The nitric oxide donor Molsidomine shows therapeutic benefit toward muscle repair after an acute impact injury, in rats.

by

Mr Christopher Nicholas Reeves

Thesis presented in fulfilment of the requirements for the degree of

"Master of Science in Physiological Sciences" in the "Science Faculty" at

Stellenbosch University

Supervisor: **Professor Kathryn H. Myburgh**

Co-supervisor: **Professor Carine Smith**

December 2016

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Abstract

THE NITRIC OXIDE DONOR MOLSIDOMINE SHOWS THERAPEUTIC BENEFIT TOWARD MUSCLE REPAIR AFTER AN ACUTE IMPACT INJURY, IN RATS.

Background: Muscle injuries are highly prevalent and arise from a multitude of situations. Trauma to the soft tissue is painful and debilitating and it requires extensive healing that often involves the formation of a fibrotic scar. Current treatments are merely management strategies, such as the RICE principle. Nitric oxide (NO) knock-out models show reduced skeletal muscle regeneration and excessive fibrosis (Filippin *et al.*, 2011 a & b), suggesting therapeutic promise for NO. NO-donation has shown therapeutic promise in mouse models of muscular dystrophy, and therefore, may be beneficial for the treatment of acute muscle injuries.

Objective: To clarify the role of treatment-derived NO on muscle trauma, using the NO-donating drug: Molsidomine (MOLS), which has been approved for use in humans.

Methods: Using a crush injury model in rats, placebo (PLA) or MOLS treatments were administered immediately and one day after the injury. MPO, MyoD, myogenin, fibronectin and TGF-β1 protein content in the injured tissue homogenates was assessed with Western blots. Collagen deposition at 21 days after injury was assessed using a Masson's trichrome stain.

Results: With MOLS, there was significantly less collagen deposition ($p < 0.05$) 21 days after injury, which was supported by less TGF-β1 protein ($p = 0.01$) and less fibronectin protein ($p < 0.005$) compared to the PLA group at this time point. Additionally, MOLS tended to modulate the amount of MPO and, therefore, the inflammatory response by 33% 5 days after injury.

Conclusion: MOLS treatment improves, and potentially hastens, tissue repair after an acute impact injury through the reduction of excessive fibrosis, as well as through enhanced clearance of inflammatory radicals from injured muscle.

Uittreksel

DIE STIKSTOFOKSIEDSKENKER MOLSIDOMIEN TOON TERAPEUTIESE VOORDELE VIR SPIERHERSTEL NA 'N AKUTE IMPAKBESERING IN ROTTE

Agtergrond: Spierbeserings is baie algemeen en ontstaan as gevolg van verskeie oorsake. Trauma aan sagteweefsel is pynlik en benadeel funksie, en dit benodig omvattende genesing wat soms met die vorming van fibrotiese letsels gepaard gaan. Tans word behandeling gerig op beheerstrategieë deur gebruik te maak van die "RICE" beginsel. Stikstofoksied (NO) geen-uitklopmodelle toon aan dat daar 'n verlaging in skeletspierregenerasie is en oormatige fibrose ontstaan (Filippin *et al.*, 2011 a & b), wat daarop dui dat daar 'n terapeutiese voordeel vir NO kan wees. In spierdistrofie muismodelle hou NO-skenking terapeutiese voordele in en kan dus potensieel ook voordelig wees in die behandeling van akute spierbeserings.

Doelwit: Om duidelikheid te kry oor die rol van behandelings-afgeleide NO in spiertrauma deur van die NO-skenkingsmiddel, Molsidomien (MOLS), wat goedgekeur is vir menslike inname, gebruik te maak.

Metode: Deur van 'n vergruisingsbesering-rotmodel gebruik te maak, is 'n plasebo (PLA) of MOLS behandeling direk, asook na een dag na besering, toegepas. MPO, MyoD, miogenien, fibronektien en TGF-β1 proteïeninhoud in 'n homogene oplossing van die beskadigde weefsel is deur Westerse blattering ondersoek. Kollageen neersetting teen 21 dae na besering is deur middel van Masson se trichroomkleuring ondersoek.

Resultate: Behandeling met MOLS het betekenisvol minder kollageen neersetting tot gevolg gehad ($p < 0.05$) teen 21 dae na besering, watverder bevestig is deur minder TGF-β1 proteïen ($p = 0.01$) en fibronektien proteïen ($p < 0.005$) vergeleke met die PLA groep by dieselfde tydpunt. Boonop het MOLS ook geneig om die hoeveelheid MPO – en dus die inflammatoriese respons - met 33% verminder, teen vyf dae na besering.

Gevolgtrekking: MOLS behandeling verbeter, en versnel moontlik, weefselherstel na 'n akute impakbesering deur oormatige fibrose te verminder, en ook deur middel van verbeterde verwydering van inflammatoriese radikale uit die beseerde spierweefsel.

Acknowledgements

Firstly, I would like to thank my wonderful girlfriend Rebecca Miller, for supporting me throughout my post-graduate journey. Getting thrown into a two-year ocean of uncertainty and problem solving was, at times, rather overwhelming; however, Rebecca, you were truly my lifeline. You kept me afloat until I eventually washed up onto the sunny beach. Thank you.

I would also like to thank my supervisors, Prof. Kathy Myburgh and Prof. Carine Smith. Kathy's style of supervision allows one to grow into a well-rounded human being. Where many students are given a padded jumping castle for the ride, as one of her students, one must navigate through muddy trails and work out for themselves which way is up, and this has made me into the man I am today. Carine (one of Kathy's previous students) compliments Kathy's style very well, and I am truly grateful for her guidance in my times of need. She has a wonderful ability to nudge one in the right direction, without giving one the answers, but allowing them to figure it out for themselves. Thank you both.

Next, big thanks, to Ashwin Isaacs, as he is the glue that holds the Department of Physiological Sciences together. Without him, I would never have begun my journey as a Physiologist, and for that, I am truly grateful. He also taught me most of what I know about Histology. I am also incredibly grateful to Dr Peter Durcan, another figure who guides without providing the answers. Peter is a laboratory magician, and he taught me the necessary skills required to complete this MSc. Thank you both.

For teaching me all of my animal handling skills, and all of the surgical techniques required for my animal work, I thank Mr Noël Markgraaf and Ms Judith Farao. These two individuals are truly talented in what they do and our department is lucky to have them.

To the National Research Foundation, thank you kindly for the financial support.

Finally, I thank my parents for all of the obvious biological reasons, as well as for shaping me into the man I am and for getting me started with my tertiary education. To you two and my sister, thank you all for being the most wonderful family. To my uncle Keith, I am tremendously grateful for your financial help throughout my studies, none of this would have been possible without you.

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List of Abbreviations

ANOVA	Analysis of variance
CD56/68/163	Cluster of differentiation 56/63/163
cGMP	Cyclic guanosine monophosphate
COX1/2	Cyclooxygenase 1/2
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic Acid
e/i/nNOS	Endothelial/inducible/neuronal nitric oxide synthase
ECM	Extracellular matrix
FAP	Fibro adipogenic progenitors
FIFA	Fédération Internationale de Football Association
GDP	Gross domestic product
GI	Gastrointestinal
GTE	Green tea extract
H_2O	Water
HClO	Hypochlorous Acid
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
JAK-STAT	Janus kinase – Signal Transducer and Activator of Transcription
LDL	Low-density lipoprotein

LLLT	Low-level laser therapy
L-NAME	Nitro-L-arginine methyl ester
MCP-1	Monocyte chemoattractant protein 1
mi-R27b	microRNA 27b
MMP	Matrix metalloproteinase
MPCs	Myogenic precursor cells
MPO	Myeloperoxidase
MRFs	Myogenic regulatory factors
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation factory
NADPH	Nicotinamide adenine dinucleotide phosphate
NCAM	Neural cell adhesion marker
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
$OONO^-$	Peroxynitrite
PARS	Poly (ADP ribose) synthetase
Pax7	Paired box 7
Pparγ1	Proliferator-activated receptors gamma
PVC	Polyvinyl chloride
RICE	Rest ice compression elevation
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIN-1	3-morpholinosydnonimine
SNAC	S-nitroso-N-acetylcysteine
TBS-T	Tris-buffered saline in Tween® 20
TGF	Tissue growth factor
TNF	Tumor necrosis factor
TxA2	Thromboxane A2
US	United States
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Units of measurement

%	Percentage
°C	Degrees Celcius
µM	Micromolar
µmol	Micromole
µg	Microgram
µL	Microlitre
µm	Micrometre
cm	Centimetre
hrs	Hours
L	Litre
Min	Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
nM	Nanomolar
nm	Nanometre
rpm	Rotations per minute
sec	Second(s)
V	Volts

Chapter 1: Introduction

A contusion injury is described as a blunt force trauma to an area of soft tissue, resulting in devastation of the musculature, which often manifests as a debilitating and painful injury that requires a lengthy healing period (Alessandrino & Balconi, 2013). These injuries may arise from a multitude of situations including most sporting activities, mishaps in the workplace, motor vehicle-related accidents and in war or civil unrest, to name a few. The duration of the healing period depends on the severity of the injury, and this is where therapeutic efforts have been directed, as the concept of speeding up recovery is attractive in many respects. Furthermore, optimal healing of the damaged muscle through regeneration and return to normal muscle architecture with minimal scar tissue formation, or fibrosis, is important.

Much work has gone into devising therapies that target traumatic muscle injuries, however, it seems the only agreed upon strategy is to apply the RICE principle – rest, ice, compression, elevation - immediately after the injury (Järvinen *et al.*, 2013). Non-steroidal anti-inflammatory drugs (NSAIDs) are possibly the most widely prescribed and utilised treatment for injuries, especially among injured athletes (Tscholl *et al.*, 2008; Warden, 2009), however, it has been suggested that they be taken at the lowest dose and for the shortest period possible (Fine, 2013). This is because NSAID use has been associated with impaired tendon healing (Dimmen *et al.*, 2009), an elevated risk of re-injury after healing (Warden *et al.*, 2006) and even reduced satellite cell activity (Mackey *et al.*, 2007), whilst NSAID use in the chronic setting increases the probability of gastrointestinal bleeding (Wallace, 1994), potentially causing unnecessary mortality through infection. Thus, extreme modulation or inhibition of the normal inflammatory response to damage at any time may not be the answer.

Although not many, some treatment strategies have been aimed at the fibrotic response to injury. Muscle heals, in part, by forming a connective tissue scar (Järvinen *et al.*, 2005) which manifests as a solid structure that may interfere with the muscle's functional recovery (Gharaibeh *et al.*, 2012), and may also be associated with recurrent muscle tears upon return to activity (Kujala *et al.*, 1997). Some antifibrotic therapies such as decorin (Foster *et al.*, 2003; Fukushima *et al.*, 2006) or γ-interferon (Foster *et al.*, 2003) have shown promise, but none has a proven evidence-base for clinical use as of yet.

It is quite possible that a dynamic therapeutic approach is required for the treatment of muscle injuries, as injury manifestation itself is a multi-faceted process, combining the skeletal muscle,

inflammatory and circulatory systems. Nitric oxide (NO) is a multi-functional molecule, known for its potent vasodilatory properties, which are exploited and used to treat many cardiovascular conditions, such as angina (Messin *et al.*, 2006). NO-based therapies slow down disease progression in mouse models of Duchenne Muscular Dystrophy (Wehling *et al.*, 2001; Zordan *et al.*, 2013; Cordani *et al.*, 2014), affecting multiple mediators in this complex disease and ultimately improving the skeletal muscle phenotype. Inhibiting NO-signalling appears to impair muscle growth and function (de Palma *et al.*, 2014). Additionally, in the context of experimental muscle damage, NO-signalling inhibition has a negative impact on the proper healing of damaged tissue, shifting the regenerative curve away from optimal repair and towards fibrosis (Filippin *et al.*, 2011 a & b; Darmani *et al.*, 2004 a & b). Therefore, NO's effects on skeletal muscle have been tested in knock-out models as well as in disease models (Muscular Dystrophy), however, the concept of using exogenous NO as a treatment for acute muscle injury is one that has not been covered in the literature.

From this evidence, it is necessary to test the potential effects, if any, of an NO-based therapy on the regeneration of acutely injured skeletal muscle. Thus, we made use of the NO-donating anti-anginal drug Molsidomine, in a rodent model of skeletal muscle crush injury and evaluated whether exogenous NO had any effect on muscle repair. Preparation for the undertaking of such a study was sufficient, in that Molsidomine is approved for use in humans, and the crush injury model has been standardised and repeated by numerous students in our laboratory (Myburgh *et al.*, 2012).

Before the presentation of the research undertaken, a brief background will be provided on the relevant physiological processes (Chapter 2), followed by a more in-depth literature review and theoretical framework of the specific topic at hand, NO and skeletal muscle regeneration (Chapter 3).

Chapter 2: Background

2.1 Normal muscle physiology

There are approximately 640 skeletal muscles in humans, accounting for a sizable proportion of total body mass; just over 30% for most women, and around 38% for men (Scharner & Zammit, 2011; Relaix & Zammit, 2012). Skeletal muscles are made up of bundles of long myofibers, which are packed with specialised contractile elements called myofibrils; and these contain the sarcomeres that are able to generate force through a contraction (see Figure 1.1). Myofibers are formed by the fusion of many myoblasts during embryonic and foetal development, and every myofiber is controlled by hundreds of myonuclei, giving them a characteristic multinucleated appearance. These myonuclei are supplied by the resident muscle stem cells – or satellite cells - during periods of growth, extensive hypertrophy or repair, giving skeletal muscle it's robust and unique regenerative capacity (Relaix & Zammit, 2012).

Skeletal muscle allows for the execution of daily tasks, such as eating, whilst additionally, and importantly, regulating involuntary actions such as posture, balance, breathing and maintaining an optimal body temperature. More complex activities, like playing ice hockey, increase the likelihood of damage induced by adverse contraction mechanics or external collision. Muscle also functions as protection for bones and organs, which also leaves it vulnerable to external impacts or injuries. This concept is the principle focus of this review, as well as of the study reported in this thesis.

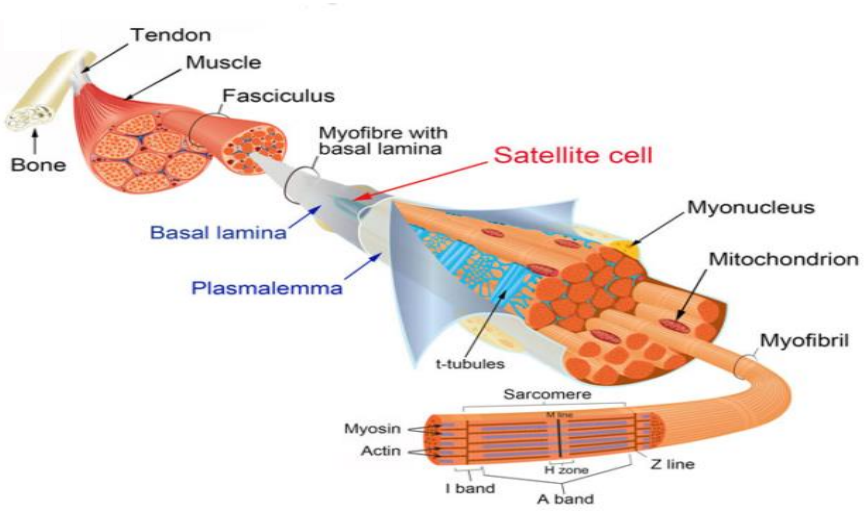

Figure 2.1 Normal skeletal muscle structure. This figure illustrates skeletal muscle structure, from an entirely functional muscle to the proteins within the myofibrils that make up the contractile apparatus. Key structures include the basal lamina, under which lie the resident muscle stem cells, or, satellite cells. The many nuclei and rich mitochondrial presence are also notable features. Figure taken from Relaix & Zammit, 2012.

2.2 Muscle injury

Any muscle of the human musculature may be injured or damaged. Broadly speaking, muscle injuries can be classified as contusions, strains, avulsions, exercise-induced injuries or muscle disease-related damage. Injuries have a high rate of occurrence, however, treatment strategies remain relatively primitive, especially in terms of speeding up recovery, let alone affecting the quality of recovered muscle. Research into muscle injuries dates back to times of major war, where projectile injuries (Hopkinson, 1963) and other conflict-related injuries were abundant (Saunders & Sissons, 1953; Sissons & Hadfield, 1953; Scully & Hughes, 1955). In the following sections, an overview of muscle injuries is presented.

2.2.1 How does injury occur?

Every day, injuries occur during the normal routine; whether it is a bump on a piece of furniture or a fall on the playground. More serious impact injuries occur with moving vehicle collisions or even an injury sustained during an earthquake or similar disaster. Thus, injuries may arise from a multitude of situations, and with varying degrees of severity, but for the most part, they require immediate attention.

The sporting field is a well-known platform for the occurrence of muscle injuries, and this is not limited to contact sports but includes most athletic events and individual sports, such as the hurdles or tennis respectively. Moreover, with moderate to severe the injury, one can expect considerable time away from activity or competition. In the professional sporting world, this is highly undesirable

for both the player and the coach or manager. Athletes are paid to play or compete, thus, when injured, they are unable to do what they are paid to do. For example, an elite football team of 25 players can expect 15 muscle injuries per season, which equates to an average of 223 days of absence, including 148 practises and 37 matches missed (Ekstrand *et al.*, 2011). These stats give a rather clear indication of the impact that sports injuries have on not only the athletes but also on all stakeholders involved.

A notable example of injury that is not often considered is an injury that occurs in the workplace or during work. Whilst this has an obvious effect on the individual's health, work-related injuries have an effect extending beyond this narrow concern, in terms of medical and legal expenses, as well as expenses from retraining, loss of productivity and an effect on the morale of fellow employees. A report from the Australian Bureau of Statistics (2011) stated that in the 2005-06 business year, there was an average injury rate of 64 per 1000 people employed, with muscular injuries, including crush injuries and strains or sprains, accounting for over one-third of these. It was estimated that this equated to 5.9% of Australia's GDP being spent on work-related injuries; a figure of 57.5 billion dollars.

More examples of injuries include casualties of disasters - both natural and unnatural, casualties of wars or civil unrest, trauma resulting with high-velocity projectiles, venom or toxin-associated damage, and also injury from immobilisation for extended periods, such as in comatose patients after head trauma and drug/alcohol overdose.

2.2.2 Models of muscle injury

Since injury can arise from so many different situations, all with varying degrees of severity, the study of muscle injury under standardised conditions is a complex undertaking. Furthermore, it is not considered ethical to inflict a direct, or traumatic muscle injury to human subjects; for example, by experimental contusion injury. Therefore, in our laboratory, models of indirect damage are utilised for our human studies, and are known as exercise-induced damage models. These include a downhill running protocol (van de Vyver *et al.*, 2015), where subjects run on a treadmill that is set on a steep decline, forcing eccentric contractions of the leg muscles for an extended period of time. The other model is a plyometric jumping protocol (Macaluso *et al.*, 2012), where subjects complete a set number of maximal vertical jumps. While both models elicit significant damage and perceived pain in the subjects, the data is relatively incomparable to traumatic impact injuries.

