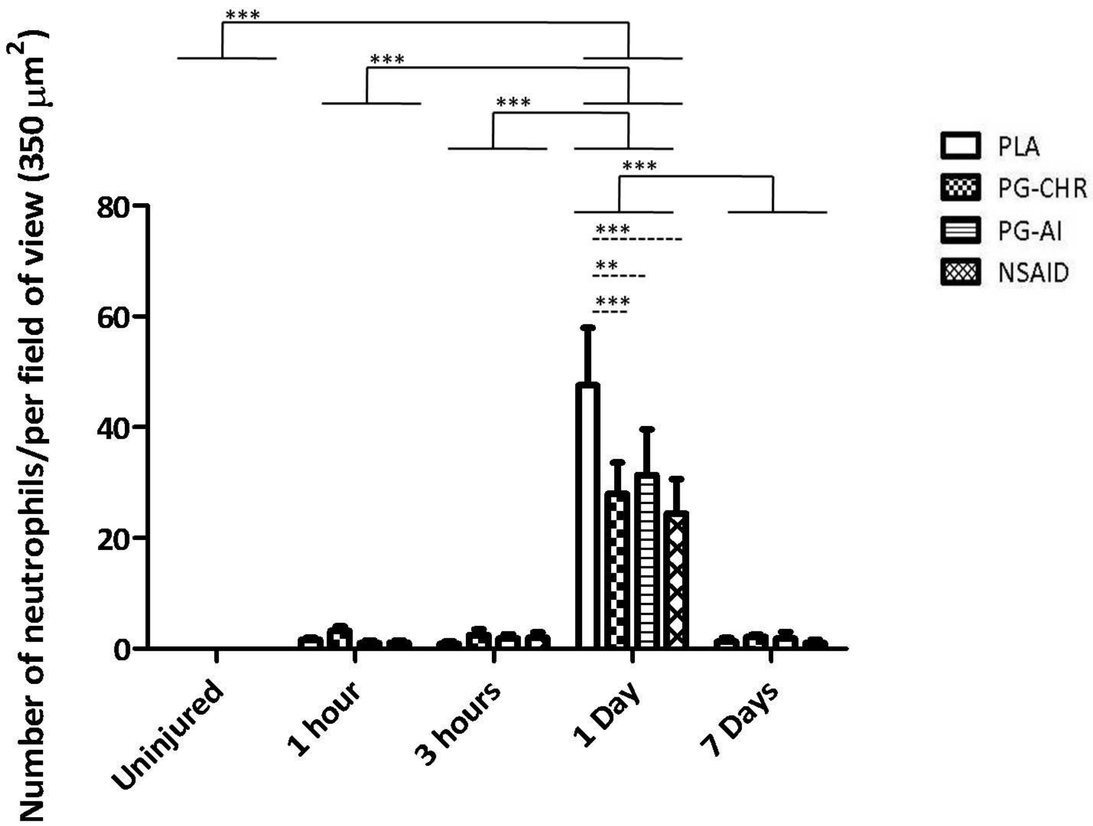


Figure 5.6: Neutrophils (His48 stain) infiltration into injured area after contusion injury and subsequent treatment. Statistical representation of data obtained. The data are expressed as mean ± SEM. Analysis were done by two-way ANOVA. n = 5 per time-point/per group; Differences over time are indicated by solid lines and broken black lines indicate group differences at specific time point; Significance: All groups: ***p < 0.0001 uninjured vs. 1 day; 1 hour vs. 1 day; 3 hours vs. 1 day; 1 day vs. 7 days. 1 Day: ***p < 0.0001 PLA vs. PG-CHR; PLA vs. NSAID; **p < 0.05 PLA vs. PG-AI

5.3.2.3 Macrophages

Similar to the neutrophil data, the presence of macrophages was undetectable in the uninjured control samples (Fig. 5.8). Of the time-points assessed, the peak number of macrophages ($p < 0.001$) present in the injured area, was 1 day after injury in all four experimental groups. These increased values had again normalised by day 7 after injury ($p < 0.001$). None of the treatments seem to have any effect on macrophage infiltration at the time points assessed. Figures 5.7 is representative of macrophage infiltration into the injured area of uninjured muscle, 1 day and 7 days after injury.

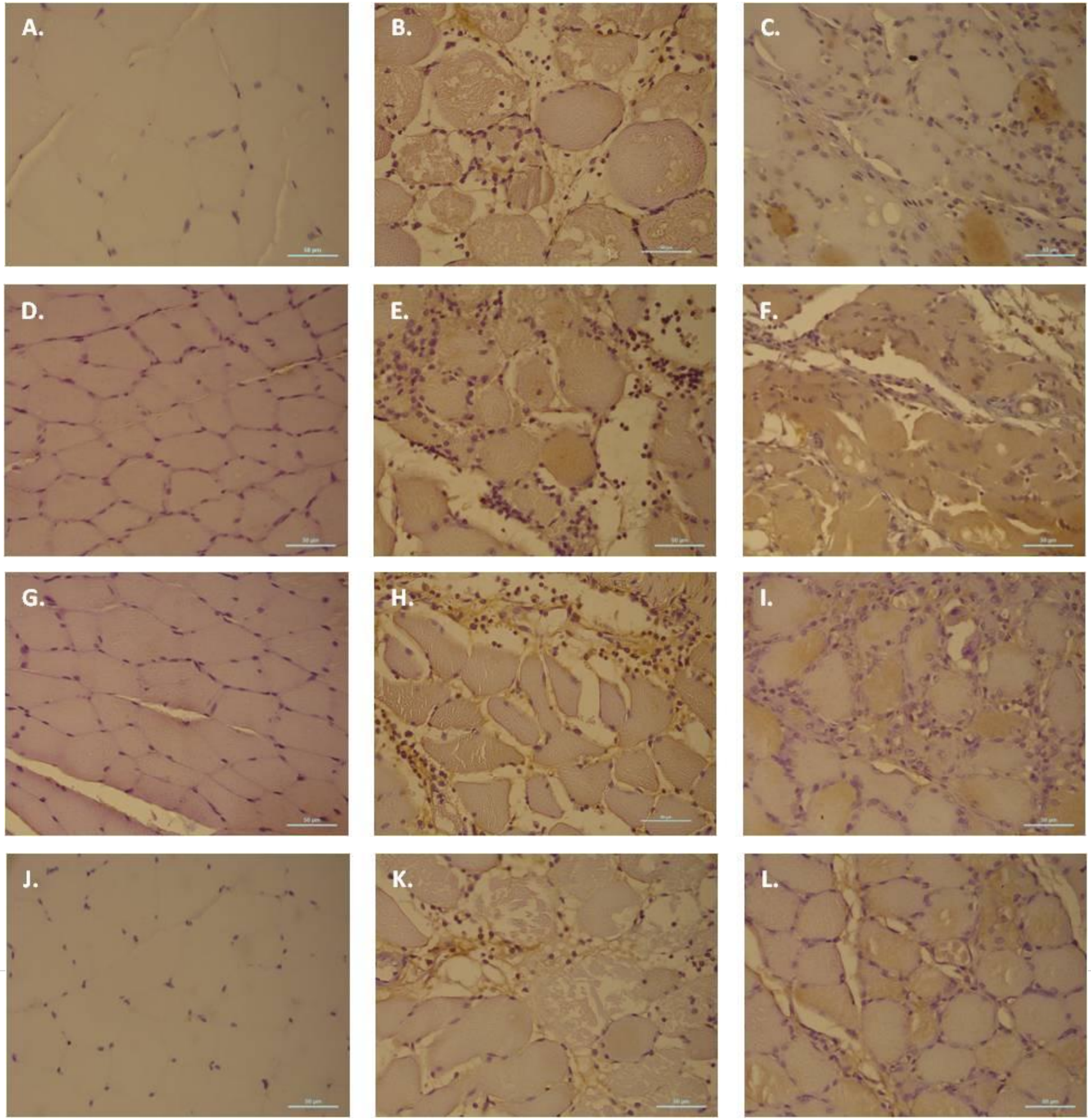


Figure 5.7: Macrophage (F4/80) expression and infiltration into the injured area of muscle of PLA (**A-C**), PG-CHR (**D-F**), PG-AI (**G-I**) and NSAID (**J-L**). Figures A, D, G and J represent samples taken from uninjured rats, Figures B, E, H and K represent samples taken 1 day post-injury and Figures C, F, I and L represent samples taken 7 days post-injury. Scale bar represents 50 µm, with the original magnification of 400x used for the image acquisition. F4/80-positive cells with a single nucleus cell and surrounding cytoplasm were counted as macrophages.

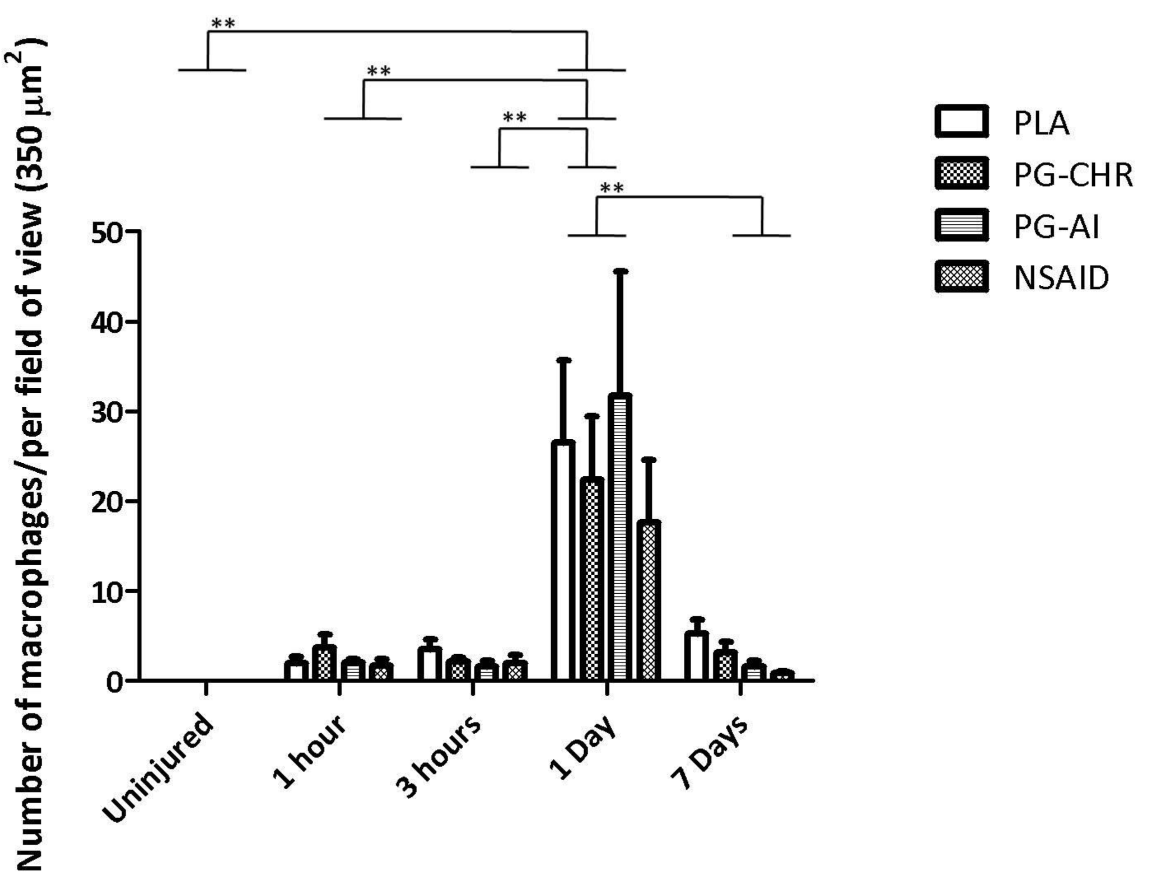


Figure 5.8: Macrophage infiltration into injured area after contusion injury and subsequent treatment. Statistical representation of data obtained. The data are expressed as mean ± SEM. Analysis were done by two-way ANOVA. n = 5 per time-point/per group; Differences over time are indicated by solid lines; Significance: All groups: **p < 0.001 uninjured vs. 1 day; 1 hour vs. 1 day; 3 hours vs. 1 day; 7 days vs. 1 day

5.3.3 Muscle recovery: Muscle regeneration

5.3.3.1 $ADAM_{12}$ expression

According to the Western blot analysis, expression of the satellite cell proliferation marker, $ADAM_{12}$, was significantly elevated from 3 hours post-injury ($p < 0.0001$) and this significant elevation persisted for at least 24 hours ($p < 0.0001$), with the expression again normalized to uninjured levels on day 7 after injury, in all experimental groups ($p < 0.0001$) (Figs. 5.10 A and B). Of all three treatments assessed, the 8-week chronic treatment with *P. glandulosa* (PG-CHR) showed the most significant effect with significantly increased ($p < 0.05$) expression of $ADAM_{12}$, on day 1 post-injury, when compared to the PLA group. Although post-injury treatment (PG-AI) seemed to suppress $ADAM_{12}$ expression at 3 hours, when compared to PLA, it was associated with a significant increase in $ADAM_{12}$ expression from 3 hours to 1 day. NSAID treatment was associated with a similarly suppressed $ADAM_{12}$ expression at 3 hours, but with this treatment, the relative suppression persisted at 1 day after injury. Indeed, the NSAID group expressed lower levels of $ADAM_{12}$, compared to the chronically treated *P. glandulosa* group at both 3 hours and 1 day post-injury, significantly so on the latter ($p < 0.001$). For the sake of clarity the statistical differences observed between the different time-points in each experimental group is illustrated in Figure 4 (C). Figure 5.9 is a representative Western blot of the different experimental groups. For the sake of convenience and clarity the statistical differences observed between the different time-points in each experimental group is illustrated in Figure 5.10 (B).