In order to conduct good research into direct injury, one has the option of using animal models. While these are also heavily regulated by Ethics Committees, they allow for the standardisation of various injury models and provide valuable data when employed correctly. Larger animals like sheep and rabbits are used, however, rodents – usually rats or mice - are easier to breed, handle and house. Furthermore, skeletal muscle proteins and morphology of mammals are highly conserved, hence, animal models are appropriate for research. One must keep in mind, however, that these are sentient animals, and that research must be conducted with utmost respect and within the stipulated guidelines.

James G. Tidball and colleagues have conducted much research into muscle damage and the ensuing inflammatory response in mice in the context of myodystrophy. Their laboratory often utilises a model of hind-limb unloading and reloading, where the hind-limb of a rodent is suspended in a specialised apparatus for a period of time, after which the limb is released from the apparatus allowing for normal ambulation for another period of time (Tidball *et al.*, 1998; Tidball, 2008; Tidball, 2014). This method allows for the examination of muscle under relatively low-grade contraction-induced injury, thus providing a strong base or starting point for studies of similar focus.

Exercise-induced damage, or contraction-induced damage, has more typically been mimicked in rats, using stretch-shortening cycle protocols (Baker *et al.*, 2006), eccentric contraction protocols (Sakurai *et al.*, 2013) or treadmill running protocols (de Palma *et al.*, 2014). The latter model is also valuable for use as a training model (Balon *et al.*, 1997) or for functional testing (Buono *et al.*, 2012; Sciorati *et al.*, 2011). Controlled muscle strains are similar to exercise-induced injury, as they are usually encountered during exercise activities or on the sports field. This model is usually applied by overloading the tibialis anterior muscle of the lower limb of anaesthetized animals (Sakurai *et al.*, 2005; Ramos *et al.*, 2012; de Paiva Carvalho *et al.*, 2013).

Another model that may be considered is ischemia-reperfusion injury, a model most utilised in cardiovascular research, which is applied by using a tourniquet (often a rubber band) on a target muscle group for an extended period of time, and then removing it, allowing reperfusion of the area (Lepore *et al.*, 1999; Liu *et al.*, 1998). This model has also been combined in order to compare the injury in both cardiac and skeletal muscle tissue (Thiemermann & Bowes, 1997). Overall, however, with all of the above-mentioned models considered, the most appropriate model for examining the effects of direct trauma appears to be a crush injury model, using the drop mass technique; which will be discussed in the following section.

2.2.2.1 Specific focus on impact injury

Mechanical injury results from an impact or a crushing force to the soft tissue and is sometimes called a contusion injury, due to the characteristic bruising of the damaged area. Contusions, or bruises, account for over 90% of all reported sports-related injuries (Järvinen *et al.*, 2007; Ekstrand *et al.*, 2011; Mueller-Wohlfahrt *et al.*, 2013) and tend to plague athletes throughout their entire career. The drop mass model, which makes use of a fixed mass free-falling from a fixed height, has been utilised in previous research on contusion injuries (Sun *et al.*, 2010; Filippin *et al.*, 2011 a & b; Myburgh *et al.*, 2012). Another variation is a controlled crush applied to the musculature with forceps (Darmani *et al.*, 2004 a & b); this method is less repeatable, as different researchers may apply different amounts of pressure to the forceps.

Impact injuries do not involve a break in the skin. The mechanical trauma of such an injury causes vessel and capillary rupture, infiltrative bleeding, oedema and inflammation, which can all vary according to the severity of the injury. If the impact is large enough, there is the possibility of severe hematoma formation, which is defined as a mass of blood (usually clotted) that forms in the tissue due to ruptured blood vessels; this is especially possible when the large, vulnerable muscle groups such as the quadriceps are involved (Ekstrand *et al.*, 2011; Mueller-Wohlfahrt *et al.*, 2013). There is also the chance of severely painful compartment syndrome, which occurs when fascial planes limit volume expansion, common in the muscles of the lower leg or forearm (Smith *et al.*, 2008). Symptoms of contusion injuries, therefore, depend on the internal changes in the skeletal muscle microenvironment, but in general, they are characterised by pain in both active and passive motion, palpatory pain, visible hematoma, or a combination of these (Järvinen *et al.*, 2007; Smith *et al.*, 2008; Ekstrand *et al.*, 2011; Mueller-Wohlfahrt *et al.*, 2013).

2.2.3 The phases of injury

After a muscle injury, the muscle tissue undergoes degeneration and inflammation, after which regeneration and remodelling occur in an overlapping manner. These events follow a fairly consistent phase pattern, which is collectively agreed upon throughout the literature; first is the destruction phase, followed by the repair phase and concluding with the remodelling phase. Interactions between skeletal muscle and the immune system play a significant role in the course of these phases, as will become apparent in the following sections.

2.2.3.1 Destruction phase

This first phase is characterised by the rupture and necrosis of the myofibers, hematoma formation between the ruptured myofibers and vast inflammatory cell infiltration; all of which depend on the severity of the injury. The injured tissue usually experiences massive vascular damage, depending on its degree of vascularization at the time of injury (Sprague & Khalil, 2009; Pugazhenthi *et al.*, 2008). Thus, not only muscle fibers but also endothelial cells and vascular smooth muscle cells at the injury site are damaged. This, combined with the activation of granulocytes and monocytes, results in the release of an array of cytokines (initially pro-inflammatory and then anti-inflammatory) and the initiation of an inflammatory response (Appleton & Lange, 1994). Cytokines are not the only active molecules released at the site of injury. Nitric oxide (NO) also seems to play a role in this acute inflammatory response, as the process is significantly reduced when NO production is inhibited by L-NAME treatment (Filippin *et al.*, 2011 a & b). The role of NO is a particular focus of this thesis and will be discussed in more detail in Chapter 3.

Neutrophils are the first responding immune cells (granulocytes) infiltrating the injury site during this initial phase. Upon injury or infection, chemotactic factors, such as fibrin/collagen or soluble factors such as cytokines, are released and these attract circulating neutrophils. The neutrophils rapidly adhere to the endothelial lining of the injured site through a process called margination, and this is regulated by L/P/E-selectins and adhesion molecules, such as VCAM-1 or ICAM-1 (Appleton & Lange, 1994). Neutrophils then migrate through the vessel walls (diapedesis) and infiltrate the injured tissue, continuing to accumulate until chemotactic signals stop (for example when the cytokine environment changes from a pro- to anti-inflammatory signal). Whilst at the injured site, they engulf and destroy any foreign matter or cell debris through non-specific pino-/phago-/endocytosis. The actions of neutrophils prepare injured muscle tissue for regeneration by removing damaged tissue and preparing the extracellular matrix (ECM) for remodelling. The first wave of neutrophils usually arrives within 30 minutes of any acute, traumatic injury, with peak neutrophil infiltration usually evident at 24 hours post-injury (Smith *et al.*, 2008). After this peak, the numbers steadily decline, which is concurrent with an increase in the number of monocytic cells. Whilst the neutrophil count does decrease, their numbers remain above baseline levels at the injury site for about 5 days, and they remain functionally active during this time (Tidball, 2008).

After, but overlapping with, neutrophil infiltration we see an increase in macrophage numbers, expressing CD68. CD68 is a glycoprotein expressed on the surface of monocytes/macrophages, which binds to low density lipoprotein. Along with these macrophages, there is also an elevation of

reactive oxygen and nitrogen species (ROS and RNS) (Filippin *et al.*, 2009). Activated macrophages are able to secrete multiple products and co-ordinate a large portion of the immune response. They engulf foreign particles, which are often larger than the targets of neutrophils (Appleton & Lange, 1994), and in the case of tissue injury, debris may be of various sizes. Many pro-inflammatory cytokines are generated by activated macrophages in the early phase (around 48 hours), namely IL-2, IL-6 and IL-12, IFN-γ and TNF-α. Upon release, cytokines bind to specific receptors and activate signalling pathways giving rise to the expression of proteins playing a role in, for example, adhesion, permeability changes and apoptosis. Cytokines may also cause ROS production through mitochondrial interactions and may also activate matrix metalloproteinases (MMPs) and integrins, which consequently degrade the composition of the ECM (reviewed by Sprague & Khalil, 2009). The ECM is made up of a number of structural proteins, such as collagen, and is synthesised by fibroblasts, present in the connective tissue. Fibroblasts become activated upon injury and, through the signalling from cytokines (for example, TGF-β) synthesise ECM components that are necessary for wound healing (Gillies & Lieber, 2012; Lieber & Ward, 2013).

Finally, an important local cell, the resident muscle stem cells, which are more commonly known as satellite cells, are activated by mechanical shear forces from an injury and are influenced by many of the other changes in their niche that have been described above. The finer details of satellite cell activation are still coming to light, however, the growth factor HGF seems to be one of the key role players (Anderson, 2000). Regardless, we do know that once they are activated they begin to proliferate rapidly, forming the muscle precursor cells that will eventually repair damaged fibers or form completely new fibers, and ultimately restore the damaged area to its former architecture.

A review by Smith *et al.*, (2008) stated that limiting the inflammatory response in the destruction phase may be clinically beneficial in terms of reducing pain and swelling, but that the depletion of macrophages may be detrimental to the healing process as it results in reduced satellite cell differentiation as well as diminished regeneration and growth of fibers. For this reason, excessive use of interventions such as non-steroidal anti-inflammatory drugs (NSAIDs; which inhibit the inflammatory response) may promote fibrosis, may delay both early and late phases of regeneration, and may ultimately leave the injured area prone to recurrent injuries. A recurring injury to the same area can be considered clinically worse than the initial injury (Järvinen *et al.*, 2007).

2.2.3.2 Repair phase

The repair phase overlaps with the latter part of the destruction phase, where any remaining necrotic tissue is removed by phagocytosis. This phase is characterised by the emergence of regenerating myofibers as well as the production of a connective tissue scar in the damaged area.

The pro-inflammatory macrophages undergo phenotypic changes that include upregulated expression of the CD163 protein. These CD163-positive macrophages are not phagocytic and persist within the injured area throughout the repair phase. This response coincides with elevated levels of IL-4, IL-10 and IL-13 which are all anti-inflammatory cytokines. The classically activated, CD68-positive macrophages, have undergone a phenotypical shift that is known as 'alternative activation'. Pro-inflammatory cytokines are reduced and IL-10 seems to be a dominant deactivating cytokine in this process as it appears to be intricately involved with the classic to alternate phenotype shift (Villalta *et al.*, 2011). The non-phagocytic CD163-positive macrophages are seen to accumulate in muscle during regeneration, and it seems that they play an active role in tissue repair; as *in vitro* coculture with rat myoblasts increased proliferation of the myoblasts (Villalta *et al.*, 2011). Alternative activation of macrophages also promotes an increased expression of genes involved in connective tissue remodelling and adequate fibrosis, essential as the next steps in the recovery of the damaged site (Tidball, 2008). Therefore, macrophage phenotypical shift is incredibly important in the response to tissue injury as it moves the process forward from the initial destructive phase, which would be detrimental if maintained over a longer period.

By this time in and around the injury site, a large number of satellite cells, positive for the paired box transcription factor (Pax7), have been expanding the satellite cell pool. Some of these cells expressing the myogenic differentiation factor (MyoD) commit to differentiation into myoblasts. Down-regulation of Pax7, maintenance of MyoD expression and the subsequent activation of myogenin indicate terminal differentiation (Relaix & Zammit, 2012). After this, they fuse with other myoblasts thus replacing the destroyed muscle fibers. Not all of the damaged fibers have necessarily been destroyed and the fusion of newly formed myoblasts with these fibers aids in their repair. This process is closely associated with the increased synthesis of surrounding ECM and ultimately restores the injured area to its previous structure, as effectively as possible.

2.2.3.3 Remodelling phase

The final phase also overlaps with its preceding phase and involves maturation of new and regenerated fibers, reorganisation of these fibers and finally revascularisation and formation of a

functionally repaired whole muscle (Järvinen *et al.*, 2007; Filippin *et al.*, 2011 a & b). Unfortunately, the regeneration phase also results in the formation of scar tissue, which is non-contractile tissue and must, therefore, be degraded during the remodelling phase.

As mentioned in the previous section, CD163+ macrophages persist in the tissue and assist with the remodelling phase. Transforming growth factor (TGF-β) is a cytokine with a large role in fibrosis and scar formation. TGF-β stimulates collagen synthesis and fibroblast proliferation whilst also inhibiting differentiation and fusion of myoblasts, all of which promote fibrotic scar formation (Leask & Abraham, 2004). This cytokine can stimulate the production of other smaller ECM proteins such as fibronectin, and also block their degradation (Brandan & Gutierrez, 2013). Scar tissue is not functional skeletal muscle, in that it is unable to contract and it is essentially a way of 'filling in the gaps' after the destruction caused by the injury. Therefore, the balance between scar formation and proper functional regeneration is important in terms of therapies and obtaining optimal recovery of the muscle. However, there is an insufficient amount of research on therapies that target fibrosis or the remodelling phase specifically, and this will be discussed in more detail in Chapter 2.

From the above, three main topics can be identified, namely inflammation, muscle regeneration and scar formation (fibrosis). Both inflammation and muscle regeneration have been studied extensively. Therefore, fibrosis was chosen as the main topic for this thesis. In the next chapter, relevant literature in the context of muscle injury and recovery will be discussed. Information on the inflammatory immune system is provided in brief for completeness. Satellite cell involvement in muscle recovery will be discussed in more detail, as relevant to the thesis topic, while fibrosis and potential intervention strategies will be discussed comprehensively.

2.2.4 Chemical messengers relevant to fibrosis

2.2.4.1 Cytokines

Cytokines are small proteins released by many cell types. They may autoregulate and carry signals locally between cells, but may also travel in the circulation and exert effects on distant cells. In the context of tissue damage, cytokines are mainly produced by immune cells, with the majority belonging to the interleukin (IL), interferon (IFN) and tumour necrosis factor (TNF) families. Cytokines are key players in the interaction between the immune system and muscle cells throughout the repair process (Sprague & Khalil, 2009).

IL-6 is a pro-inflammatory cytokine released from granulocytes, mainly neutrophils, and from monocytes, mainly mature macrophages. IL-6 is also called a myokine, due to the fact that it is

released from skeletal muscle in response to muscle contraction during exercise, for example (Pedersen & Febbraio, 2008), but also by the injured muscle itself very soon after injury. One of the many functions of IL-6 is to signal and regulate the degradation of necrotic tissue after muscle injury and enhance lymphocyte proliferation (Smith *et al.*, 2008). IL-10, on the other hand, is an anti-inflammatory cytokine released primarily from monocytes and has the ability to directly down-regulate the production of pro-inflammatory cytokines. As mentioned earlier, it plays a major role in the macrophage phenotype shift, but it also appears to attract the later invading, alternatively activated M2 type macrophages to the injured site, thus promoting an environment of repair and regeneration (Villalta *et al.*, 2011). At least one, but probably a combination, of the anti-inflammatory cytokines, namely IL-4, IL-10 and IL-13, is responsible for the shift in activation state of macrophages from classical (M1) to alternative (M2).

In addition, expression of IL-4 in muscle is elevated during regeneration, with the myotubes themselves acting as the predominant source of the cytokine (Tidball, 2008). IL-4 has a pro-migratory function, where emerging myotubes recruit myoblast fusion through the secretion of IL-4 (Horsley *et al.*, 2003). IL-4 seems to be required for proper fusion between myoblasts and new myotubes, as IL-4 inhibition diminishes migration, which may retard proper muscle regeneration and growth (Lafreniere *et al.*, 2006).

In summary, it is most likely that not one, but all of the aforementioned cytokines are capable of inducing alternative activation of macrophages, with the additional possibility of a combined expression and signalling effort.

2.2.4.2 Fibronectin

Fibronectin is a glycoprotein, secreted predominantly by fibroblasts and chondrocytes, and is a major component of the ECM. It functions to enhance the attachment of cells to the ECM through the formation of fibronectin-collagen complexes, for example, thus acting as a bridge between the cell and its surrounds (Ruoslahti, 1981). Other compounds that fibronectin has an affinity for include fibrin, heparin, DNA and actin, and through these interactions, the protein plays a major role in wound healing and growth (Carsons, 1989). Fibronectin exists as a high molecular weight (~220kDa) protein, however, its integrity may become compromised through alternative splicing or protease degradation, where it breaks into multiple fibronectin fragments with varying molecular weights from 10 – 200kDa (White & Muro, 2011). MMP-13 and -14 could be responsible for the enzymatic formation of fibronectin fragments (Zhang *et al.*, 2012). Fragments seem to retain some functional

similarities to the intact protein, for example, in an inflammatory synovial fluid, the fragments in the 50 to 100kDa range were able to mediate synoviocyte chemotaxis, and thus may be important in establishing chemotactic gradients at inflammatory sites (Carsons, 1989).

2.2.4.3 Growth factors

Growth factors are proteins that bind to receptors on the cell surface, resulting in the activation of cellular proliferation and/or differentiation and/or growth. Following muscle injury, growth factors are important in maintaining the balance between proliferation and differentiation of satellite cells as well as the remodelling of newly formed tissue. Below is a brief overview of two growth factors that are most relevant to the regulation and repair of muscle injury.

Hepatocyte growth factor (HGF) has been elucidated as an important and essential factor, especially in the acute phase of injury where it is implicated in satellite cell activation (Anderson, 2000; Filippin *et al.*, 2009). It is thought that mechanical shear forces mobilise HGF from the ECM. Matrix metalloproteinases (MMPs) activate HGF by cleavage. It binds to the c-Met receptor and activates the satellite cells, which themselves produce more HGF in an autocrine manner (Anderson, 2000; Filippin *et al.*, 2009). To date, HGF is the only known growth factor with the ability to stimulate and activate quiescent satellite cells *in vivo* (Sakuma & Yamaguchi, 2012).

Transforming growth factor beta (TGF-β) is also released by injured muscle, as well as most inflammatory cells, and plays a prevailing role in connective tissue synthesis or scar formation, through the synthesis of collagen, fibronectin and proteoglycans (Border & Noble, 1994). It is proposed that excessive TGF-β expression leads to fibrosis through fibroblast activation and proliferation, which results in sustained collagen and matrix synthesis (Filippin *et al.*, 2009; Rovere-Querini *et al.*, 2014). TGF-β expression is influenced by many factors in the injured niche. It has been shown that inhibiting NO production results in elevated TGF-β levels early on following crush injury (Filippin *et al.*, 2011 a & b) whilst additionally causing a chronic overexpression of this growth factor (Darmani *et al.*, 2004 a & b), thus promoting fibrosis instead of complete muscle regeneration. Fibrosis is of particular interest for this thesis, particularly therapies that target the condition in skeletal muscle, as literature on this subject is more limited than that which covers myogenesis. Therefore, fibrosis will be discussed in more detail in the following chapter along with the growth factors mentioned above.

Chapter 3: Literature review

3.1 Inflammation in response to muscle injury

Upon trauma, damaged muscle cells initiate the inflammatory response through the release of pro-inflammatory cytokines. Shortly after this, inflammatory cells populate the damaged area and propagate the rest of the response. The interaction between the immune system and skeletal muscle is a critical regulatory process, controlling many key events over many days during repair and remodelling. Circulating platelets may adhere to the damaged vasculature, becoming activated as a result and releasing more pro-inflammatory mediators such as 5-hydroxytryptamine (serotonin), histamine and thromboxane A2 (TxA2). Mast cells in the injured tissue may also release histamine, which increases the blood flow to the injury site, allowing blood-borne inflammatory cells to gain direct access to the injured area (Jancar & Sánchez Crespo, 2005; Sprague & Khalil, 2009).