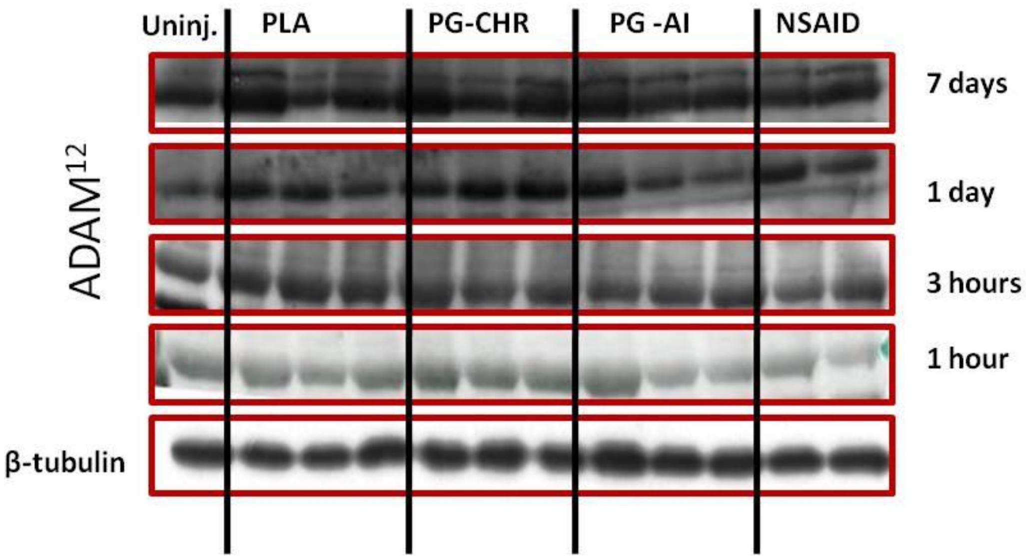


Figure 5.9: Representative Western blots of $ADAM_{12}$ expression in skeletal muscle following a contusion injury. The top 4 bands represents $ADAM_{12}$ expression at the different time points after injury and the bottom bands represents β-tubulin expression. Stripped blots from the different groups were reprobed with an antibody against β-tubulin to confirm equal loading of the protein.

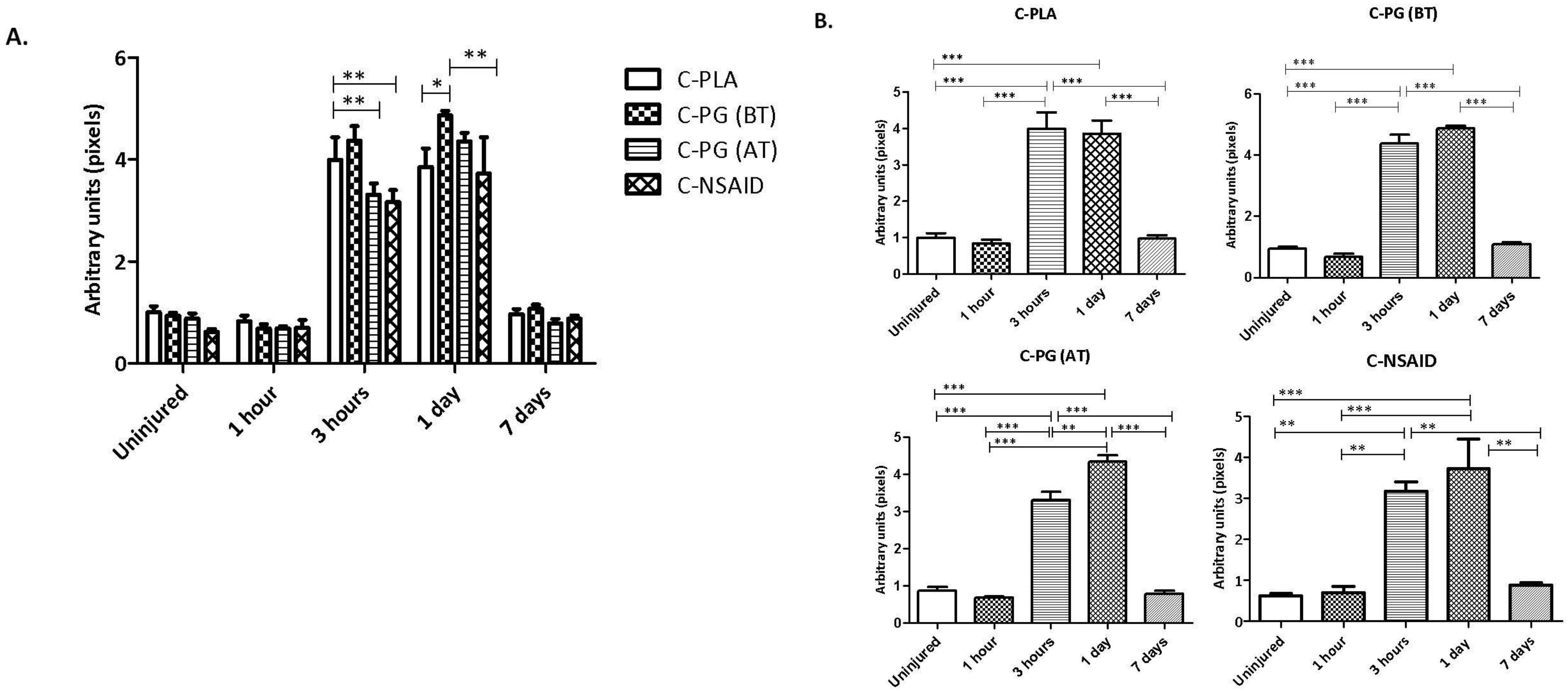


Figure 5.10: ADAM$_{12}$ expression in skeletal muscle following a contusion injury. (A) Represents the combined data for all the different groups and (B) represents the statistical differences observed between the different time-points in each experimental group. Values are expressed relative to the uninjured values. The data are expressed as mean ± SEM; Analysis by two-way ANOVA; n = 5 per time-point/per group; Differences over time are indicated by solid lines and broken black lines indicate group differences at specific time point; Significance: *** p < 0.0001, ** p < 0.001 and * p < 0.05

5.3.3.2 Desmin expression

Desmin expression was found to steadily increase after injury, with highest values at the 7 days post-injury time point, in all four different experimental groups. At the 7-day post-injury time-point, the chronically treated *P. glandulosa* group (PG-CHR) displayed significantly elevated desmin expression compared to all other groups (Fig. 5.12). While post-injury *P. glandulosa*-treatment had no effect on the expression of desmin, the NSAID-treated group displayed significantly decreased desmin expression, when compared to all other groups, indicative of delayed regeneration.

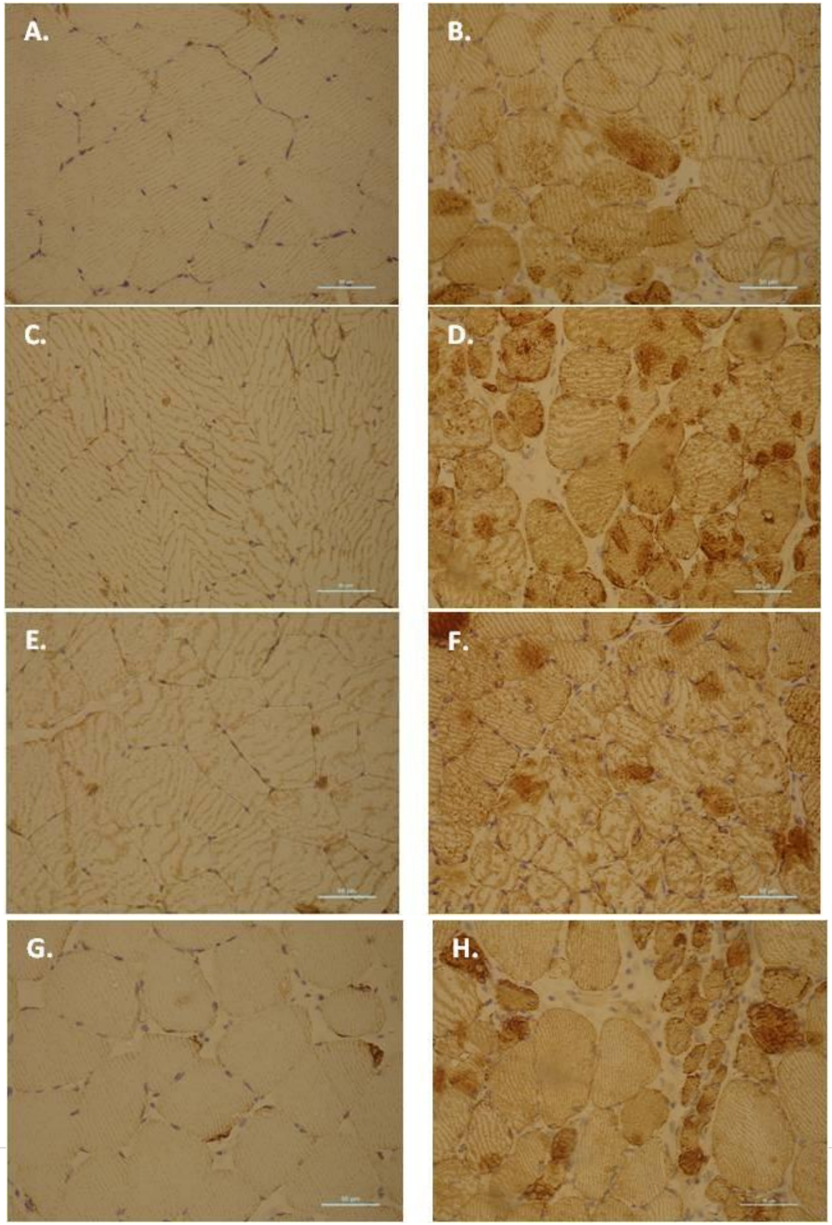


Figure 5.11: Desmin expression in the injured area of muscle of PLA (**A-B**), PG-CHR (**C-D**), PG-AI (**E-F**) and NSAID (**G-H**). Figures A, C, E and G represent samples taken from uninjured rats, Figures B, D, F and H represent samples taken 7 days post-injury. Scale bar represents 50 µm, with the original magnification of 400x used for the image acquisition. Positive desmin stains brown at the Z-disks.

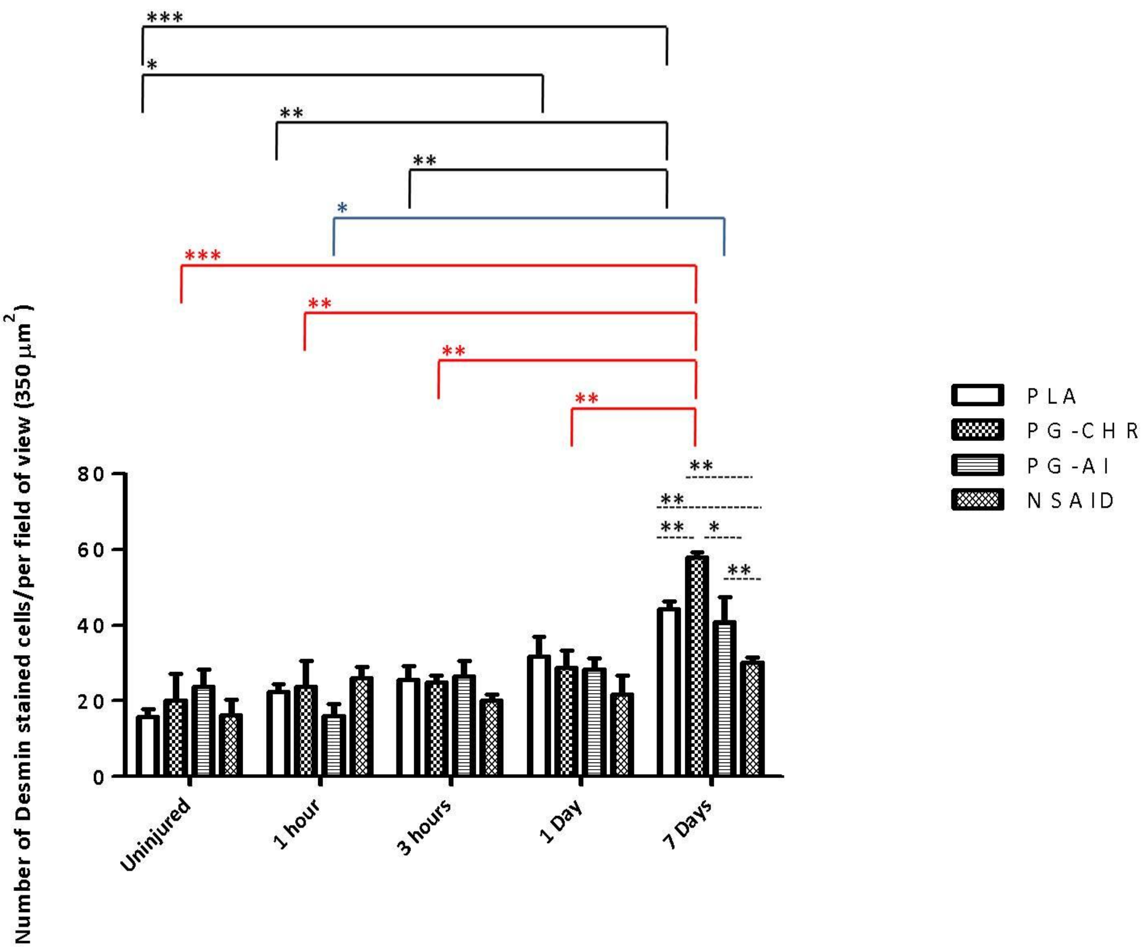


Figure 5.12: Desmin expression in skeletal muscle following a contusion injury. The data are expressed as mean ± SEM; Analysis by two-way ANOVA; n = 5 per time-point/per group; Differences over time are indicated by solid black lines (PLA), solid blue lines (PG-CHR), solid red lines (PG-AI) and broken black lines indicate group differences at specific time point; Significance: *** p < 0.0001, ** p < 0.001 and * p < 0.05

5.4 DISCUSSION

When muscles are injured, such as during active stretch or contusion injury, chemotactic factors are released by the myocytes and other surrounding cells, which results in immune cell mobilization and attraction to the injured area [Cannon and St Pierre, 1998; Fielding *et al.,* 1993; Smith *et al.,* 2008]. In this part of the study we focussed mainly on the effect that chronic *P. glandulosa* treatment had on neutrophil and macrophage infiltration into the injured area, as well as the effect thereof on markers of regeneration, $ADAM_{12}$ and desmin. In addition to the 8-weeks chronically treated *P. glandulosa* group, we also had a group of animals which was only placed on *P. glandulosa* treatment after injury as well as a group of rats that were placed on NSAID treatment (diclofenac) post-injury. The latter group of rats were used as positive controls, as the effects of NSAID's on immune cell infiltration and the progression of regeneration have been researched before. The post-injury *P. glandulosa* group were used to determine whether *P. glandulosa* could be used as a possible treatment option after injury or whether *P. glandulosa* treatment is only effective if used prior to injury. Muscle injury was induced by contusion injury, which was accomplished by the strike of a blunt object to the gastrocnemius muscle of a rat.