The actions of neutrophils – which were discussed in the previous chapter - are a necessary and normal response, however, it is a rather 'messy' and nonspecific process, causing early, rapid removal of necrotic muscle fibers and fiber fragments. Neutrophil invasion is associated with the release of high concentrations of free radicals that target the debris for removal by phagocytosis; this process is termed 'the respiratory burst' and occurs through activation of the enzyme NADPH oxidase (Filippin *et al.*, 2009). Proteases are released, which further degrade debris and ECM, whilst additional pro-inflammatory cytokines, also triggered by neutrophils, strengthen the inflammatory response. In mild skeletal muscle injury, the bulk of the damage to the muscle plasma membrane does not result from the primary injury itself, but rather from secondary injury. It is hypothesised that superoxide/myeloperoxidase (MPO)-dependent mechanisms, early invading neutrophils and the respiratory burst play important roles in this process (Tidball, 2008). Secondary damage is an important consideration for any study using an injury model. MPO is an enzyme specific to neutrophils and catalyses the production of hypochlorous acid (HClO); a highly oxidative and cytolytic free-radical. These neutrophil-derived free-radicals may, through oxidative modification of membrane lipoproteins, lead to sufficient membrane damage and prolong the degeneration phase (Tidball, 2008). After the neutrophil numbers start to decline, the injured area is invaded by two subpopulations of macrophages, which were mentioned in the previous chapter, thus converting the damaged area from a pro- to an anti-inflammatory state. It seems then, that a controlled neutrophil response, rather than an inhibited one, may be more beneficial in terms of therapeutic

strategies. Figure 3.1 depicts the time course of inflammatory cell infiltration into the injured muscle, and this time-course is briefly discussed in the next section.

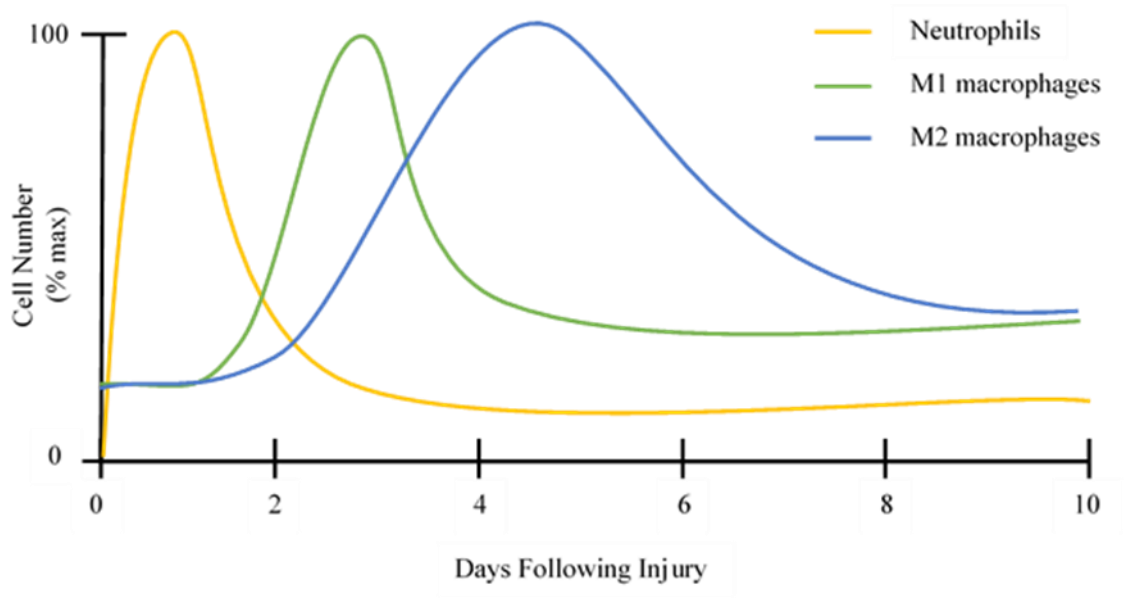

Figure 3.1 Chronological illustration of immune cell involvement after injury. 100% cell presence indicates that those cells reached a peak response at that specific time-point after injury. This figure is adapted from a review by Smith *et al.*, 2008.

Recently, Saclier *et al.*, (2013) released a paper, which provides the first *in vitro* time-course analysis of inflammation in a model of muscle regeneration. This model allows for the analysis of each step of *in vitro* myogenesis, including; commitment to myocyte, migration and fusion. At each of these steps, macrophages were shown to have essential, orchestrating roles on myogenic precursor cells (MPCs), thus illuminating an intricate relationship between inflammatory cells and effective skeletal muscle repair; knowledge that until now, was known but not clarified in sufficient detail. According to Saclier and her group, macrophages are said to act differentially on MPCs, according to their activation state; eliciting trophic effects through the secretion of soluble cytokines and growth factors. M1 macrophages migrate toward and stimulate MPC proliferation while preventing premature differentiation through the secretion of IL-6, IL1-β, high TNFα and IL-13. Later in the time-course of regeneration, M2c and M2a macrophages are known to be present. The *in vitro* experiments showed that, through TGF-β and low TNF-α secretion, M2c and M2a macrophages attracted MPCs and stimulated their commitment to myocytes and earlier subsequent formation of mature myotubes.

The latter was a novel finding of this study, where M2c and M2a macrophages decreased MPC motility and were better at attracting the MPCs and possibly delivering pro-differentiation cues. These particular macrophages also strongly promoted MPC commitment into myocytes - which

precedes fusion - and subsequent formation of large myotubes, as opposed to M1 macrophages, which inhibited MPC fusion. IL-4 was proposed as a possible effector for this stimulation of fusion by M2c and M2a macrophages. This study is a strong demonstration of the intricate relationship between inflammatory signals and the muscle itself, especially after an injury.

3.2 Muscle regeneration following injury

Skeletal muscle has an astounding ability to regenerate after any sort of injury. The components of skeletal muscle tissue orchestrate its own regeneration through inter- and intracellular signalling and repair processes, however, this is a complex process as it combines the actions of multiple physiological systems. For the purpose of this review, the following sections will discuss some of the skeletal muscle constituents, including satellite cells, that make regeneration possible.

3.2.1 Muscle cells

The muscle cell, or myocyte, is a long, cylindrical, multinucleated structure. Whilst the multi-nucleation is unusual, they share the fundamental properties of most other cells. The bundled myofibrils within a muscle cell are bound by a plasma membrane, which is a specialised layer known as the sarcolemma; and this is surrounded by an overlying membrane called the basal lamina. Between these two membranes lie the satellite cells, and this is discussed in the next section.

The sarcoplasm is comparable to the cytoplasm of non-muscle cells, however, due to a constant energy and frequently high metabolic demand, it contains a very large amount of glycogen, which is stored in granular form as glycosomes. It also contains high volumes of myoglobin, the primary oxygen-carrying protein within muscle tissue. Importantly, myoglobin may be used as a marker of muscle damage, and its presence within the blood is unusual, and may indicate an increase in sarcolemma permeability, and thus damage to the muscle fibers (Radak *et al.*, 2012).

The individual muscle cells are, like the myofibrils they contain, further bundled into fasciculi (see Figure 1.1) and grouped accordingly to make up fully functional, whole muscle bundles. The cells are provided with structural support by a large complex network known as the ECM, which has been mentioned earlier in this review. The ECM is made up of fibrous proteins, such as collagen, that are connected by glycosaminoglycans, which are essentially protein-bound polysaccharides (Michel *et al.*, 2010). Additionally, the ECM is separated from the sarcolemma of each muscle cell by the basal lamina. The ECM also plays a dynamic biochemical role in a variety of cellular functions, such as cell survival, cell adhesion, differentiation and proliferation, as well as allowing for cellular cooperation in a multicellular environment (Behonick & Werb, 2003). The ECM acts as a store since

it sequesters a variety of growth factors, making it possible for very rapid, local, growth-factor-mediated responses without having to synthesise them from anew. It follows from this then, that the formation of the ECM is essential in the process of repair and regeneration, and ECM synthesis is actually achieved by the muscle cells themselves, where ECM components are produced intracellularly and secreted into the existing ECM via exocytosis (Behonick & Werb, 2003; Engler *et al.*, 2006). However, the fibroblasts (myofibroblasts) support this function.

The ECM and formation thereof is incredibly important to muscle regeneration after an injury. In the early phases of injury, after the destruction phase, the ECM functions as a sort of scaffolding maintaining the structure of the muscle whilst new fibers are being formed and old fibers are being repaired (Lieber & Ward, 2013). Once the muscle tissue is regenerated, remodelling of the muscle begins and the ECM is gradually degraded by MMP enzymes (Gillies & Lieber, 2012) until normal muscle architecture is achieved. However, in some severe cases of injury, too much ECM remains, or not enough is degraded, and what remains is a fibrotic scar which is not beneficial for the overall health of the muscle. This scenario of fibrosis will be discussed towards the end of the chapter.

3.2.2 Satellite cells

Wedged between the sarcolemma and the basal lamina lies the resident stem cell of skeletal muscle, the satellite cell (see Figure 2.1). Satellite cells can differentiate into myoblasts, which can contribute to the growth, homeostasis, hypertrophy as well as repair of this tissue. It is because of these cells that skeletal muscle displays such great regenerative plasticity, distinguishing it from cardiac muscle. Following muscle damage, satellite cells become activated; after which they begin to proliferate, differentiate and fuse with other myoblasts, or even with damaged muscle fibers, in order to repair muscle fiber ultrastructure. It follows from this that active satellite cells are very scarce in healthy muscle, but become proliferative and more abundant upon mechanical stress to the musculature, regardless of the severity. A unique characteristic of satellite cells is the ability to self-renew and maintain their populations, which fulfils one of the definition criteria of a stem cell (see Scharner & Zammit, 2011, for an extensive review).

Satellite cells do not represent a unique cell type, but rather, a heterogeneous population of muscle precursor cells in various stages of activation, proliferation and differentiation. Furthermore, although some satellite cell markers, such as Pax-7, CD56 or Ki67, are expressed at various stages during injury, the expression patterns differ according to their respective activation state. Additionally, not all of the molecular markers are unique to these cells in particular, for example,

CD56 is a membrane glycoprotein, also known as a neural cell adhesion molecule (NCAM), and is present on a number of other cell types. This makes the selection of satellite cell markers a relatively complex process.

In adult muscle, quiescent satellite cells can be characterised by the expression of paired box 7 (Pax7). Conversely, activated satellite cells begin to express CD56 and Myf5, indicating their commitment to a muscle progenitor fate. Myogenic Factor 5 (Myf5) is part of the myogenic regulatory factor (MRFs) protein family and has a key role in the regulation of myogenesis through its function as a transcription factor. Pax7 belongs to a large Pax family of transcription factors. Pax7-null skeletal muscle shows a complete absence of satellite cells, and mice with this mutation have a significantly reduced body weight (Seale *et al.*, 2000). Moreover, Pax7 induction in foetal muscle stem cells promotes satellite cell development specifically, through restriction of alternate developmental programmes that these stem cells may follow (Seale *et al.*, 2000). This highlights the notion that satellite cells and muscle stem cells are distinctly different cell populations. Furthermore, ablation of Pax7-expressing cells renders damaged muscle completely unable to regenerate, with a failure to recover this ability (reviewed by Relaix & Zammit, 2012). The progression of satellite cell myogenesis is depicted in Figure 3.2 along with the myogenic markers that are expressed at each stage of myogenesis.

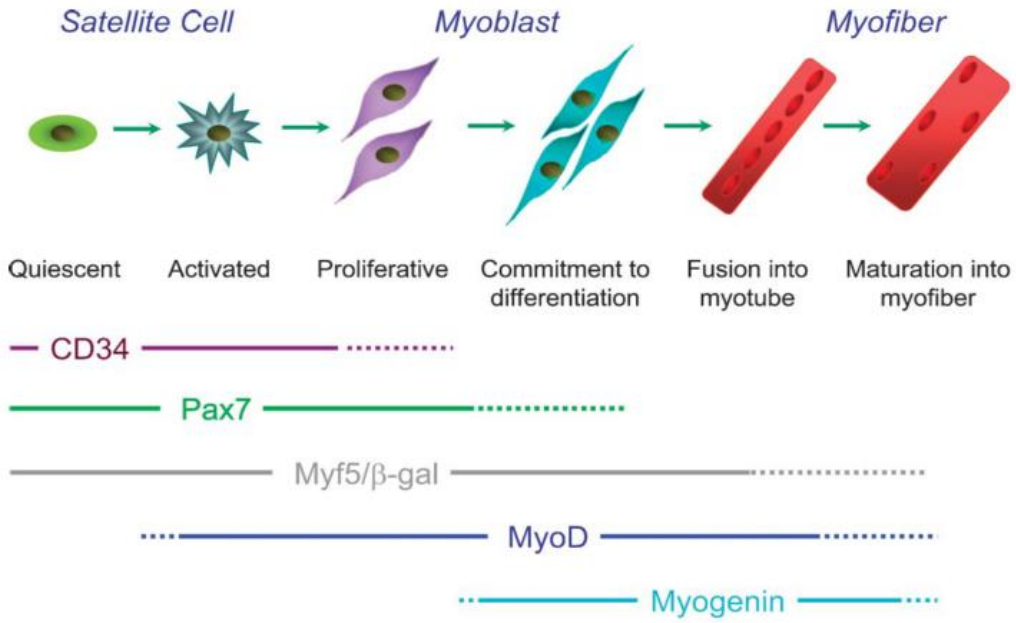

Figure 3.2 Schematic of myogenic progression of satellite cells. This diagram provides an overview of the progression of satellite cell fate after activation, and the eventual formation of new myofibers. Also depicted are the myogenic markers and their respective expression at each stage of the process. Figure taken from Zammit *et al.*, 2006.

Little is established with regards to how satellite cells remain in a quiescent state, and the exact mechanisms behind satellite cell activation also remain elusive. The first factor to be established as a satellite cell activator was hepatocyte growth factor (HGF - Allen *et al.*, 1995). HGF is normally found in the ECM and is liberated upon mechanical stretch or injury, activating satellite cells through co-localisation with the c-met receptor – on the satellite cell surface. More recently in models of mechanical shear forces acting on the muscle, it has been shown that NOS enzyme activity is required for initial activation of satellite cells (Anderson, 2000), with HGF as another, later stage activator (Filippin *et al.*, 2011 a & b; Martins *et al.*, 2011). This information may well lead to unravelling the mechanisms behind satellite cell activation.

What is known regarding activated satellite cells is that MyoD, a transcription factor, is rapidly induced in almost all satellite cells at this stage, with these cells then proliferating as Pax7+/MyoD+- myoblasts. Subsequently, most of these cells then down-regulate Pax7, but maintain MyoD, thus committing to a state of differentiation, through activation of another MRF, myogenin. Some remaining cells do the opposite, maintaining Pax7 but down-regulating MyoD, and in doing so, they withdraw from the cell cycle and return to myogenic quiescence (Relaix & Zammit, 2012). This co-expression of Pax7 and MyoD upon activation seems to be a universal feature of all satellite cells before the decision to differentiate or self-renew. However, there has been some doubt about the

homogeneity of satellite cell characteristics at different stages, due to the differing data reported from the various muscle research groups that work in the field.

Some proliferating satellite cells do not form exactly equivalent cells upon division. In such cases it has been observed that one progeny differentiates into a myonucleus whilst the other can remain a satellite cell (Relaix & Zammit, 2012); however, understanding is lacking as to which progeny undergoes self-renewal and which differentiates, but, Wnt signalling has been implicated (Scharner & Zammit, 2011). It was recently suggested that Wnt signalling must be activated for differentiation and fusion of myoblasts into myotubes to occur, whereas Notch signalling presides during the proliferation of myoblasts (Sakuma & Yamaguchi, 2012). An interesting hypothesis regarding the 'plane of cell division' and daughter cell fate has emerged and is summarised in a review by Bryson-Richardson & Currie (2008). More specifically, cells dividing along a planar axis, i.e. parallel to the basal lamina, give rise to daughter cells with similar Myf5 expression. On the other hand, cells undergoing apical-basal division – perpendicular to the basal lamina – give rise to cells with differing expression of MRFs. In doing this, satellite cells uphold the maintenance of a pool of uncommitted or quiescent cells (MRF negative), which is characteristic of these cells. The roles of the ECM and various factors in the niche remains to be explored.

Unfortunately, skeletal muscle's regenerative capacity is not without flaws, and a portion of the healing is achieved through scar tissue formation (Alessandrino & Balconi, 2013). Therefore, the process of myogenesis was not the primary focus of this thesis but rather the events that occur 2-3 weeks post injury, namely fibrosis.

3.3 The fibrotic response to muscle injury

The ECM is a crucial structural component of skeletal muscle with additional roles in muscle homeostasis, adaptation as well as in repair; and, there is evidence that the ECM bears most of the muscle passive load (see Gillies & Lieber, 2012 for a review). The latter point may indicate that perceived stiffness, after exercise or injury, may stem from the ECM itself. Furthermore, nearly all pathological changes in muscle, such as those seen in disease states like Duchenne Muscular Dystrophy (DMD), result in some form of ECM fibrosis. Importantly, the requirement for ECM formation during healing does not allow for therapies that block or inhibit the process. Rather, therapies that minimise the scar or speed up the degradation of fibrotic scar tissue during the remodelling phase should be used. The next sections will focus on fibrosis, particularly in the context of injury to skeletal muscle, as well as the potential of nitric oxide as a therapy for this condition.

3.3.1 What is fibrosis?

Fibrosis is a term used to define the pathological state of excessive connective tissue, or ECM, deposition; in response to an injury, this is called scarring. Myofibroblasts – or, activated fibroblasts within the muscle tissue - are responsible for the production of connective tissue in skeletal muscle fibrosis, and sources of these cells include inflammatory cells, fibroadipogenic progenitor (FAP) cells and the resident fibroblasts themselves (see Lieber & Ward, 2013 for a review). The formation of a solid connective tissue structure in the damaged area is a part of the normal wound response, and after 10 days post-injury this structure begins to disappear due to remodelling, thus leaving only a small scar by 21 days post-injury (Järvinen *et al.*, 2005). The muscle's capacity to regulate regeneration and connective tissue formation is compromised, shifting the balance toward excessive scar formation and fibrosis.

The ECM has a strong mechanical influence in muscle function and the effect of fibrosis on proper function is both detrimental and clinically relevant. Fibrosis results in decreased fiber size as well as increased collagen content, myofibroblast number, inflammatory cell number and myofibrillar disorganisation (Lieber & Ward, 2013). The degree of fibrosis varies with the severity of the pathological state, for example, DMD, which is characterised by large amounts of connective tissue between the fibers; results in the muscle being exposed to a chronic state of fibrosis, which worsens with time. However, in the case of an injury, the muscle may only be exposed to a short period (~3-5 weeks) of fibrosis, where the excess ECM is eventually degraded by matrix remodelling enzymes such MMPs, which degrade collagen and fibronectin (Gillies & Lieber, 2012). However, repetitive injury as well as factors such as ageing, may offset the balance of remodelling by these enzymes; therefore, it is this matrix remodelling phase that is of particular therapeutic interest to this thesis, as the concept of speeding up scar degradation, or even producing a smaller scar, is a very attractive therapeutic option.