Indeed, we present compelling evidence for an effect of *P. glandulosa* at the tissue level in the early phase response to injury.

5.4.1 Neutrophil and macrophage infiltration

It is well known that an early response to muscle damage is the recruitment of neutrophils to the site of injury [Smith *et al.,* 2008]. However, not much data is available with regards to the effect and specific time frame of immune cell infiltration after a contusion injury. The general idea is that neutrophil numbers peak predominantly 1 day after injury, which is followed by the resolution of inflammation roughly around day 5 after injury [Smith *et al.,* 2008]. However, as mentioned in the introduction above, the time points at which neutrophils start to infiltrate the injured area and resolution of inflammation, differs with different injury models. For example, in rabbits, excessive stretch injury resulted in neutrophils infiltration from 4 hour after injury and undetected 48 to 72 hour after injury [St. Pierre Schneider *et al.,* 2002]. Conversely, Marsolais *et al.* (2001) found neutrophils were still elevated for up to 5 days after injury in a rat model of Achilles tendon injury. Similar to previous studies on contusion injury [Kruger and Smith, 2012; Myburgh *et al.,* 2012], we found the infiltration of neutrophils within 1 hour after injury and the neutrophil numbers reaching a peak at ± 24 hours post-injury (Fig. 5.6). The neutrophil numbers then decreased rapidly and were essentially undetectable by day 7 post-injury. In our study the time-points between 2 and 6 days after injury were not measured, so the numbers of neutrophils at those time points are not known. However, one can extrapolate from the studies mentioned above by Kruger and Smith (2012) and Myburgh *et al.* (2012), in which the same injury model was used. Similar to our results they found that the neutrophil numbers in the control group, after contusion injury, were significantly elevated in the injured area 1 day after injury and in addition they found a significant decrease in neutrophil number from day 3 post-injury onward.

The macrophage phase of inflammation follows the neutrophil phase, with macrophage cell numbers only peaking roughly around day 5 post-injury [Tidball and Wehling-Henricks, 2005; Kruger and Smith, 2012 Myburgh *et al.,* 2012]. These two phases overlap and cell numbers are inversely related, with significant numbers of macrophages appearing after day 2, when neutrophil numbers start to decline [Tidball and Wehling-Henricks, 2005]. From the time points measured in our study, macrophages were already present 1 and 3 hours after injury, in low numbers, similar to results obtained from other studies using the same injury model [Kruger and Smith, 2012; Myburgh *et al.,* 2012]. In addition, we found the number of macrophages to be highest 1 day after injury, compared to all the other time points; however we did not evaluate the presence of macrophages at time-points between 2 and 6 days after injury, so it remains unknown whether the number of macrophages further increased to reach a peak around day 5 post-injury as the literature states [Tidball and Wehling-Henricks, 2005; Kruger and Smith, 2012; Myburgh *et al.,* 2012]. Previous researchers using the same injury model, found a significant elevation in macrophage number 3 to 5 days post-injury, followed by the significant reduction in macrophage number by day 7 post-injury [Kruger and Smith, 2012 Myburgh *et al.,* 2012]. The significant reduction in macrophage number 7 days after injury observed in the above-mentioned studies is similar to the results found in our study.

One of the profound findings in our research was that both 8-week chronic pre-treatment with *P. glandulosa* and *P. glandulosa* treatment post-injury resulted in significantly fewer (2-fold less) neutrophils in the injured area, 1 day after injury (Fig. 5.6), a crucial time-point during the inflammatory phase. This neutrophil blunting effect observed in our study corresponds with previous studies, albeit with another herbal substances [Kruger and Smith, 2012; Myburgh *et al.,* 2012; Donnelly *et al.,* 2004; Andrade *et al.,* 2011]. Kruger and Smith (2012) and Myburgh *et al.* (2012) pre-treated rats with procyanidins (PCO) for 14 days prior to injury and for up to 14 days post-injury and found that the number of neutrophils present in the injured area was significantly lower in the PCO-treated group, compared to the untreated control group. Similarly, Donnelly *et al.* (2004) found that the grape-derived, resveratrol, acted as an anti-inflammatory agent by partially inhibiting the activation of the immune cells and Andrade *et al.* (2011) found guabiju extract inhibited neutrophil chemotaxis, inevitably decreasing the number of neutrophils at the site of injury.

This effect of *P. glandulosa* on neutrophil blunting suggests that treatment either caused a (i) lesser degree of activation of these immune cells, (ii) a lesser capacity for extravasation of the neutrophils or (iii) an increased functional capacity of neutrophils. Even though we did not measure the number of neutrophils in circulation (only at tissue level), it does not seem as if *P. glandulosa* altered the mobilization of the neutrophils, as the number of these immune cells at the site of injury was similarly elevated in all groups at both 1 hour and 3 hours after injury (Fig. 5.6). It was only on day 1, post-injury, that the number of neutrophils stayed elevated in the PLA group, when compared to the treated groups. Kruger *et al.* (2012), who conducted a similar study to our study using the same injury model, did a follow-up study in which they found that the significant reduction in neutrophil presence in the injured area was as a result of reduced neutrophil extravasation from the blood [Kruger *et al.,* 2013]. In this study they investigated the effect of PCO on circulating neutrophils and macrophage populations and *in vitro* neutrophil migration, by utilized primary cultured neutrophils, obtained from control animals, which they incubated in media with 20% conditioned plasma. In this study they found that on day 1 post-injury, circulating neutrophil numbers were significantly lower in the control group, compared to the PCO-treated group, suggesting the extravasation from the blood was reduced in the treated group. Concurrently, the data obtained from their *in vitro* studies established that neutrophil migration was blunted in the presence of PCO-conditioned plasma from supplemented rats. Earlier findings by other researchers have demonstrated that neutrophil attraction, adhesion and migration can be influenced by an increase in ROS generation [Fialkow *et al.*, 2007]. For example, Lewis *et al.* (1988) showed that H_2O_2 increases neutrophil adhesion and Judge and Dodd (2003) demonstrated that blocking xanthine oxidase, an enzyme that generates ROS, results in decreased neutrophil infiltration. One can therefore infer that treatment with an antioxidant, which scavenges ROS, would alleviate the excessive ROS production and thus decrease the attraction, adhesion and migration of circulating neutrophils. Since previous studies conducted in our laboratory demonstrated that *P. glandulosa* does not seem to elicit its effects by antioxidant mechanisms (Fig. 3.2), we turned our attention to alternative mechanisms of action for *P. glandulosa* that could affect infiltration of neutrophils into the site of injury.

Another potential mechanism by which *P. glandulosa* may exert its neutrophil-blunting activity may be by reducing the ability of the neutrophils to migrate to the site of injury by reducing the expression of adhesion molecule on the circulating neutrophils or reducing the chemotactic process. Since there is no other known literature on the effect of *P. glandulosa* or any of the other *Prosopis* species with regards to neutrophil response to skeletal muscle injury, we draw inference from the effects other herbal substances have on this response. Garbacki *et al.* (2004) for example, found that treatment with an extract from blackcurrant leaves reduced neutrophil infiltration and proposed this to be due to neutrophil-endothelial cell interaction that might have reduced the process of extravasation. Kalin *et al.* (2002)

conducted a study on systemic sclerosis and reported that treatment with activin, a grape seed-derived PCO, was able to reduce circulating soluble adhesion molecules, intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM), endothelial (E)-selectin and platelet (P)-selectin, which consequently led to the reduced number of neutrophils migrating to muscle. Tong *et al.* (2013) also demonstrated that the water-soluble polysaccharides from *Bupleurum chinense* could significantly impair *in vivo* neutrophil infiltration and inhibit the activation of the chemoattractant, formyl-methionyl-leucyl-phenylalanine (fMLP) and clustering of β2 integrin.

In addition to the aforementioned chemotactic factors, cytokines also act as chemotactic factors, attracting both neutrophils and macrophages to the site of injury. Bunn *et al.* (2004) demonstrated that a mild contusion injury (100g from a height of 13 cm) resulted in an increase in pro-inflammatory cytokines 4 days after injury and after moderate injury (200 g from a height of 13 cm) the peak in pro-inflammatory levels was at day 8. In the study by Warren *et al.* (2002), in which a freeze-injury model was used, they found an increase in TNF-α mRNA expression occurred within 5 hours following injury and peaked at 24 hours after injury, followed by a gradual decline from days 3-7 and a return to control levels by day 13. Studies investigating the effects of cytokine production after a contusion injury, such as Kruger and Smith (2012), found that the pro-inflammatory cytokines, TNF-α and IL-6, was significantly reduced in the PCO-treated group, compared to the untreated group, 3 days after injury and Myburgh *et al.* (2012) found that the anti-inflammatory cytokine, IL-10, was significantly increased in the PCO-treated group, 3 days after injury. This type of results opens up the possibility that treatment with *P. glandulosa* might also blunt the pro-inflammatory cytokine response and enhance the anti-inflammatory response, contributing to the earlier recovery of damaged muscle.

5.4.2 $ADAM_{12}$ as marker for regeneration

The level of expression of $ADAM_{12}$ in gastrocnemius muscles, at different time-points after injury, has not been measured before. Most muscle regeneration studies in which $ADAM_{12}$ was used as a marker for regeneration, were conducted on C2C12 cells, which is a mouse myoblast cell line, frequently used as a model for myogenic differentiation *in vitro* [Cao *et al.,* 2003]. By comparing our data with the data obtained from these *in vitro* studies, our results are in agreement, since our data also show that the expression level of $ADAM_{12}$ is very low in differentiated muscle fibers (Fig. 5.10) [Bornemann *et al.*, 2000; Gilpin *et al.,* 1998; Yagami-Hiromasa *et al.,* 1995]. From Figure 5.10 it is clear that the expression level of $ADAM_{12}$ was low and there was no significant difference between the uninjured and 1 hour post-injury groups, seeing that regeneration of muscle fibers has not yet commenced at that stage. According to the literature, the amount of $ADAM_{12}$ protein is dramatically increased in regenerating muscle compared to differentiated muscle [Cao *et al.,* 2003]. In the study by Cao *et al.* (2003) they found that during differentiation of C2C12 cells, $ADAM_{12}$ mRNA and protein expression were elevated in undifferentiated cells and during early stages of differentiation and both the mRNA and protein levels were decreased to low levels as the process of differentiation ensued. Similar results were obtained in our study, albeit in a different model, as we observed that the expression of $ADAM_{12}$ was significantly increased 3 hours after injury and persisted at this elevated level of expression for at least 24 hours, while expression was significantly decreased on day 7 after injury (Fig. 5.10 (A)). This data indicates that regenerative processes have already started 3 hours post-injury and persisted for at least 1 day after injury. This result coincides with research such as the one by Rantanen *et al.* (1995), a study in which they found the first signs of myogenic differentiation, as indicated by an increase in myogenin mRNA expression, occurred between 3 and 8 hours after injury. In our study, at day 7 after injury, the expression of $ADAM_{12}$ was significantly reduced compared to the 3 hour and 1 day time period, which suggests that most satellite cells have differentiated and fused to form differentiated myotubes by day 7. Our desmin data supports this interpretation (section 5.4.3). Similarly, Cao *et al.*, 2003 found that the expression of $ADAM_{12}$ was decreased in myotubes of their C2C12 cultures.

In the above-mentioned study by Cao *et al.* (2003), they reported reduced differentiation in the C2C12 cells after $ADAM_{12}$ expression was inhibited by small interfering RNA, depicted by lower expression levels of both quiescence markers (retinoblastoma-related protein p130 and cell cycle inhibitor p27) and differentiation markers (myogenin and integrin alpha7A isoform. Their results coincided with a previous report by Yagami-Hiromasa *et al.* (1995)**,** in which C2C12 clones stably transfected with an $ADAM_{12}$ antisense mRNA construct, showed decreased formation of myotubes. Yagami-Hiromasa *et al.* (1995) interpreted their findings as a direct impairment of cell-cell fusion in $ADAM_{12}$-deficient myoblasts and Cao *et al.* (2003) indicated that the decreased expression of $ADAM_{12}$ led to inhibition of an early step of differentiation that involves expression of myogenin. Conversely, it has been reported that increased expression of $ADAM_{12}$ accompanies myoblast fusion and myotube formation *in vivo* [Cao *et al.,* 2003]. However, in the same study, when $ADAM_{12}$ was over-expressed in these C2C12 cells under conditions that promoted cell cycle progression, it led to the upregulation of p130 and p27, cell cycle arrest and the downregulation of MyoD. MyoD belongs to a family of proteins known as MRFs and it is a protein with a key role in regulating muscle differentiation [Cao *et al.,* 2003]. Thus, they hypothesised that enhanced expression of $ADAM_{12}$ induces a quiescence-like phenotype and does not stimulate differentiation. In our study we found that chronic treatment with *P. glandulosa* significantly augmented the expression of $ADAM_{12}$ 1 day after injury. In essence what our results might indicate is that the increased expression of $ADAM_{12}$ observed in the chronic *P. glandulosa* treated animals resulted in the possible triggering of more satellite cells to differentiate, increasing fusion of myoblast and therefore myotube formation, which subsequently could have resulted in accelerated regeneration. However, in this study we did not investigate the activity of satellite cells and this therefore warrants further investigation.

5.4.3 Desmin as marker for regeneration

Unlike many of the other myogenic markers, which are only expressed during myogenesis, desmin (a myoblast maturation marker) is also expressed in normal adult skeletal muscle. It plays a vital role in the maintenance of the mechanical and structural integrity of the contractile apparatus, by stabilizing the sarcomeres and is responsible for transmission of muscle contractile force between separate myofibrils and the sarcolemma [Russ and Grandy, 2011]. Vaittinen *et al.* (2001) demonstrated that desmin expression increased significantly during myogenesis and that it remained at high levels in mature myofibers, as it is located near the Z-discs of sarcomeres, to keep myofibrils intact. In the context of muscle injury, myofibers are destroyed and the assumption would therefore be that the proportion of desmin-expressing myoblasts would decrease immediately after injury as a result of proteolysis of the intermediate filament network. Equally, due to the activation and maturation expected after contusion injury, the proportion of desmin-expressing myoblasts would be expected to increase as regeneration progresses. According to Przewoźniak *et al.* (2013), on day 7 post-injury myoblasts fuse to form myotubes and reconstruct damaged myofibers. At the same time the first myotubes with centrally located myonuclei are observed, which is an observation made in our study too. Numerous other studies have also reported on the significant increase in desmin-positive staining during regeneration, such as Vater *et al.* (1992) who demonstrated an intense desmin expression two days after a severe muscle injury, brought on by toxin injection. Their results validated the presence of regenerating myotubes. Additionally, Creuzet *et al.* (1998) reported that desmin expression disappeared in necrotic mouse myofibers after freeze lesions to the pectoralis major muscle, but satellite cells and newly formed myofibers in the injured area showed increased desmin-staining 2 to 4 days after injury.