TGF-β is a prominent cytokine in both initiating and terminating this normal wound repair (Border & Noble, 1994) and its actions are summarised in Figure 3.3. However, when the injury is severe or when it is a repeated injury, TGF-β production is sustained. TGF-β is a prominent pro-fibrotic mediator that is released by inflammatory cells and the damaged muscle itself, and once activated TGF-β induces fibroblasts to synthesise ECM components as well as to suppress MMPs (Gillies & Lieber, 2012). Collagen and fibronectin are two major constituents of fibrotic connective tissue that are synthesised by the TGF-β superfamily, with fibronectin functioning to bind collagen to the

muscle cell (Pankov & Yamada, 2002). These two proteins were of particular interest to this thesis, and TGF-β is a major target of some anti-fibrotic treatment strategies which will be looked at in the next section.

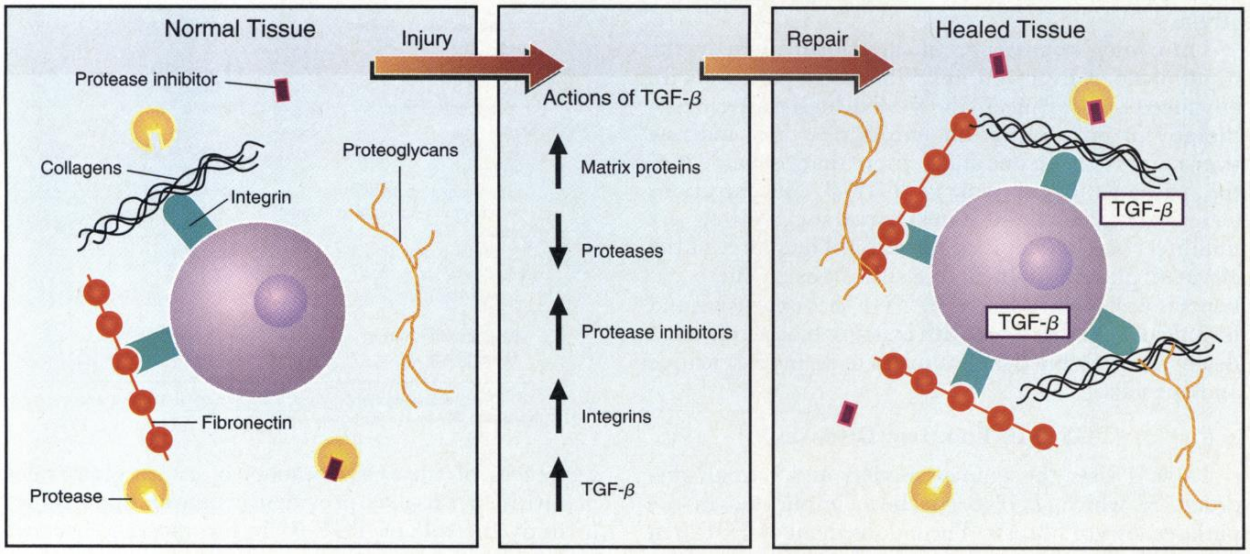

Figure 3.3 The role of TGF-β in normal wound repair. This figure illustrates how TGF-β orchestrates wound healing when it is transiently up-regulated. In pathological states this process is overstimulated and excessive matric proteins are synthesised. Figure taken from Border & Noble, 1994.

It is clear from the above that satellite cells play a vital role in maintaining structural homeostasis within the muscle tissue, especially after an injury where they are responsible for skeletal muscle's ability to regenerate and repair itself and return to a state of homeostasis. What was also discussed was the intricate relationship between satellite cells, muscle cells and the immune system and how they combine as a "repair and restore" response upon injury. With regards to therapies for muscle injury, targeting the inflammatory response has been a primary focus over the years; with desired outcome measures including the reduction of swelling and minimising pain. Given the undesired clinical outcomes – such as recurrent muscle tears (Kujala *et al.*, 1997) – linked to excessive fibrosis, ideally, a treatment should not only beneficially modulate stem cell and/or inflammatory responses, but also this later, more permanent outcome. The following section provides a brief summary of the previous, current and prospective therapies for muscle injuries.

3.4 Therapeutic strategies for muscle injury

Whatever damage may occur, the action taken often involves one or more strategies, including; topical ointments or creams, ingested analgesics (pain-killers) and anti-inflammatories or a trip to the doctor, physiotherapist, chiropractor or other health care personnel. Much research and funding has, and is still being supplemented into these strategies, however, no solid therapeutic strategy has

been universally employed for treatment of soft tissue damage (Järvinen *et al.*, 2007; Mueller-Wohlfahrt *et al.*, 2013), especially strategies that target fibrosis.

3.4.1 Traditional strategies

Treatment principles for muscle damage/injury follow a relatively standardised procedure, and may rather be regarded as 'management' techniques. The most commonly used acute treatment strategy; RICE. This stands for Rest, Ice, Compression and Elevation and is a practical principle, where all four methods aim to minimise bleeding into the damaged area and stimulate deep tissue blood flow for repair. There are no clinical trials to confirm the effectiveness of the RICE-principle, however, there is evidence for the appropriateness of each component (Järvinen *et al.*, 2007). This strategy is practically based, allowing for normal healing processes to continue with optimal, but slow, recovery of the damaged area. For actual muscle tears, the first step is immobilisation for the first few days following the injury, in order to allow the injured tissue to build sufficient strength to withstand contractile forces without re-rupturing. Re-rupture injuries are considered the most severe type of skeletal muscle injury, causing an excessively long recovery period.

The common problem with current treatment principles seems to be the rate of recovery from an injury, which is ultimately slow; or at least too slow for the casualty and those concerned with him/her. This has led to widespread use of NSAIDs, which are some of the most commonly prescribed, as well as self-administered, class of drug for any extent of muscle damage/injury (Kim *et al.*, 2014). They are usually advised immediately following an injury and are often combined with other therapies such as RICE (Järvinen *et al.*, 2007). Still, there is no guarantee of a full recovery, where the optimal function is restored in the damaged muscle(s).

3.4.2 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are available as many different classes, differing in terms of strength, inhibitory targets and mechanism of action. However, traditional NSAIDs act by inhibiting the cyclooxygenase (COX) enzymes, with downstream inhibition of the production of prostaglandins that mediate pain and inflammation (Kim *et al.*, 2014). More recently, NSAIDs were altered to selectively inhibit the COX-2 enzyme, as COX-1 is important to gastrointestinal homeostasis, thus, its inhibition is undesirable (Shah *et al.*, 2001). Even with their proven efficacy and suitability for use in humans, they can elicit various side-effects such as gastric ulcers (Fine, 2013). Despite this, they remain the most utilised type of drug in the treatment of moderate sports injuries (Almekinders, 1999; Rahusen, 2004; Ramos *et al.*, 2012).

3.4.2.1 Current use of NSAIDs

In a study by Tscholl *et al.*, (2008), the use of drugs was examined in the 2002 and 2006 FIFA World Cups. NSAIDs comprised almost half of all reported medications used, with more than 10% of players using NSAIDs before every match. Additionally, this study reported that 86% of professional Italian football players were consistently using NSAIDs.

The sporting field is but one example of the frequency of NSAID use, and given the potential of these drugs to interfere with proper muscle healing (Smith *et al.*, 2008) as well as their gastrointestinal side-effects (Wallace, 1994), it is necessary to explore more appropriate therapeutic alternatives.

3.4.2.2 History of NSAID use

The literature on NSAID use dates back to before the 1950's, where they have since been continually prescribed for arthritic conditions. It was around the 90's when their use came under more rigorous scrutiny, due to the high probability of gastrointestinal bleeding from chronic consumption (Wallace, 1994) (the pathogenesis of which will be discussed in more detail in section 3.4.2.3 below). For this reason, various strategies were employed to develop drugs that would limit these unwanted side-effects, for example; the enteric-coating of drugs, the use of prodrugs and selective COX-2 inhibitors, to name a few.

3.4.2.3 NSAID mechanisms of GI injury

In 1998, it was reported that the gastrointestinal (GI) side-effects resulting from NSAID use cause an estimated 16 000 deaths and 107 000 hospitalisations each year in the US (Keeble & Moore, 2002). These numbers are staggering when one considers that the drugs were being consumed for non-fatal conditions. From the literature, it is clear that the mechanisms involved in NSAID-induced GI injury are multi-factorial, but a general reduction in mucosal defences may be key in the pathogenesis of the adverse side effects, clinically characterised by peptic ulcer (gastric and duodenal) formation and bleeding. An earlier review by Wallace (1994) highlighted the role of prostaglandin suppression in the development of gastric injury, and also reported evidence of increased leukocyte (mainly neutrophils) adherence to the vascular epithelium in the gastric microcirculation. He suggested that the latter resulted from TNF release in response to NSAIDs, leading to a rapid up-regulation of adherence molecules, such as ICAM-1. The resulting neutrophil adherence might obstruct the capillaries and reduce mucosal blood flow, as well as be accompanied by activation of the neutrophils, with the subsequent respiratory burst causing epithelial and endothelial injury and exacerbated inflammation. Wallace also suggested that NSAIDs might

combine with bile in the small intestine, created a cytotoxic mixture which further impaired the mucosal defences. Following from these key mechanisms, a subsequent review (Rainsford, 2001) added that NSAIDs were responsible for the reduction of NO and the increased activity of caspases 1 & 3 - which facilitate pro-inflammatory cytokine release and apoptosis. Therefore, the addition of NO to these NSAID compounds began, with the rationale being that NO would increase gastric mucosal blood flow as well as reduce the adherence of neutrophils to the vascular endothelium of the gastric microcirculation (Davies *et al.*, 1997). It was subsequently shown that NO-NSAIDs were highly effective in reducing gastric side-effects, as well as maintaining their anti-inflammatory and analgesic properties (Cuzzolin, 1995; Davies *et al.*, 1997). Wallace and his group were the forerunners into NO-NSAID research, with extensive and thorough publications in multiple models, using various NO-drug combinations.

Interestingly, in contrast to the initial effect, NSAIDs were proposed to induce NO at a later phase of the same response, when it binds to oxygen radicals to form peroxynitrite, resulting in an NFκB-mediated inflammatory response. This inhibition and the later induction of NO is most likely due to the fact that NO can be synthesised by three different enzymes, which makes it a multi-functional molecule involved in many different processes (see section 3.5 for more details on NO). Overall, however, NO is probably more beneficial than detrimental in reducing NSAID-induced GI ulceration and bleeding, a notion that became apparent in studies that use NO-linked NSAIDs (Muscará *et al.*, 1998; Muscará & Wallace, 2006).

3.4.2.4 NSAIDs and muscle damage

In an early review of anti-inflammatory treatment of muscle injuries, Almekinders (1999) reported that NSAIDs appear to have some beneficial anti-inflammatory effects early in the post-injury period, which may transform into an initial clinical improvement. This inflammatory mediation is because of the COX inhibition and subsequent decline in prostaglandin production, which was already well established by some big names in the field, such as Wallace and Davies (Davies *et al.*, 1997; Wallace, 1994). Additionally, Almekinders noted that, although NSAIDs have early benefits including increased contractile force sooner following injury, they may be associated with unwanted effects later on, such as delayed degradation of damaged tissue as well as slowed regenerative capacity. It was subsequently concluded that there was no compelling case for aggressive NSAID use in the treatment of muscle injuries. A compromise was reached amongst sports medical personnel that if NSAIDs were to be used, it should be early in the post-injury period and discontinued once the acute inflammatory response has peaked (within ~48 hours) in order to

minimise any adverse effects later on in the healing process. Given the lack of consensus and insufficient research, the use of NSAIDs is a rather dated approach.

3.4.3 More recent therapeutic strategies

From the above sections, it is clear that the treatment of muscle injuries is still very juvenile in its methodology, and much is yet to be understood. For this reason, research has branched out to explore a multitude of potential therapies and it is even a notion that different therapies that target different parameters could be used.

One strategy is the use of melatonin (Pugazhenthi *et al.*, 2008). Treatment was found to up-regulate COX-2 activity and protein levels immediately post-injury. It was also suggested that this increase may advance the healing process due to prostaglandin's involvement in fibroblast proliferation and promotion of collagen synthesis. Melatonin also accelerated angiogenic processes, through increased VEGF expression, and was seen to decrease iNOS activity acutely and then increase its activity during the resolving phase – which is similar to the action of NSAIDs. These results (Pugazhenthi *et al.*, 2008) however, were reported in a dermal incision wound model; therefore, the mechanisms may differ to models of mechanical muscle damage, where the formation of excessive collagen may not be desirable. Several other treatments have been tried in other models.

One study tested the effects of atorvastatin – which reduces LDL and triglycerides, but promotes HDL in the blood. It improved the function and morphology of infarcted swine hearts, primarily through eNOS activation (Song *et al.*, 2013), which most likely improved cardiac perfusion through NO-mediated vasodilation. In a skeletal muscle strain injury model, however, simvastatin seemed to increase the extent of damage in terms of gross muscle morphology (Tomazoni *et al.*, 2012).

In a hindlimb suspension model in aged rats, green tea extract (GTE) – which contains caffeine – reduced muscle wasting and atrophy and attenuated the loss in unloading-associated force production. GTE also enhanced satellite cell proliferation and differentiation as well as decreased oxidative stress (Alway *et al.*, 2014).

An emerging strategy in the treatment of muscle injury is the use of LLLT or low-level laser therapy. LLLT is an infrared (810 nm), a non-pharmacological alternative to commonly used therapies and is similar to ultrasound therapy. Lower doses of LLLT, up to about 6 Joules, showed significantly improved functional and performance outcomes following a muscle strain injury (Ramos *et al.*, 2012); whilst the 3 Joule dose improved the rodent walking track index (a functional measure) score.

A follow-up study compared the 3 Joule dose, with a traditional NSAID which was applied topically in one group and intramuscularly in a second group (de Paiva Carvalho *et al.*, 2013). LLLT reduced blood levels of inflammatory prostaglandins more than the two NSAID treatments, and also showed a better-improved walking track analysis.

So, there has been much research into the field of muscle injury, but as of yet, still no ground-breaking, universally applicable therapy exists. This is probably because therapies should not inhibit any natural responses involved in healing. Rather, modulating those processes that have the potential to be excessive, such as the inflammatory respiratory burst or the overproduction of ECM components that leads to fibrosis should be the goal.

3.5 Therapeutic strategies that may target fibrosis specifically

The inhibition of fibrosis is a highly attractive target for improving muscle regeneration and has clinical implications for any sort of injury as well as for disease states like the muscular dystrophies. This section will cover some of the anti-fibrotic therapeutic strategies that have been researched previously, although none have been made clinically available.

Due to the prevailing role of TGF-β in the fibrotic response, this cytokine is a possible therapeutic target. γ-Interferon is a TGF-β pathway inhibitor, and treatment with this cytokine down-regulated TGF-β protein expression *in vitro*, and reduced the area occupied by fibrosis in vivo, in tissue cross-sections (Foster *et al.*, 2003). The latter result was in a murine laceration-injury model, and the same model was used to show that decorin, another TGF-β inhibitor, also improved histological parameters of fibrosis as well as function (Fukushima *et al.*, 2006). Whilst these studies show promise for cytokine inhibitors as anti-fibrotic therapies in animals, the translation of the results to humans is hindered by other quite severe side-effects (Lieber & Ward, 2013). However, losartan is an anti-hypertensive therapy that blocks angiotensin II receptors and is used in human patients. It is an alternative for patients who are intolerant to ACE inhibitors – another blood pressure medication. Losartan also seemed to reduce – histologically - the extent of the fibrotic area in lacerated mouse muscle (Bedair *et al.*, 2008), but the additional effect of reduced blood pressure must be considered here. It seems that an optimal environment is required for efficient repair after an injury, and one way to create such an environment may be to maintain proper circulation to the area, especially after a crush injury, where vasculature may be damaged. In terms of the latter parameter, a prospective candidate for the treatment of injuries may be nitric oxide, which is a potent vasodilator. This concept is of particular interest to the proposed thesis and is discussed later in the following section.

The aforementioned experimental treatments indicate that fibrosis can be effectively modulated, however, there is still a lack of one that is clinically applicable. In addition, similar to the case of the traditionally used NSAIDs - discussed in section 3.4.2 - it seems that complete inhibition of pro-fibrotic signalling pathways is too extreme a strategy, leading to unwanted side-effects and ultimately hindering regeneration. The rationale behind the use of NSAIDs was to eliminate an inflammatory response, which was successful but did not translate into an overall benefit and had some serious side-effects; and the same result is apparent with the use of TGF-β inhibitors. Potentially then, a partial modulation is the answer, where normal signalling and responses could occur, but to a reduced extent.

Albeit scarce, evidence has emerged that NO may protect against fibrosis in the tunica albuginea and in cardiac and kidney tissue (Ferrini *et al.*, 2002; Kilic *et al.*, 2014). This makes a strong case for the investigation of a NO-based therapy for skeletal muscle fibrosis, especially when combined with the antioxidant and anti-inflammatory effects (Wallace, 2005; Gupta *et al.*, 2008) as well as the muscle repair improvements (Buono *et al.*, 2012) shown by some NO-donating agents such as Molsidomine. Knowledge on mechanisms by which NO may interact with fibrotic signals are lacking, however, when NO synthesis is inhibited (using nitro-L-arginine methyl ester: L-NAME) the result is an excessive expression of TGF-β in the tendons and muscles of crush-injured rats (Darmani *et al.*, 2004 a & b; Filippin *et al.*, 2011 a & b). So, it seems that NO may play a role in the regulation of TGF-β signalling and, therefore, scar formation after injury. Overall, there is a need for the further investigation of the effects of an NO-donating therapy in skeletal muscle injury and fibrosis.

3.5.1 Nitric oxide donors

The concept of NO-donation as a therapy has been around since the early 1900's, at least, however, there seemed to be some hesitancy before its use; probably due to the notion that too much NO or reactive nitrogen species (RNS) may be toxic in some biological environments. Indeed, the presence of too much NO/RNS has proved to be detrimental in some cases, including the down-regulation of antioxidant systems in skeletal muscle (Lawler & Song, 2002), however, these contraindications are primarily experienced under chronic overproduction of NO. The small amount of literature covering NO's role in skeletal muscle does, however, suggest that this molecule is important in all phases of injury and repair; whether it be through direct effects or through signalling effects. The potential benefits and the potential detriments of NO-donation based therapies in skeletal muscle injury require a better understanding of NO, in general.

3.5.2 Nitric oxide – what is it?

NO is a gaseous messenger, best known for its vasodilating and relaxing actions in the vasculature. NO is highly reactive, has a very short half-life and can only travel short distances before being oxidised. NO determines endothelial function, influences metabolic and vascular health. NO is instrumental in coordinating hemodynamic responses and is therefore exploited as a therapy for angina, heart failure, pulmonary hypertension and even erectile dysfunction. It is also a potent antioxidant, anti-inflammatory agent and antithrombotic factor whilst playing additional signalling roles in the nervous and immune systems. Furthermore, in skeletal muscle, NO signalling is implicated to have an influence on muscle metabolism, contractility, immune function, cell growth and neurotransmission (Dutka *et al.*, 2011). It seems then, that this 'super-molecule' is a highly attractive therapeutic candidate for a multitude of conditions.