In our study we did not observe the significant decrease in desmin-expressing cells immediately after injury, as the desmin-expressing cells numbers stayed constant for at least 24 hours after injury and only significantly increased 7 days after injury (Fig. 5.12). A possible explanation might be that our injury was not severe enough to cause substantial destruction of the myofibers, however when investigating the H&E stains (Figure 5.3), that explanation does not seem plausible as the damage incurred was severe enough to cause significant destruction to the ultrastructure of the muscle. This lack of desmin staining immediately after injury has been observed before, although in a different model of injury. Yu *et al.* (2002) showed, with high-resolution immunohistochemistry, that at 1 hour post-exercise, the staining of desmin did not differ from the controls. They ascribed this phenomenon to desmin remodelling taking place after exercise-induced muscle damage (eccentric exercise) in humans. They reported that desmin does not only present at the Z-discs, but single longitudinal desmin strands could be seen aligned next to the myofibers, linking several Z-discs [Yu *et al.,* 2002]. Even though eccentric exercise does not cause severe muscle damage, the results of this study could possibly explain the lack of positively desmin-stained cells present immediately after injury.

A novel finding in our study was that *P. glandulosa* treatment significantly elevated the desmin-expressing cells in the PG-CHR group, compared to the PLA group. This might indicate that a significant percentage of cells in the chronically treated *P. glandulosa* group were more mature, i.e. more myotubes had formed from the fused myoblasts, compared to PLA group, 7-days after injury. This data also coincides with our $ADAM_{12}$ data, where we found $ADAM_{12}$ expression to be significantly elevated in the chronically treated *P. glandulosa* group, 1 day after injury, compared to the untreated controls. This increased $ADAM_{12}$ expression might have resulted in the triggering of more satellite cells to differentiate, increased fusion of myoblast and therefore myotube formation, with the latter now seen as increased desmin-staining at 7 days post-injury.

5.4.4 NSAID's as positive control

As mentioned previously, we used the group treated with NSAID's as a positive control for our model. NSAID's are commonly used after sports-related injuries and they are frequently prescribed by clinicians, however in most cases for their analgesic purposes rather than for enhanced healing, since the latter presents with conflicting results (section 2.6.5.1). The most common clinical approach is to start treatment two days after injury, when swelling caused by inflammation results in pain and to continue treatment for up to 7 days. Just as previous research has shown [Mackey *et al.,* 2007; Mikkelsen *et al.,* 2009; Almekinders and Gilbert, 1986], we have also demonstrated that NSAID treatment, when treatment starts immediately after injury, results in a decreased inflammatory response. We found that the NSAID-treated group displayed a significantly lower number of neutrophils compared to the untreated group. The results obtained in this current study are similar to the results found by Bondesen *et al.* (2004), Mishra *et al.* (1995) and Vignaud *et al.* (2005). In the study by Bondesen *et al.* (2004), localized freeze injury was induced in the tibialis anterior muscles of mice chronically treated with either a COX-1- or COX-2-selective inhibitor (SC-560 and SC-236, respectively), starting before injury. Mishra *et al.* (1995) made use of a different injury model, eccentric contraction-induced muscle injury, where rabbits were treated with an oral administration of flurbiprofen, 2 times a day for 6 days. Vignaud *et al.* (2005) utilized two injury models, namely, myotoxin injection and crush-injury and in both models found that NSAID treatment (diclofenac, DFM, DMTU, DMSO, indomethacin and PDTC) resulted in a decreased accumulation of inflammatory cells in the damaged muscle. In all the above-mentioned studies the authors found that NSAID treatment resulted in the inhibition of the inflammatory phase, decreasing the capacity for regeneration and thereby delaying muscle regeneration, if NSAID's were taken prior to injury or immediately after injury.

Of greater importance is that the number of neutrophils present in the injured area of the *P. glandulosa*-treated groups did not significantly differ from the NSAID group, i.e. *P. glandulosa* treatment acted similarly to a known anti-inflammatory agent [Lapointe *et al.*, 2002; Marsolais *et al.,* 2003]. In addition, we found that NSAID treatment resulted in delayed muscle regeneration [Järvinen *et al.,* 1992; Mishra *et al.,* 1995], as depicted by the significant decrease in desmin-expressing tissue found in this group (Fig. 5.12). A novel finding in our study was that *P. glandulosa* treatment seems to have been more effective than the known NSAID, as we found no effect of NSAID on the expression of $ADAM_{12}$ 1 day after injury, when comparing it to the untreated controls (PLA). However chronic *P.*

glandulosa treatment resulted in a significant increase in $ADAM_{12}$ expression at the same time point, compared to the PLA and more importantly the expression of $ADAM_{12}$ was significantly higher in the chronically treated *P. glandulosa* group, compared to the NSAID-treated group, rendering it more effective than the known NSAID.

In summary, the main findings from this part of the study was that chronic *P. glandulosa* treatment as well as post-injury treatment led to the significant reduction in neutrophil infiltration into the injured area, therefore resulting in decreased inflammation [Tidball, 1995; 2005; Kruger, 2011]. We also found that chronic *P. glandulosa* treatment significantly increased the expression of both $ADAM_{12}$ and desmin, which were the markers used to validate muscle regeneration, at 1 day ($ADAM_{12}$) and 7 days (desmin) after injury. These results indicate that chronic *P. glandulosa* treatment may result in an accelerated regenerative process. We also confirmed the reports of previous studies, stating that NSAID treatment, administered to soon, can lead to decreased inflammatory cell infiltration into the site of injury as well as delayed regeneration. Finally, we have shown that *P. glandulosa* treatment is more effective than the known NSAID. In conclusion, our data indicates that *P. glandulosa* might be an effective pre- and post-injury treatment option; however this is but the tip of the iceberg as much more research is needed.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 CONCLUSION

Africa has a remarkable biodiversity and a rich cultural tradition of plant use for the treatment of various diseases. There are numerous advantages to using medicinal plants, such as (i) being cheaper and more easily accessible than prescription drugs, particularly for populations in developing countries, (ii) some cultural groups have a preference for using natural remedies that are in line with their indigenous knowledge and (iii) religion might have an additional impact, as the belief exists that, where an area gives rise to a particular disease, it will also provide the plants to cure it. Due to this particular belief system, the use of plant-related medicines could result in better compliance in the taking of long-term medication, which would in turn have a positive outcome on disease treatment. In view of the need for effective medication to control various disease states, the utilization of plant-based therapies is strongly advocated. Since, plant-based therapies offer potentially cost-effective management; their effects need to be scientifically validated.

The data obtained from this study is novel as there is no known literature on the effect of *P. glandulosa* on insulin resistance, force generation after muscle stimulation, fatigue tolerance or muscle recovery after injury. Given the current evidence, it seems as if *P. glandulosa*, after short-term use, might be beneficial as a dietary supplement. However, due to the large scope of this study much more in-depth research into all three bigger sub-divisions of this study needs to be conducted to determine possible side-effects after long-term use as well as the mechanism/s responsible for the observed effects.