3.5.3 Sources of NO

The nitric oxide synthase (NOS) enzyme has three distinct isoforms that are responsible for NO production in many different tissues, and these are neuronal, endothelial and inducible NOS (nNOS, eNOS and iNOS respectively). Of the three, nNOS and eNOS are constitutively expressed, whereas iNOS is induced in response to stimuli, such as inflammation. eNOS is calcium-dependent and produces relatively low amounts of NO (Sprague & Khalil 2009). In the context of muscle, its expression has also been correlated with mitochondrial content (Kobzik *et al.*, 1995) perhaps due to the relationship to capillarization. nNOS is more abundantly expressed in skeletal muscle and interestingly, this is mainly in type II fibers, where it is localised to the sarcolemma and interacts directly with the dystrophin complex (Kobzik *et al.*, 1995), suggesting mechanical influences. iNOS plays an important role in the immune system and is expressed by leukocytes (Sprague & Khalil, 2009). iNOS is induced by cytokines (Rovere-Querini *et al.*, 2014) and produced in vast quantities under inflammatory conditions. NO production through iNOS is thought to be a protective adaptation to injury as it reduces leukocyte infiltration and establishes an effective homeostatic inflammatory response. This has been confirmed through iNOS null studies (Darmani *et al.*, 2004 a & b; Gupta *et al.*, 2008), which reported increased infiltrating neutrophils and the persistence of macrophages (Rovere-Querini *et al.*, 2014).

3.5.4 NO response to muscle injury

As mentioned earlier, primary muscle injury is combined with an inflammatory response that causes NO production at the site of injury in a number of ways. Through calcium-dependent mechanisms,

eNOS synthesises a constant, but relatively small amount of NO into the vasculature. However, it has been shown that increases in levels of TNF-α – released by injured muscle fibers - decreases the translation of eNOS mRNA (Caldwell *et al.*, 2010; Rubinstein *et al.*, 1998), reducing the bioavailability of NO (Levine *et al.*, 2012). The primary NOS isoform expressed in skeletal muscle is nNOS, however, and quite intriguingly, after an acute injury (by cardiotoxin) nNOS expression was shown to disappear, almost entirely, and remain well below normal levels for over two weeks (Rigamonti *et al.*, 2013). In contrast, the same research group showed that iNOS, expressed by infiltrating macrophages, was significantly elevated after injury in a completely opposite expression pattern to nNOS, and was identified as the primary source of NO after acute injury. It was also clear from this study that iNOS-derived NO contributed to muscle regeneration, stimulating satellite cell activation as well as differentiation. iNOS induction is said to reach a maximum at around 6 hours post-injury (Rubinstein *et al.*, 1998; Darmani *et al.*, 2004 a & b; Filippin *et al.*, 2011 a & b), indicating an important role for NO in the acute phase of injury. NO's importance in the acute phase is further promoted by the fact that inhibiting NO synthesis with L-NAME resulted in a massive transcription of iNOS mRNA (Filippin *et al.*, 2011 a & b), most likely due to lack of autoregulation.

3.5.5 Opposing results from NO studies

In very high concentrations, NO-induced apoptosis in skeletal muscle myoblasts that were cultured from rat muscles (Stangel *et al.*, 1996). This is relevant in the case of a crush injury, where considerable amounts of NO may be present due to the large induction of iNOS shortly after injury (Zhang *et al.*, 2011; Filippin *et al.*, 2011 a & b), as well as slightly elevated levels of eNOS (Rubinstein *et al.*, 1998). Having said this, there has been some controversy surrounding the study of exogenous NO *in vitro*, as these studies are conducted in the presence of oxygen and may use oxygenated buffers, which could react with NO and alter its signalling effects. Nevertheless, it has also been proposed high NO may cause serious damage to muscle through excessive vasodilation and subsequent 'hyperperfusion' of the muscle tissue. Rubenstein *et al.* (1998), an *in vivo* study using an experimental crush injury in rats, looked at all of the NOS isoforms and found that an induction of eNOS was associated with increased capillary perfusion and vasodilation that occurred post-injury. This could be a two-sided effect, with the possibility of maintaining tissue viability on the one side and the possibility of oedema and hypovolemic shock on the other. Interestingly, before the work of Rubenstein and colleagues (1998), it was thought muscle damage occurring from crush injury resulted from capillary occlusion and subsequent hypoxia and ischemia of the area. Their data

suggests the opposite. This leads to the question of whether or not NO may have any other effects that could offset hyperperfusion (if hyperperfusion is indeed a problem).

3.5.6 Roles of NO that may influence muscle recovery

NO has been shown to elicit many anti-inflammatory effects (Wallace, 2005). NO decreases endothelial cell (EC) activation by leukocytes through reducing the expression of EC-leukocyte adhesion molecules, namely VCAM-1, ICAM-1 and MCP-1. A possible mechanism for this may be through NO's indirect inhibition of NFκB, which is a pathway that would otherwise elevate the expression of these adhesion molecules. TNF-α – released during inflammation - activates the NFκB pathway via ROS; however, NO is able to scavenge, compete with and inactivate ROS, therefore reducing NFκB signalling. Importantly, macrophages, endothelial cells and vascular smooth muscle cells generate NO at the site of inflammation, thus enabling this inhibition (Sprague & Khalil, 2009).

Interestingly, a 2011 study by Filippin (also mentioned earlier in the text) found that NO played an important role in the normal healing process following acute muscle trauma with a crush injury and treatment with L-NAME. Although there was evidence of increased oxidative and nitroxidative stress, there was still improved regeneration of the injured fibers without treatment. Therefore, it could be that maintenance of blood flow to the injured area is important. Finally, NO decreased scar formation following trauma, by decreasing TGF-β expression, therefore lowering collagen deposition and shifting away from fibrosis and towards normal regeneration. Furthermore, the use of this inhibition model resulted in an acute and a chronic upregulation of TGF-β following an experimental crush injury in rats (Darmani *et al.*, 2004 a & b). Thus, the inhibition of NO synthesis with L-NAME treatment demonstrates the importance of this molecule (NO) in the balance of fibrosis and normal healing.

Finally, recent work has elucidated some very important functions of NO in the regulation of satellite cells in muscle repair. NO was shown to stimulate the proliferation of satellite cells via cGMP generation, whilst also maintaining the reserve pool of Pax7+/Myf5- satellite cells through the Vangl2-dependent Wnt signalling pathway (Buono *et al.*, 2012). From this it is clear that NO is a key messenger in the control of satellite cell number and fate; promoting activation and fusion, and ultimately preventing the exhaustion of the reserve pool, all of which are very beneficial to optimal healing.

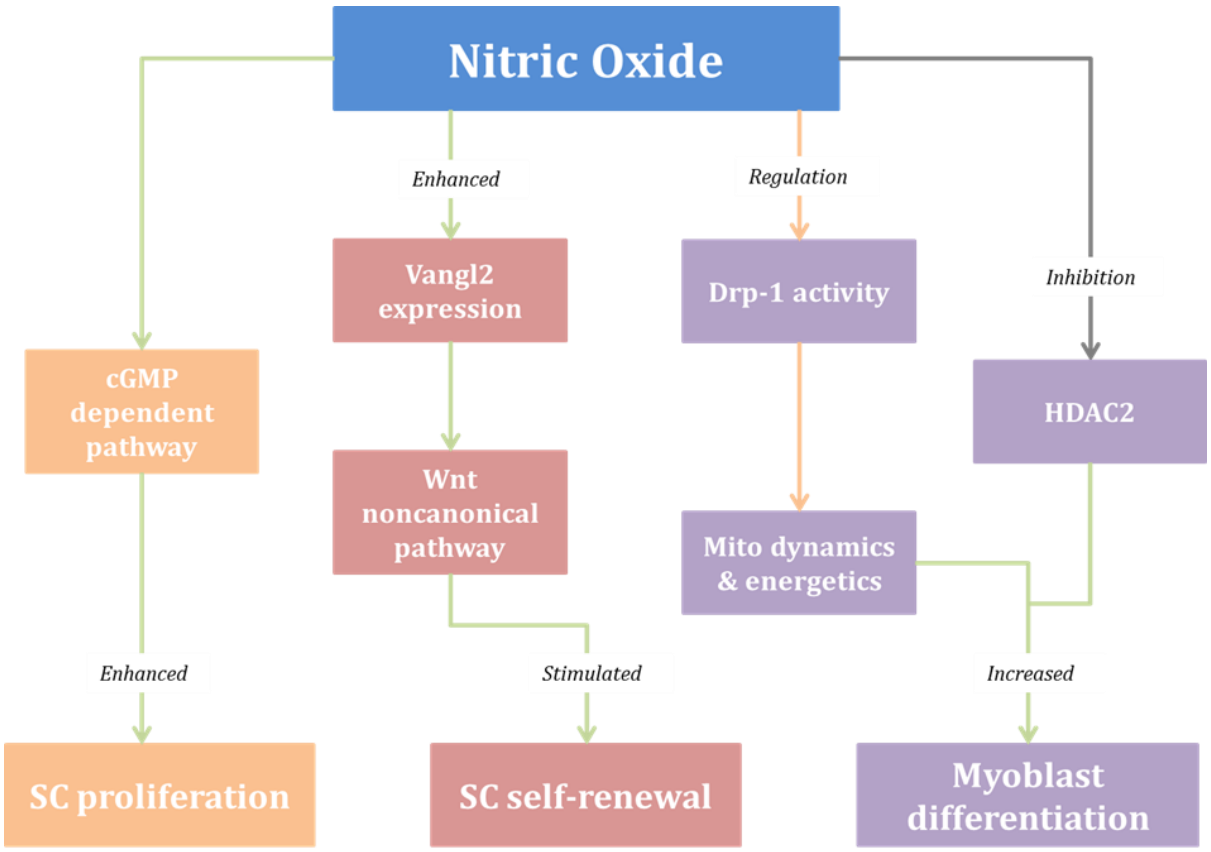

Figure 3.4 NO-mediated repair of damaged tissue. NO plays an intricate role in the repair of damaged muscle/tissue, predominantly through influences on survival, proliferation and differentiation of satellite cells. The figure above summarises a review put forward by Rovere-Querini and colleagues (2014).

3.5.7 NO-donors and lessons from other models

In cardiovascular research, much work has been done on NO and NO-donating agents in ischemia-reperfusion injury models, with some studies including a model of reperfusion of skeletal muscle. Reperfusion results in elevations of ROS products as well as NO and its associated products. The actions of NO are protective at very small (pico/nanomolar) concentrations, but may become cytotoxic at higher concentrations through derivatives such as peroxynitrite (Pacher *et al.*, 2007; Liaudet *et al.*, 2009). These radicals are responsible for causing strand breaks in DNA and may cause apoptosis through excessive poly (ADP-ribose) synthetase (PARS) activation. PARS is an essential DNA repair enzyme, which is beneficial only in moderate amounts. In a model of ischemia-reperfusion of the hearts or the skeletal muscles of rabbits, Thiemermann (1997) showed that PARS-dependent cell death of cardiomyocytes did not occur through NO and peroxynitrite, however in skeletal muscle myocytes, nNOS inhibition substantially reduced (+/-50%) infarct size, indicating a role for NO-mediated cell death - if present in large amounts - most probably through excessive PARS activation. This also indicates differential NO signalling in cardiac and skeletal muscle.

In other models, where NO-donation and reperfusion injury were studied, NO protected contractile function (Chen *et al.*, 1998), whilst additionally attenuating mitochondrial swelling and degeneration. S-nitroso-acetylcysteine (SNAC), an NO donor, was also shown to maintain microvascular perfusion. NO donors, therefore are able to reach the tissues, cells and organelles. Infusion of L-arginine increased NO production and inhibited superoxide production, showing that L-arginine may enhance eNOS activity (Balon *et al.*, 1997).

To examine what happens when NO is not present, L-NAME is often used to inhibit the activity of the NOS enzymes. In particular, iNOS inhibition has been shown to result in an extraordinarily large adherence of inflammatory cells to damaged tendon and muscle, three days following a crush injury to the digital flexor tendon (Darmani *et al.*, 2004 a & b). This was also associated with an imbalance between NO and TGF-β, indicating then, and this has been subsequently proposed (Filippin *et al.*, 2011 a & b), that NO is essential for a normal inflammatory response.

3.5.8 Limitations of in vitro studies investigating NO Donors

We have identified an NO donor, Molsidomine, to use in this context of our injury model. Molsidomine is an NO-donating anti-anginal drug that is approved for use in human patients. This drug is ingested and metabolised in the liver to liberate SIN-1, which then dissociates spontaneously in aqueous environments to liberate NO (Kilic *et al.*, 2014). Many research groups have used Molsidomine and it has effects other than vasodilation, such as non-hepatic glucose uptake in hyperglycemic and hyperinsulinemic conditions in fasted dogs (An *et al.*, 2008). The afore-mentioned study made use of SIN-1 as an NO donor, but it is also used in some cases as a peroxynitrite donor (Kanazawa *et al.*, 2000; Hemanth Kumar *et al.*, 2013), which may seem confusing until explained by the chemistry behind it.

In an oxygenated buffer, SIN-1 dissociates into both superoxide and NO, and these spontaneously form peroxynitrite (OONO-) (Huie & Padmaja, 1993). The reaction between NO and superoxide is an extremely fast reaction, therefore, NO is scavenged rapidly, and, in such a situation, would be unable to exert any beneficial effects as a lone-standing molecule. Without the presence of superoxide, NO might not be scavenged so rapidly, and may be 'allowed' to exert vasodilating and anti-inflammatory effects. However, *in vivo*, or in the presence of biological electron acceptors (e.g. heme proteins like myoglobin), SIN-1 acts as an NO donor rather than a peroxynitrite donor (Singh *et al.*, 1999). In other words, when oxidising agents other than molecular oxygen are present, which

is true for *in vivo* conditions, these agents may compete with oxygen to oxidise SIN-1 and form NO without concomitant superoxide production (see Figure 3.5).

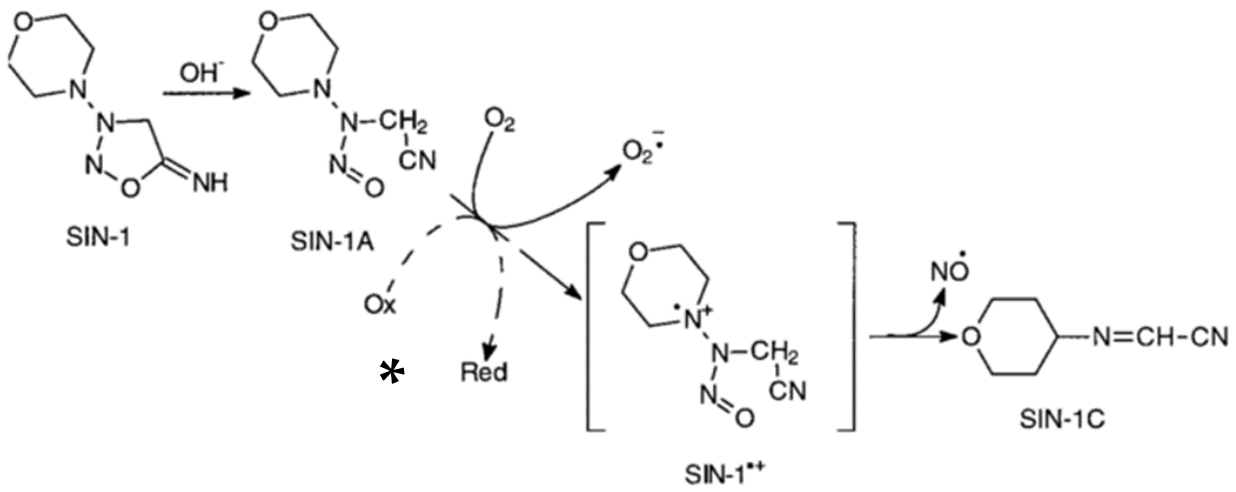

Figure 3.5 The oxidisation of SIN-1. The asterisk (*) indicates where oxidising agents (e.g. myoglobin) may compete with molecular O_2 and form the SIN-1 $^{\bullet +}$ cation radical and NO without forming superoxide ($O2^{\bullet -}$). Diagram taken from Singh *et al.*, 1999.

In a biological system (organism), it is most likely that SIN-1 generates both peroxynitrite and NO, with the ratio depending on the oxygen concentration as well as the concentration of endogenous oxidants. Beneficial effects of SIN-1 in reperfusion injury studies (Yeh *et al.*, 2004) suggest that NO mediates the attenuation of such an injury by scavenging oxygen. However, peroxynitrite has been shown to exacerbate reperfusion injury and it is thus unlikely that SIN-1 produced large amounts of this product. Subsequently, it is advised that the use of SIN-1 as either an NO donor or a peroxynitrite donor cannot be determined by the chemical interactions of SIN-1 are fully understood in the system being studied (Singh *et al.*, 1999). Additionally, a review of the literature by Liaudet *et al.*, (2009) elucidated a dysregulatory role for peroxynitrite in a multitude of signalling pathways; ultimately triggering the development of cardiovascular pathologies through excessive tissue damage. However, the review emphasised that these effects had only been confirmed in *in vitro* studies.

Due to the above-mentioned discrepancies, we chose to evaluate SIN-1 (in the drug form of Molsidomine) in an *in vivo* model of skeletal muscle injury and regeneration. Molsidomine is taken orally and must be enzymatically cleaved in the liver to release the active metabolite, SIN-1. In this way, our study is not compromised by any oxygenated buffers or atmospheric interactions and ultimately provides a robust evaluation of the actions of this drug *in vivo*.

3.6 Hypothesis

After a thorough review of the available literature as discussed above, I have formulated the following research question:

- Does exogenous NO improve tissue recovery in acutely injured muscle, compared to placebo-matched controls?

My hypothesis was that acute post-injury NO supplementation by *in vivo* treatment with an NO-donor will modulate inflammation, improve myogenesis and reduce the degree of fibrosis after a muscle-damaging crush injury to the gastrocnemius muscle of rats.

In order to test this hypothesis, I have formulated the following objectives:

- To induce an experimental crush injury in rats.
- To assess the effect of acute Molsodomine treatment (NO-donation) on the following:
    - Extent of inflammation, as assessed by MPO protein levels in injured muscle tissue
    - Effect on processes of reparative satellite cell proliferation and differentiation
    - Effect on the degree of fibrosis, as indicator of the quality of tissue repair
    - Potential mechanisms involved in fibroblast deposition

Chapter 4: Research Methods

4.1 Ethical considerations

All experimental procedures were approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University's Research Development Division (Ref. #: SU-ACUM14-00008). Care was taken to minimise the number of animals used while allowing for sufficient statistical power. All experiments on animals were carried out in accordance with the guidelines of the Medical Research Council.

4.2 Experimental animals

For this study, young-adult (2½ – 3 months), male Wistar rats (n = 58) – species *Rattus Norvegicus* - weighing between 320g and 420g were used. Rats had access to standard rat chow (Imbani Nutrition, Western Cape, South Africa) – for which nutritional information is presented in Table 4.1 – and tap water ad libitum while being exposed to a 12hr light/dark cycle (lights on at 6:30 a.m.). The temperature in the housing facility was controlled at 21°C and ventilated at 10 air changes per hour.

Table 4.1 Nutritional information of rodent chow.

Protein	Moisture	Lipids	Fibre	Phosphorus	Calcium
160 g/kg	120 g/kg	25 g/kg	60 g/kg	7 g/kg	18 g/kg
Rat Chow Ingredients (Imbani Nutrition)					

The arrival of animals at the department's small laboratory animal facility and entry into the study protocol was staggered, due to limited capacity in the facility. On arrival, animals were kept in large cages in groups of six for approximately one week, after which they were randomly assigned to smaller cages, in groups of three for the remainder of their intended study period. For enrichment, a small section of PVC piping was placed in the cage, through which the animals could walk and in which they could sleep. Rats are naturally communal animals, thus, they were not housed individually.

Table 4.2 Average rat weights (g) between post-injury time points.

Day 1 (n=22)	Day 3 (n=12)	Day 5 (n=12)	Day 21 (n=12)
352,09 (± 20,65)	356,75 (± 15,36)	363,83 (± 26,53)	372,79 (± 28,53)
Average Weight (± SD)			

Once in their appropriate cages, experimental animals were randomly divided into their respective experimental groups (total number of animals = 58). 10 animals were used as uninjured controls receiving either Placebo treatment or Molsidomine treatment (n = 5 per group). The remaining 48 animals were also randomly divided into Placebo or Molsidomine treatment groups and were euthanized at four post-injury time-points (n = 6 per time-point per treatment group). The rats were further divided into four groups of six rats for each time point. The researcher was not blinded to the cage allocations. The time-points for sample collection were 1 day, 3 days, 5 days and 21 days post-injury. (At the time that this protocol was planned, it was not common practice to use control groups for every time-point examined, therefore, we opted for the use of fewer animals). Average body mass measured at different time points are provided in Table 4.2, and a breakdown of the experimental animal groups is depicted in Figure 4.1.

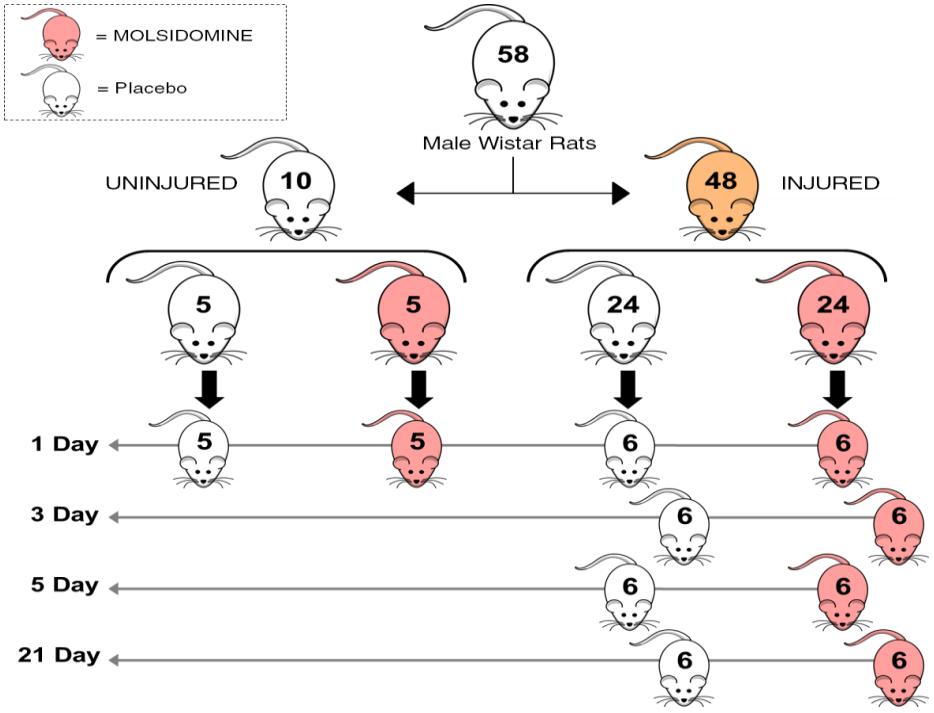

Figure 4.1 Experimental animal grouping. The figure above illustrates how the experimental animals were divided into the necessary study groups. A total of 58 animals were used for the study.

4.3 Drug intervention

As mentioned already, since the aim of the study was to investigate the role of NO in muscle regeneration following a crush injury (discussed in the following section), an NO-donating, anti-anginal drug that delivers the molecule into the circulation – Molsidomine – was chosen as intervention drug. Molsidomine (Cat. #: M2901, Sigma-Aldrich, USA) is a pro-drug for the formation

of NO (see Figure 4.2). Upon ingestion, it is rapidly absorbed and hydrolyzed in the liver into the active metabolite 3-Morpholinosydnonimine, or SIN-1. SIN-1 has a half-life of 1 to 2 hours, however, the total duration of therapeutic action seems to be longer than expected (See Rosenkranz *et al.*, 1996, for pharmacokinetics of Molsidomine). Administration of Molsidomine was by oral consumption of jelly blocks, at a dose of 3mg/rat. Each block was individually prepared by dissolving the drug in pre-set jelly (1 block = 1 dose). In our opinion, this route of administration was less stressful to experimental animals in comparison to e.g. oral gavage. For dosage, a paper by Singh *et al.* (1999) found that there was no difference in effects obtained for doses from 0.1mg, up to and including a dose of 1mg *in vitro*, thus, we chose a dose relevant to a clinical situation, and similar to other studies in rodents (Öztürk *et al.*, 2009; Zordan *et al.*, 2013).

Commercially available dessert jelly (Moirs) was purchased and prepared as per supplier instructions. An extra tablespoon of gelatine (Moirs) was added to the mixture, so as to provide extra structure to each block. Jelly blocks were prepared in an ice tray (~2mL each). A 3mg dose of Molsidomine was dissolved in 500µL of dH_2O and mixed into each block. The tray was then placed back in the fridge (4°C) and left overnight to cool and to be used post-injury and one day after the injury. Thus, Molsidomine-infused jelly blocks were kept for a maximum of two days.

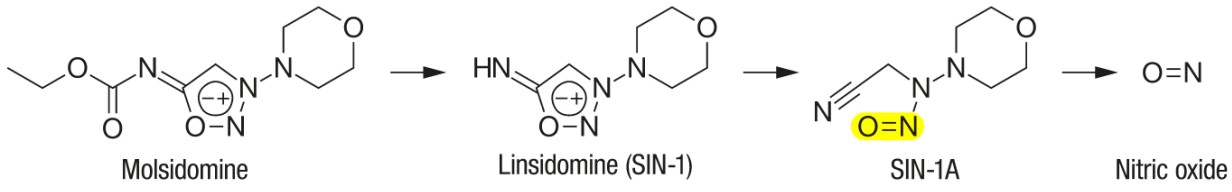

Figure 4.2 The formation of NO from Molsidomine *in vivo*. This figure shows the progressive metabolism of Molsidomine once it is ingested. The drug is broken down by the liver into SIN-1 which enters the circulation and liberates nitric oxide. Adapted from Rosenkranz *et al.*, 1996.

4.4 Injury intervention

The crush injury, or drop mass model, has been utilised extensively in our department, in order to study muscle damage (e.g. Myburgh *et al.*, 2012), and was chosen as it effectively elicits a reproducible and quantifiable injury. The model involves dropping a fixed mass onto the desired muscle group of the animal from a fixed height. In this study, a 250g, flat-bottomed, cylindrical weight was dropped onto the medial surface of the right gastrocnemius muscle of each rat, from a

height of 50cm. This procedure induces a contusion in a non-invasive manner, with no bone involvement. Figure 4.4 clearly depicts the apparatus used for this procedure.

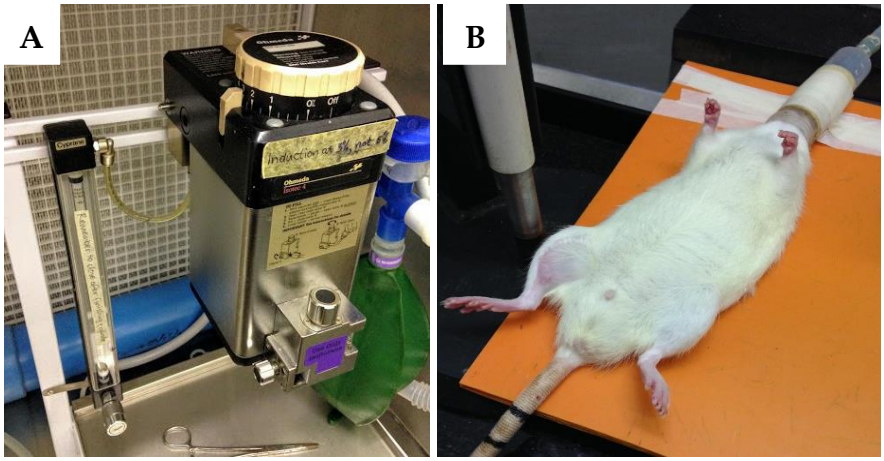

Figure 4.3 Apparatus utilised for anaesthesia. (A) Ohmeda Isotec 4 Isoflurane Vaporiser (OMED of Nevada, USA). (B) Rat under anaesthesia with gas mask. Note the shaved right leg.

Animals were anaesthetised before the injury intervention with 3% isoflurane gas in oxygen and anaesthesia was maintained throughout the injury process with a funnel fill isoflurane vaporiser (Ohmeda Isotec 4, OMED of Nevada, USA; Figure 4.3 A). Our apparatus is set up with a falcon tube at the end of the gas tube, which acts as the face-mask for the animal (Figure 4.3 B). The animal's lower leg was then shaved using a battery-powered razor (Sassoon Appliances, Model VSTR8161), in order to view the site more clearly (see Figure 4.3 B). Animals were positioned on their back, in a supine position, and their right lower leg was placed on the crush-platform. The drop-weight was then rested, lightly, on the desired injury site to ensure correct placement of the limb. We aimed to target the medial portion of the gastrocnemius muscle group. In order to standardise the procedure, a length of approximately 1.5cm above the heel was chosen as the site for delivery of injury. The animal's leg was supported in place by the researcher, using the index finger to keep the knee in place while the thumb and middle finger held the animal's foot for support (all with the left hand). Once ready, the researcher lifted the weight up by means of a string attached to the weight, and then secured the weight in place (at 50cm) with a pin (all with the right hand, maintaining the left hand's supporting position). The pin was then pulled out, allowing the weight to fall freely down the tube and onto the animal's leg, for only one repetition. Animals were then placed into individual cages until fully recovered. This exact procedure was used for each animal of the injury groups.

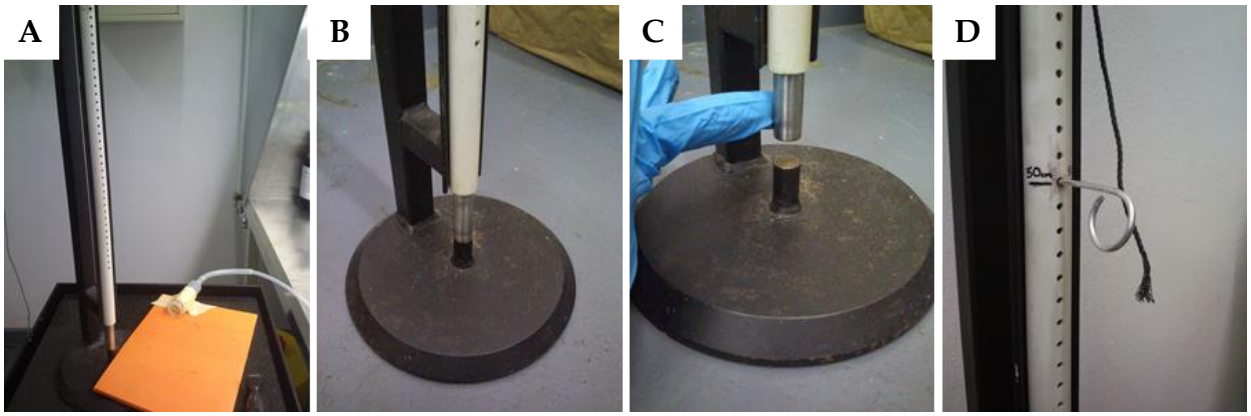

Figure 4.4 Apparatus utilised for the crush-injury intervention. Depicted above; (A) the full length of the drop tube together with anaesthesia set-up, (B) a closer view of the crush-platform and weight, (C) the 250g weight and (D) the removable trigger pin set at a 50cm height.

4.5 Sample collection

All animals were placed under general anaesthesia by inhalation of 3% isoflurane gas (ISOFOR, Safe Line Pharmaceuticals (Pty) Ltd., South Africa) in oxygen, prior to sample collection, and reactions/reflexes were tested with a toe pinch. Anaesthesia was maintained at 3% until the animal was non-responsive, which was approximately 10 minutes. Blood collection occurred first, after which the animal was humanely euthanised by removal of the heart, and the gastrocnemius muscles from both legs were then swiftly extracted. A summary of the protocol is presented in Figure 4.5.

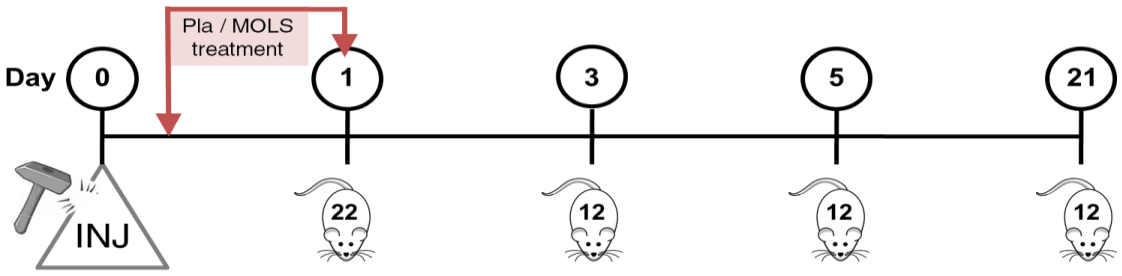

Figure 4.5 Study design for injury, treatment and sample collection. Either Placebo or Molsidomine treatment occurred immediately and 24 hours post-injury and study groups were euthanized at 1, 3, 5 and 21 days after injury. The number of animals used for each experimental time-point is indicated on each rodent illustration.

4.5.1 Muscle

The gastrocnemius muscles from both left and right legs of each animal were collected (although only muscle on right leg was exposed to contusion) and snap-frozen in a cryoprotectant for later analysis. To do this, the skin of the lower leg was removed to expose the lower-leg muscles. Using a scalpel, the fascia was removed along with the surrounding tissue, until the gastrocnemius was clearly visible. The scalpel was then placed behind the Achilles' tendon and drawn along the tibia with the blunt edge facing forward, freeing the tissue from the bone and allowing for easy extraction

of the entire muscle. The sample was then excised, cut into two pieces each of which was snap-frozen and stored at -80°C. The injury site was clearly visible in the injured animals (see Figure 4.6), and the injured site was separated into two halves to allow for more rapid freezing throughout the tissue (see Figure 4.7 for an illustration of excision details). For snap-freezing, 2-Methylbutane (or Isopentane – Cat. #: 277258, Sigma-Aldrich, USA) was placed into an aluminium can, which was rested in a polystyrene container, filled with liquid nitrogen. Once the Isopentane had just begun to freeze (determined by white solidification of Isopentane around the sides of the container), the excised section of muscle was submerged in it for approximately 20 seconds before being placed in a 5mL stand-alone reaction vial for storage at -80°C. Holes were punched in the lids of the tubes to eliminate pressure build up.

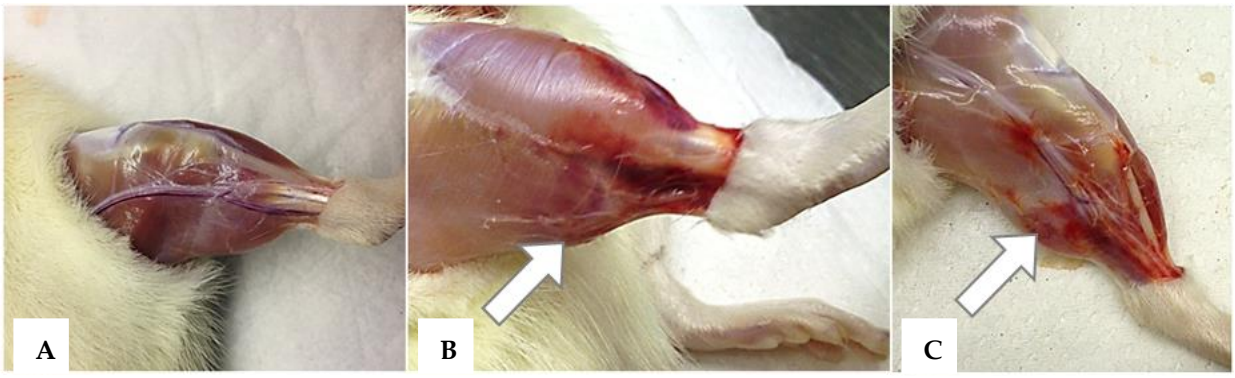

Figure 4.6 Depiction of the injury zone after injury intervention. Here we compare an uninjured animal (A) to an injured animal (lateral view in (B) & medial view in (C)) 24 hours after the crush injury. A contusion is clearly visible in the injured area in (B) & (C). The site of injury is indicated by a white arrow.

4.6 Sample analysis

4.6.1 Determination of NO metabolites in injured tissue

Tissue lysates were prepared according to Appendix III. Lysates were spun for 30 minutes at 14 000g, through a filter with a nominal molecular weight limit of 3kDa (Merck KGaA, Amicon Ultra-0.5mL Centrifugal Filter Unit, Cat. #: UFC500324, Germany), in order to remove proteins; which may otherwise give falsely elevated levels of NO metabolites, or NO.

Subsequently, levels of nitrite and nitrate were determined, as indicators of the presence of NO, using a colorimetric Nitrate/Nitrite assay kit (Cat. #: 23479, Sigma-Aldrich, USA). The kit detects total NO metabolites using the Griess reaction (Granger, 1996), which involves azo coupling between diazonium species. The absorbance of the azo compound was measured spectrophotometrically at a wavelength of 540nm using a Microplate Spectrophotometer (Powerwave HT, BioTek Instruments, Inc., VT, USA), running Gen 5 Data Analysis Software. Values

were extrapolated from nitrite/nitrate standard curves that were prepared according to the manufacturer's instructions. The detection limit of the assay was 10μM, or 0.46 ng/μL for nitrite and 0.62 ng/μL for nitrate.

4.6.2 Histological analysis of muscle morphology

4.6.2.1 Hematoxylin & Eosin staining

In order to view the overall muscle structure, we made use of an H&E stain. Hematoxylin (basic/positive) binds to and stains all DNA/RNA (acidic/negative) structures purple/blue. Eosin (acidic/negative) counterstains all proteins of the tissue (basic/positive) to shades of pink; examples of proteins include cytoplasmic filaments, intracellular membranes and extracellular fibers (Bancroft & Cook, 1994).

Cross-sectional, 10μm cryosections were prepared with a cryostat (Leica CM1860 UV, Leica Biosystems Nussloch GmbH, Germany) at -25°C. For each piece of injured muscle tissue, approximately 1cm of tissue was cut away from the distal end of the sample. This was an attempt to standardise the sectioning procedure, and it was agreed that the injury zone was most distinct at this particular area of each sample. The sample preparation procedure is depicted in Figure 4.7. About 4 to 6 muscle sections per sample were placed on an uncoated microscope slide and allowed to air-dry at room temperature for approximately one hour. Slides were either stained after the one hour of drying, or they were stored at -20℃ for later staining. In the case of the latter, slides were allowed to thaw at room temperature for 5 minutes before staining.