6.2 RECOMMENDATIONS FOR FUTURE RESEARCH

6.2.1 General recommendations into future research of *P. glandulosa*

To our knowledge, no studies besides our own have been conducted on the mechanisms involved in the effects of *P. glandulosa* and thus the active compound/s thereof has yet to be identified. Due to the promising results observed in previous studies [George *et al.,* 2011; Huisamen *et al.*, 2012] and results obtained from this study, it seems feasible, as it does to all researchers in the field of medicinal plants, to identify and possibly extract the active component(s) and package it in the form of capsules instead of the raw plant material. Since *P. glandulosa* consumption elicits these multiple effects in our animal models, there may be different active components involved in the different effects observed. Therefore, isolating these active components and determining their bioavailability, can help determine whether the effects observed are as a result of one active substance or the synergistic interplay of numerous components. In addition, the activities in the rat model must be tested and verified in humans. However, this is likely to prove a time consuming, costly and difficult process.

6.2.2 Recommendations regarding insulin sensitivity

As discussed in Chapter 3, insulin resistance, as a result of hyperphagia-induced obesity, was accomplished by placing a group of rats on a diet that contained a high-sucrose content (DIO diet in Table 6.1). Even though the animals in our study were insulin resistant after this 16-week high-sugar diet, verified by the significantly increased body weight, intraperitoneal fat weight, fasting blood glucose and -insulin levels, as well as an increased HOMA-IR index of DIO (D-PLA) versus control (PLA) rats (Table 3.2), no significant differences were observed with regards to the glucose uptake by the soleus muscle of the DIO animals when compared to the control animals. From unpublished data obtained from our laboratory, it has since become apparent that the fasting glucose levels of animals on the high-sucrose diet are not as pronounced as found to be in rats on the high-fat diet. This high-fat diet (DIO + Holsum in Table 6.1), which contains all the ingredients found in the high-sucrose diet, with the addition of Holsum (trade name of cooking fat made from Malaysian Palm oil), contains significantly higher amounts of fat and cholesterol and the amount of kJ per 100g is significantly higher in this diet. These pilot studies conducted in our laboratory, have also shown that animals on the high-fat diet gain significantly more weight during a 16 week period. Therefore, it might be a viable option to place the animals on the high-fat diet, in which the insulin resistant characteristic seems to be more pronounced and see what effect *P. glandulosa* treatment has on glucose uptake in skeletal muscle as the non-significant effects observed might be a short-coming related to the animal model used rather than the treatment with *P. glandulosa*. This proposal is also underscored by the observation that the cardiomyocytes prepared from the DIO animals have been sensitized towards the action of insulin by *P. glandulosa* treatment [George *et al.,* 2011].

Table 6.1: Macronutrient composition of three different diets

	Fat (g/100g)	**Cholesterol (mg/100g)**	**Sucrose (g/100g)**	**% Protein**	**% CHO**	**kJ/100g**
Control	4.8	3	6.6	17.1	34.6	1272
DIO	4.6	10	27.7	9.4	45.8	1173
DIO + Holsum (fat)	11.5	13	24.4	8.3	42	1354

6.2.3 Recommendations regarding muscle fatigue

The muscle fatigue experiments in our study were conducted on the slow-twitch, soleus muscles of animals that received *P. glandulosa* treatment for 10 weeks. As mentioned before, the motivation for using the soleus muscles was, (1) because it is a fairly homogenous muscle type, consisting of 84% slow-twitch muscle fibers [Ariano *et al.*, 1973] and (2) because it is a fairly small muscle. It has been found that larger muscles (> 2 mm in thickness) tend to become anoxic at the core sooner than a smaller muscle, due to the larger diffusion distances [Allen *et al.,* 2008]. Since our animals had to be on a 10-week treatment program, after which they weighed about 438.00 g (± 14.97 g), we opted for a smaller muscle. For future research, the fatigue experiments can be redone, however instead of using the soleus muscle, the extensor digitorum longus (EDL) muscle can be used. The EDL muscle is also a fairly small, homogenous muscle, consisting of 98% fast-twitch fibers [Armstrong and Phelps, 1984]. By utilizing the EDL muscle, the effects of *P. glandulosa* on fatigue induction and increased force production can be tested in fast-twitch fibers.

In addition, it might also be of great value to analyze both the soleus and the EDL muscle, to determine whether *P. glandulosa* does induce the transition of fiber types, i.e. from slow- to fast-twitch fibers or *vice versa*. This can be done by Western blotting analysis, using different monoclonal antibodies representing the different myosin heavy chain isoforms [Kim *et al.,* 2013]. As mentioned in the literature review, myosin heavy chain isoforms have been considered as makers for muscle fiber types. The presence of different fiber types can also be analyzed immunohistochemically, by probing the different myosin heavy chain isoforms and viewing them histologically [Cornachione *et al.,* 2011].

6.2.4 Recommendations regarding muscle injury

The bigger scope of research is ultimately to investigate whether *P. glandulosa* can act as an anti-inflammatory agent and the mechanism of action. The first step in the quest to answer these questions was to evaluate the effect of *P. glandulosa* on the immune cells (neutrophils and macrophages) infiltrating the muscle after injury. With regards to our muscle injury experiments, we evaluated injured tissue at 4 different time-points after injury, namely, 1 hour, 3 hours, 1 day and 7 days after injury. According to the literature, macrophages usually peak around day 5 post-injury; therefore for future research one could include time points between 1 and 7 days post-injury, such as day 3 and day 5 post-injury, to give a more comprehensive picture of the effects of *P. glandulosa*. The main focus of our study was to examine the effects of *P. glandulosa* at the site of injury (muscle tissue); however a recommendation for further research would be to add the effects that *P. glandulosa* has on neutrophils and macrophages in circulation as well. This can be done by flow-cytometry, in which one can determine the number of the different immune cells present per µl blood. For future research, neutrophil migration assays can also be conducted, as we found that chronic *P. glandulosa* treatment resulted in a significant decrease in neutrophil infiltration into the injured area, by still unknown mechanisms. Neutrophil migration assays works on the basis of allowing neutrophils to migrate in the presence of chemotactic factors. In addition to understanding the effect of *P. glandulosa* seems to have on neutrophil infiltration, it might also be advisable to assess the effects it has on cytokine production, both pro- and anti-inflammatory cytokines, such as IL-10 (anti-inflammatory) and IL-6 and TNF-α (pro-inflammatory), both at tissue level and in circulation.

As a result of the positive results obtained with regards to the regenerative process, it would by worthwhile to examine the activity of satellite cells, as these cells are crucial in the regenerative process in muscle after injury. This can be done by evaluating markers of satellite cells, such as Pax-7, CD34 and CD56 [Myburgh *et al.,* 2012; Smith *et al.*, 2008], by means of immunohistochemistry. Finally, to confirm $ADAM_{12}$ and desmin as regenerative markers, one can also evaluate the presence of the embryonic or foetal myosin heavy chain isoform (MHC_f), which is expressed predominantly in developing skeletal muscles, but can also be detected in the adult muscle in regenerating fibers, where central nuclei are apparent [d'Albis *et al.,* 1988]. With all this in mind, more research is needed to gain a more comprehensive picture as well as inferring mechanism.

CHAPTER 7: REFERENCES

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