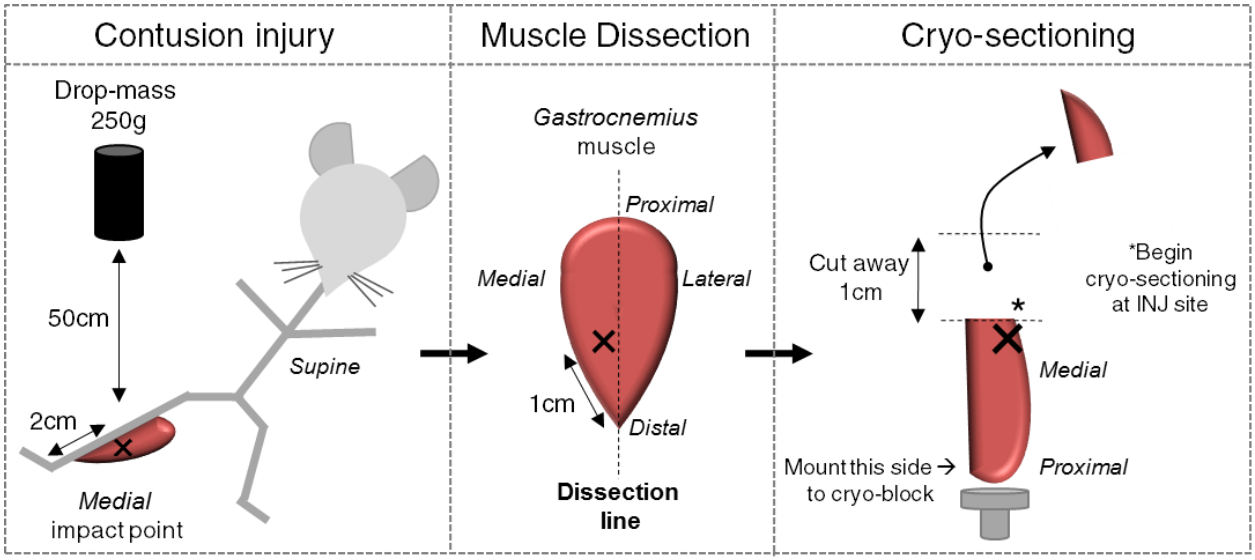

Figure 4.7 Depiction of sample handling, excision and positioning for cryosectioning. Each injured muscle sample was handled in the manner illustrated above. The injury was induced to the same area of the gastrocnemius muscle for each animal, which was approximately 1cm from the distal end of the muscle. Uninjured muscle samples were prepared in exactly the same way.

The H & E staining procedure was done using an automatic slide staining apparatus (Leica ST4020, Leica Biosystems Nussloch GmbH, Germany – see Figure 4.8). The slides were submerged in freshly prepared reagents for two minutes per reagent, in the following progression: tap water, two changes in Mayer's haematoxylin, warm tap water (26°C), Scott's tap water, tap water, Eosin (5% in 95% Ethanol), tap water, 95% Ethanol, 100% Ethanol and finally, clearance in xylene. Once stained, the slides were mounted with DPX mountant (Cat. #: 06522, Sigma-Aldrich, USA) and covered with a cover-slip for viewing. See Appendix I for a detailed protocol.

Slides were viewed in bright-field on a microscope (Nikon ECLIPSE E400), mounted with a camera (Nikon DS-Fi2), and processed through a Nikon Digital Sight DS-U3 processor (Nikon, Japan). Image processing was done on NIS elements v4.10, on a Dell desktop computer (Dell, USA) running Windows 7 (Microsoft, USA).

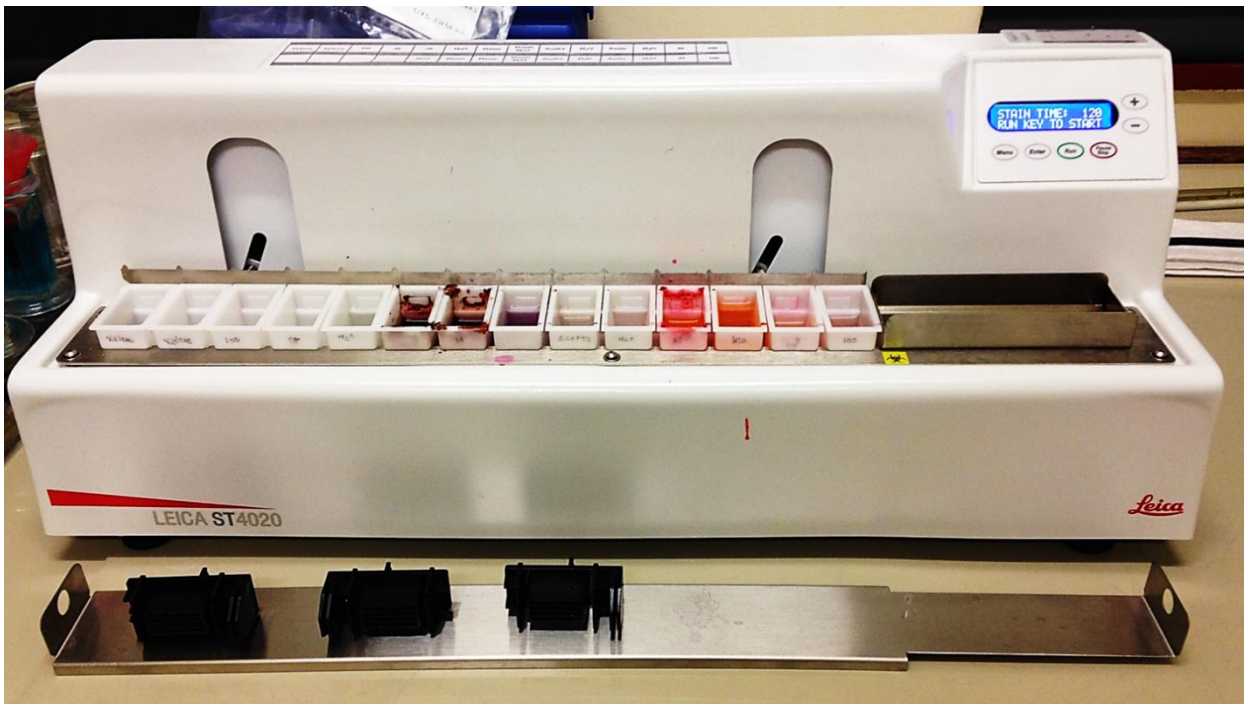

Figure 4.8 Automatic slide staining apparatus. The above apparatus (Leica ST4020, Leica Biosystems Nussloch GmbH, Germany) was utilised for H & E staining procedures.

4.6.2.2 Trichrome staining for connective tissue

The trichrome stain is a broad term used for a stain comprising three colour components, one of which is a nuclear stain. For this particular study, a Masson's trichrome stain was employed for the histological visualisation of collagenous connective tissue fibers in frozen tissue sections of rodent gastrocnemius muscle cross-sections. In brief, collagen is stained blue, muscle fibers are stained red and nuclei are stained black/purple.

For this stain, 10µm cross-sections of injured muscle were again prepared. Prior to sectioning, microscope slides (Lasec, South Africa) were coated with 0.1% (w/v) Poly-L-Lysine (Sigma-Aldrich, Cat. #: P8920, USA) in H_2O and allowed to dry. About 4 to 6 muscle sections were placed on each microscope slide and the slides immediately frozen and stored at -20°C for later analysis.

The stain was carried out according to Appendix II, and the collagen stained area was quantified.

4.6.2.3 Image analysis of Masson's trichrome stain

Stained slides were viewed in brightfield as previously described in section 4.6.2.1. Using the NIS software, images of the tissue were captured at 4x magnification and stitched together (with the stitching tool) to create an image of the entire piece of tissue (see Figure 5.6 A-C). Because rat muscle is relatively large, the muscle fibers and the blue-stained collagen were easily visible at this magnification.

Once images were acquired, they were analysed using Image J software (Schneider *et al.*, 2012). Briefly, the background was subtracted and brightened to make each colour stand out. The image was converted to a red/green/blue (RGB) stack and the analysis measurements set, namely 'area', 'area fraction', 'limit to threshold' and 'display label'. The threshold was then adjusted to optimise the red and blue filters and then measured, with the result being the area of blue colour as a percentage of the area of the entire piece of tissue. Entire muscle sections from six animals were analysed in this way for both the Molsidomine and the placebo groups, and four uninjured and untreated animals were used as controls.

4.6.3 Analysis of tissue lysate protein expression

4.6.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Tissue lysates were prepared and an SDS-PAGE carried out according to the protocol in Appendix III. Briefly, hand-cast polyacrylamide gels were run in a standard bench-top electrophoresis unit (Bio-Rad Mini-PROTEAN® Tetra Cell, USA), and consisted of a 4% stacking gel and either an 8% or a 12% separating gel, depending on the target protein. Prior to sample loading, tissue lysates were mixed with 5µL of Laemmli solution and boiled for 5 minutes at 95°C. A Precision Plus Protein™ Kaleidoscope™ marker (Bio-Rad, Cat. #: 161-0375, USA) was used obtain protein band sizes. Gels were run at 70V for approximately 3 hours. Each gel compared samples from the injured legs (right legs) of the Placebo (CX) and the Molsidomine (MX) groups, n = 6 for each group per time point. Also, present in each gel was a reference sample, which was tissue homogenised from a single animal that had not been treated or injured. Once run, the gels were prepared for Western blotting.

4.6.3.2 Western blotting

Western blot analysis was carried out according to the protocol in Appendix III. SDS-PAGE proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Life science, RPN 3032D, UK) via a transfer system (Turbo-blot, Bio-Rad, USA). Blots were transferred at 25V and 2.5A for 20 minutes, and the successful transfer was confirmed by Ponceau S. staining. Membranes were then blocked with 5% fat-free milk (Parmalat SA (Pty) Ltd., South Africa) for one hour at room temperature, and treated with primary and then horse-radish peroxidase (HRP)-linked secondary antibodies, which are presented, along with their dilutions, in Appendix IV. After secondary antibody incubation, membranes were washed 4 x 5 minutes with 1% Tris-buffered saline in Tween® 20 (TBS-T), followed by addition of enhanced chemiluminescence substrate (SuperSignal® West Femto Maximum Sensitivity Substrate, Cat. #: 34095, Thermo Scientific, USA). Membranes were subsequently imaged using the Chemidoc MP (Bio-Rad, USA) that was supported by Image lab software 4.0 (Bio-Rad,

USA). Immunoreactive proteins were normalised against the Ponceau S. staining and further normalised with the uninjured reference sample, which allowed for the comparison of protein expression of all samples, irrespective on the particular gel they were analysed on.

4.7 Statistical analyses

All data are represented as means ± standard deviation of the means (SDs). Student's t-tests were performed for comparisons between simple sets of parametric data and paired Mann-Whitney U test for non-parametric data. Additionally, two-way ANOVA was performed to compare effects of treatment and time on the two groups, with Fischer-LSD posthoc tests being performed where appropriate. (Since different rats were used for different time points, repeated-measures ANOVA was not appropriate.) All statistical tests were performed in consultation with an experienced biostatistician (Prof Martin Kidd, Stellenbosch Statistical Consultation Centre, Stellenbosch University, South Africa) using Statistica (13th edition, StatSoft, Sandton, South Africa), and all graphs were prepared using Prism 5 (GraphPad Software, Inc., USA). A p-value of less than 0.05 was accepted as statistically significant.

Chapter 5: Results

5.1 Validation of nitric oxide delivery by Molsidomine

In order to confirm that Molsidomine caused an increase in nitric oxide, nitric oxide's metabolites were measured in filtered tissue homogenates one day after injury in all four experimental groups. At this time point, all animals would have received two doses of either Placebo- or Molsidomine-infused jelly blocks.

Unfortunately, NO metabolite (nitrate and nitrite) values for all samples were below the detection limit of the kit used. Therefore, we can make no conclusion from the data and it will be omitted from the discussion.

In future, it would be important to include a positive control for this assay, such as *E.coli* (positive for nitrate reduction) and *A.baumannii* (negative), that that we may validate whether there was a problem with the assay, since at the moment, there is no literature with information on the expected normal levels of these parameters in skeletal muscle tissue.

5.2 Tissue morphology following a crush injury

Injured tissue was qualitatively evaluated at all time-points in Placebo and Molsidomine groups by H&E staining. Figures 5.1 and 5.2 depict a timeline of injury-regeneration progression from 1 day to 21 days after an acute crush injury. Time course seemed similar for both Placebo and Molsidomine treated groups when visualised at a low (4x) magnification. At a higher magnification (40x), the damaged fibers are visible at 1 day, and these are characterised by poorer uptake of the eosin dye (see black arrows in Figure 5.2 B and C). By the 3-day time point, much of the debris is cleared away, but in the Placebo group, the fibers are still undergoing necrosis. Inflammatory cells are still abundant at day 5, but it is possible to see a reorganisation of the tissue and a clear area of regeneration, with small, lightly stained new fibers. Three weeks after the injury, the injury site is still visible, with newly formed fibers arranged into bundles.

Despite the similar time course for recovery, some distinct differences were observed in the qualitative comparison of Placebo and Molsidomine groups. Firstly, the magnitude of inflammatory infiltrates (5 days) seemed to cover a smaller area in the Molsidomine treated group, with larger fibers on the border zone. Secondly, better reorganisation and regeneration at the site of injury was evident 5 days after Molsidomine treatment. Additionally, at 21 days, centrally nucleated

regenerating fibers were larger and arranged closer together in the Molsidomine treated group. These observations are in accordance with other findings presented in the following sections.

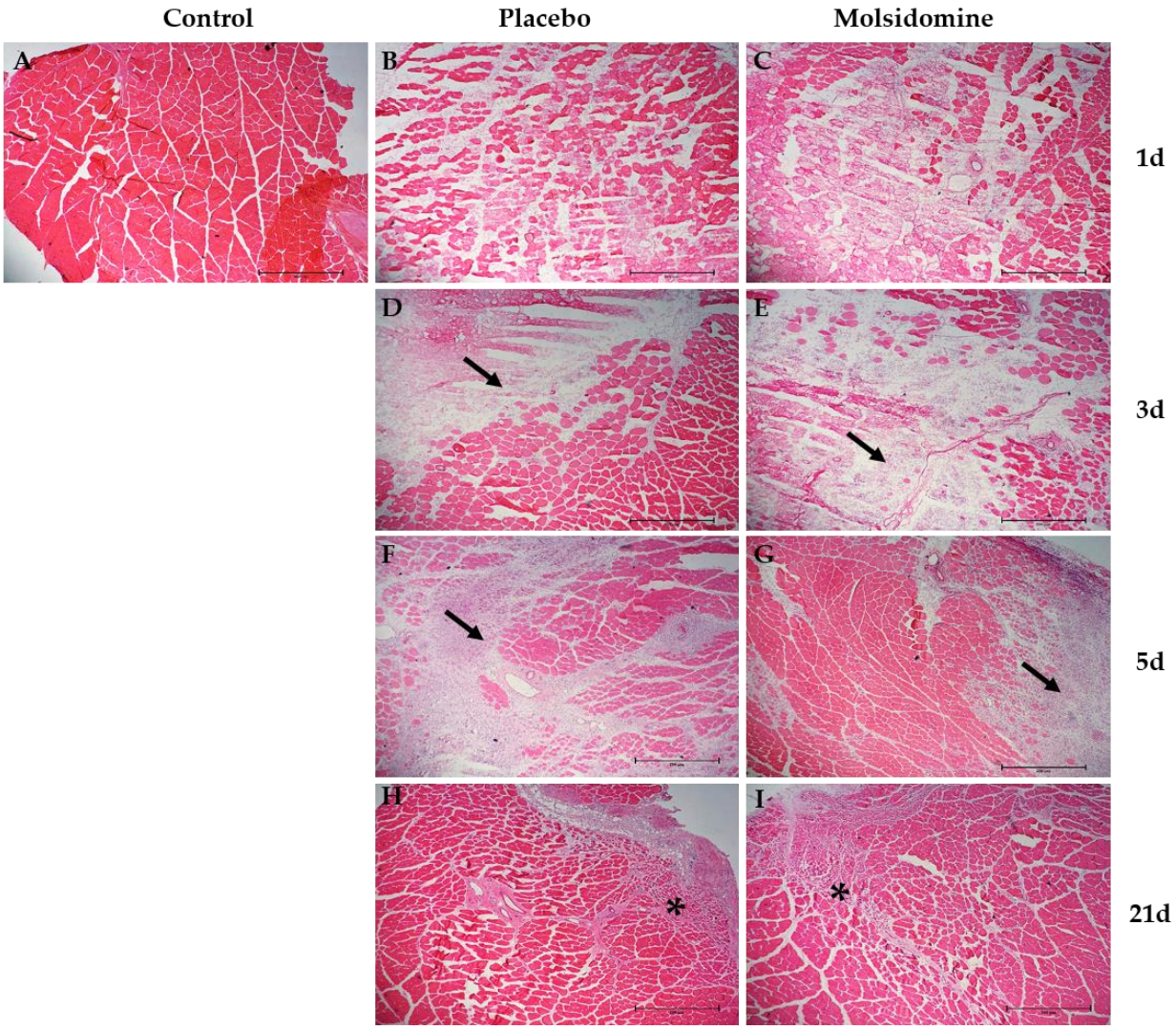

Figure 5.1 H&E stained images at 4x magnification. (A) Control uninjured sample with normal muscle architecture. (B, D, F, H) Injured and Placebo-treated muscle tissue. (C, E, G, I) Injured and Molsidomine treated muscle tissue. Black arrows indicate inflammatory infiltrate. Black asterisks indicate newly regenerated muscle tissue in the injury zone. Scale bar represents 200μm.

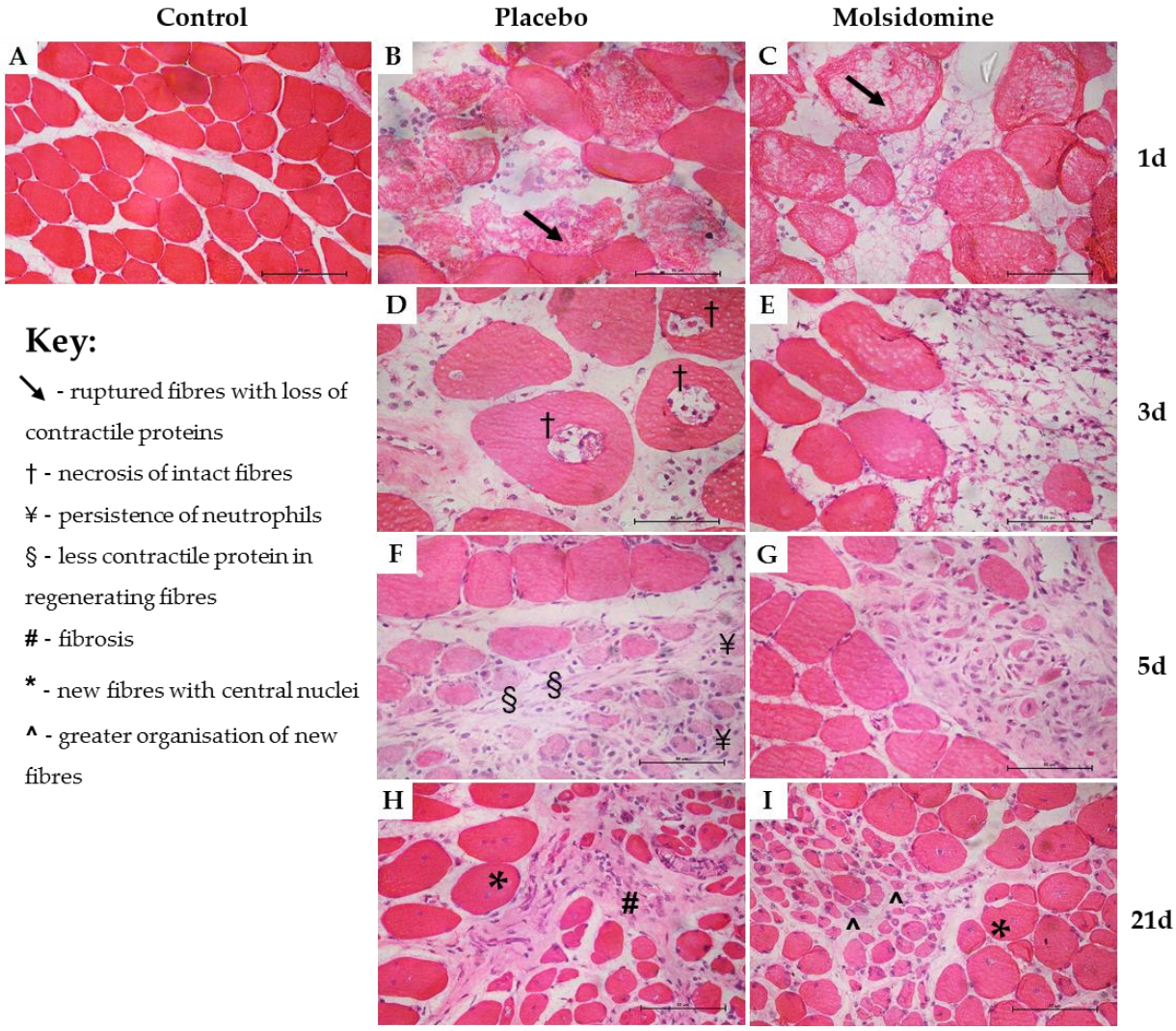

Figure 5.2 H&E stained images at 40x magnification. (A) Control uninjured sample with normal muscle architecture. (B, D, F, H) Injured and Placebo-treated muscle tissue. (C, E, G, I) Injured and Molsidomine treated muscle tissue. The figure key provides an explanation of the image annotations. Scale bar represents 50μm.

5.3 Post-injury MPO release

Western blots analysis of MPO content in gastrocnemius muscle homogenates revealed a main effect of time (ANOVA $p < 0.0001$), where MPO protein content was significantly elevated on day 5 post-injury, irrespective of treatment. Although no treatment effect was observed statistically, the peak in MPO content 5 days after injury was approximately 33% lower in Molsidomine treated animals (Figure 5.3).

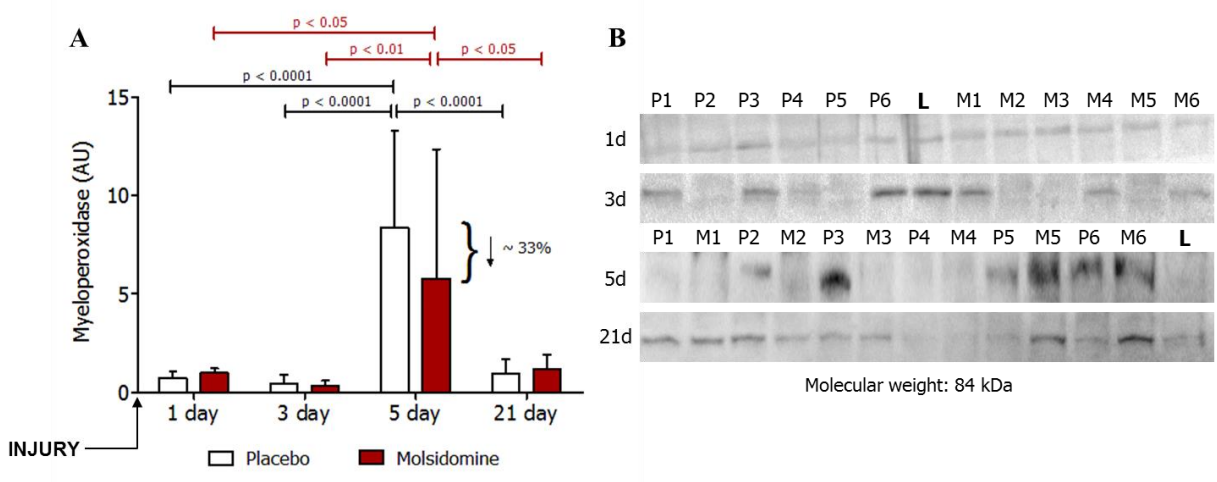

Figure 5.3 Western blot quantification of total MPO content as an indicator of inflammation in crush-injured rat gastrocnemius muscle. (A) Densitometry was performed on the blots using Bio-Rad Image Lab 4.0 software, to yield semi-quantitative results. P = Placebo, M = Molsidomine. Data expressed as mean ± SD; n=6 per group. Statistical analysis using 2-way ANOVA. Significances indicated are within group effects of time (black = Placebo, maroon = Molsidomine). (B) Representative Western blots for MPO from all four time points. Each row represents 1 Western blot. Ponceau S. staining was used as an internal loading control, after which samples were further normalised with an uninjured, untreated reference sample that was run on all gels (**L**). Numbers 1-6 represent individual samples in each treatment group. See Appendix V for representative Ponceau stains.

5.4 Myogenic regulatory factor response to injury

Western blots for MyoD and Myogenin total protein content were performed as an indication of muscle satellite cells proliferation and differentiation into myotubes, respectively.

There was a main effect of treatment (ANOVA $p = 0.05$) to lower MyoD expression. Post-hoc tests revealed the significant difference between Placebo and treatment groups was present at day 5 only (Figure 5.4). In the Placebo group, MyoD content peaked at day 5 post-injury, while in the Molsidomine group, MyoD content was not significantly different from the earlier or the later time-points.

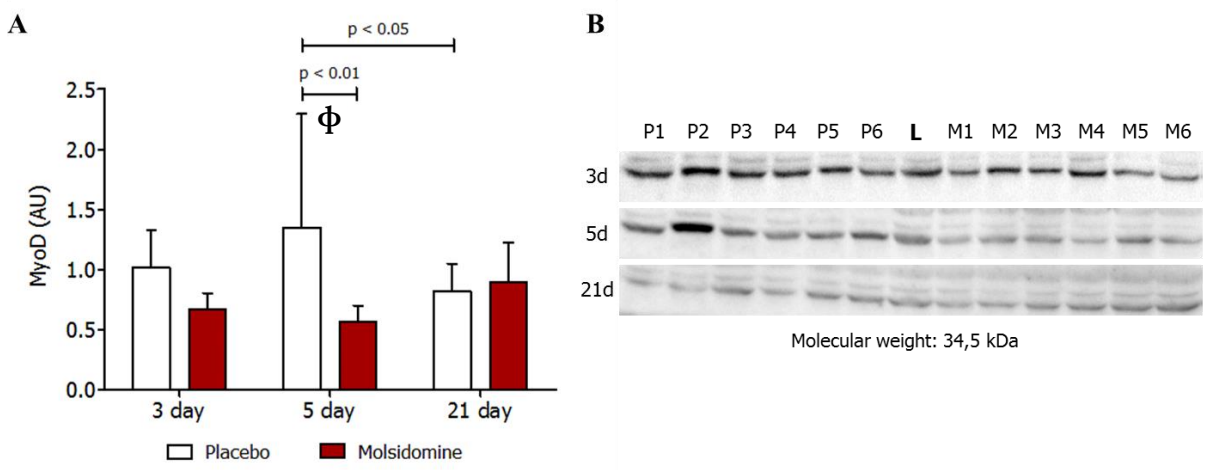

Figure 5.4 Western blot quantification of total MyoD protein content as an indicator of satellite cell proliferation in crush-injured rat gastrocnemius muscle. (A) Densitometry was performed on the blots using Bio-Rad Image Lab 4.0 software to yield semi-quantitative results. Data expressed as mean ± SD; n=6 per group. ϕ = Molsidomine treatment effect. (B) Representative Western blots for MyoD on tissue lysates from all four time points. Each row represents 1 Western blot. Ponceau S. staining was used as an internal loading control, after which samples were further normalised with an uninjured, untreated reference sample that was run on all gels (**L**). Numbers 1-6 represent individual samples in each treatment group. See Appendix V for representative Ponceau stains.

In the analysis of total myogenin protein content, a main effect of time (ANOVA $p < 0.01$) was revealed, this time, irrespective of treatment (Figure 5.5). Myogenin content was significantly lower on day 5 compared to day 3 for both groups.

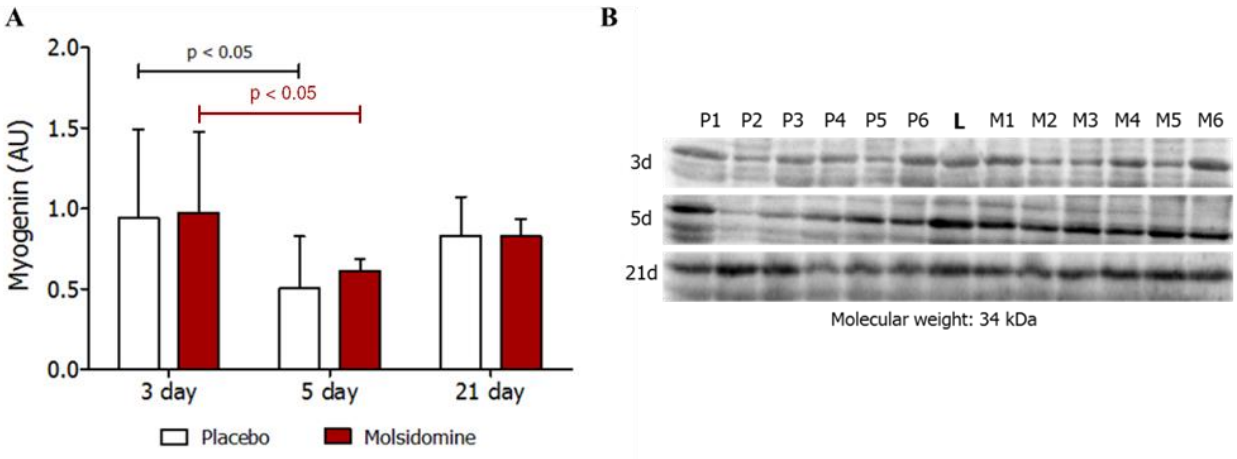

Figure 5.5 Quantification of total Myogenin protein content as an indicator of satellite cell differentiation in crush-injured rat gastrocnemius muscle. (A) Densitometry was performed on the blots using Bio-Rad Image Lab 4.0 software to yield semi-quantitative results. Data expressed as mean ± SD; n=6 per group. (B) Representative Western blots for Myogenin on tissue lysates from three time points. Each row represents 1 Western blot. Ponceau S. staining was used as an internal loading control, after which samples were further normalised with an uninjured, untreated reference sample that was run on all gels (**L**). Numbers 1-6 represent individual samples in each treatment group. See Appendix V for representative Ponceau stains.

5.5 The extent of fibrosis in regenerating muscle tissue: modulation of TGF-β signalling and fibronectin content.

In terms of the extent of fibrosis after injury, representative images of collagen deposition are presented in Figure 5.6. Non-injured muscle shows relatively small amounts of collagenous tissue (Figure 5.6 A & D). Injured tissue shows far more collagen (Figure 5.6 B, C, E & F), especially in-between newly formed fibers, which are indicated by centrally located nuclei (Figure 5.6 E & F). Molsidomine treatment seemed to not only reduce the amount of collagen, but it also seemed to have more uniformity to the collagen deposition, in that it seems to be organised around the new muscle bundles and not between the individual fibers, as seen in the Placebo group (Figure 5.6, F vs. E).

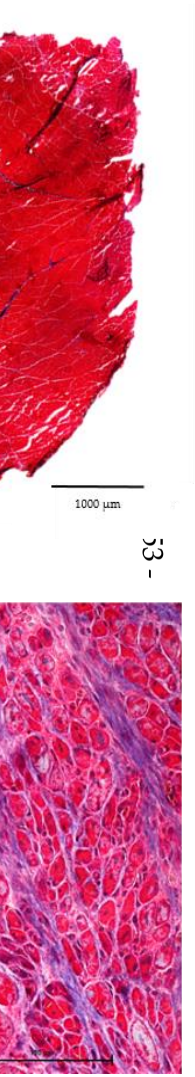

Figure 5.6 Masson's trichrome staining 21 days after crush-injury. Cross-sections of muscle samples at 4x (A, B, C) and at 20x (D, E, F). Muscle fibres are stained red/pink, nuclei are stained black and collagen is stained bright blue (trichrome method). Scale bars represent 1000 µm (A-C) or 100 µm (D-F).

To enable statistical comparison, images were not only qualitatively analysed, but also subjected to automated image analysis. Quantitative data and statistical results (expressed relative to uninjured controls) are depicted in Figure 5.7. As expected, collagen content was significantly higher after 21 days of recovery when compared to controls (an average of 437% and 295% higher for the Placebo and Molsidomine groups respectively). Mann-Whitney U tests confirmed that in Molsidomine treated animals, collagen deposition was significantly lower when compared to Placebo-treated animals, at 21 days after injury ($p < 0.05$, Figure 5.7).

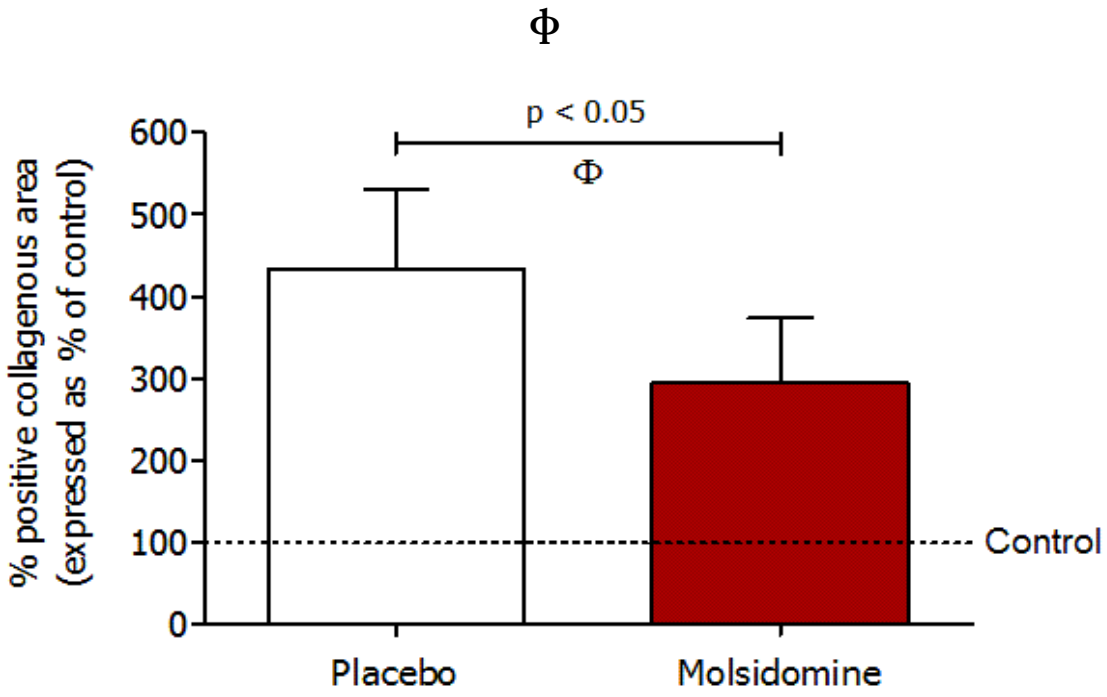

Figure 5.7 Collagen content at day 21 after injury. Images at 4x magnification were processed in ImageJ using the colour threshold function, and blue staining expressed as a percentage area of red staining. Data expressed as mean ± SD, relative to control (uninjured) values. n=6 per group. **Φ** = Molsidomine treatment effect.

TGF-β1 is involved in extracellular matrix synthesis and remodelling. There was a main effect of time (ANOVA $p < 0.005$), with total TGF-β1 protein content higher at the later time point of recovery. Additionally, a time-treatment interaction effect ($p < 0.05$), indicated that TGF-β1 increased significantly over time in the Placebo group only (Figure 5.8). Post-hoc testing confirmed the significant difference between the Placebo and treatment groups on day 21 ($p = 0.01$).

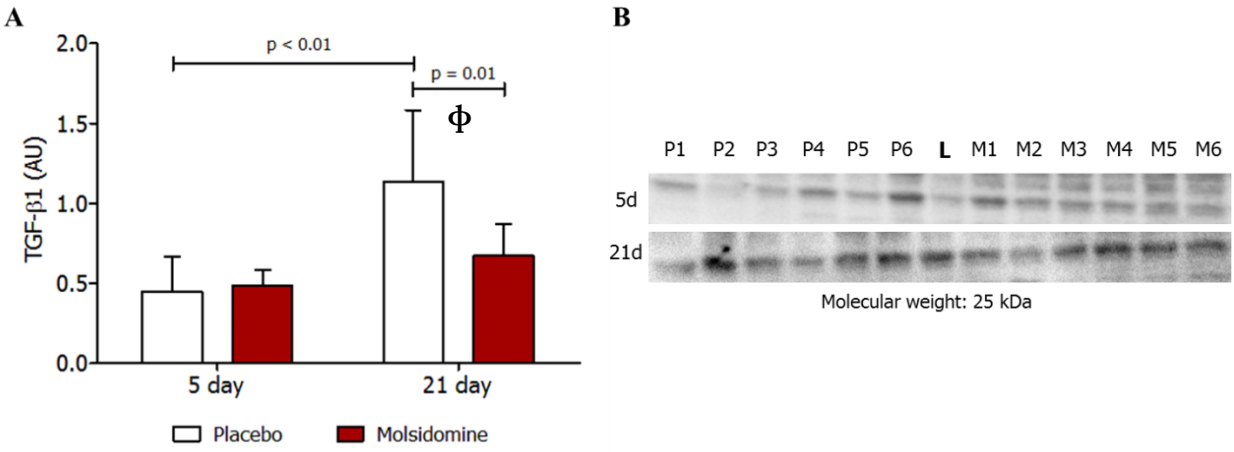

Figure 5.8 Quantification of total TGF-β1 protein content as an indicator of collagen formation in crush-injured rat gastrocnemius muscle. (A) Densitometry was performed on the blots using Bio-Rad Image Lab 4.0 software to yield semi-quantitative results. Data expressed as mean ± SD; n=6 per group. **Φ** = Molsidomine treatment effect. (B) Representative Western blots for TGF-β1 on tissue lysates from 5 and 21 day post-injury time points. Each row represents 1 Western blot. Ponceau S. staining was used as an internal loading control, after which samples were further normalised with an uninjured, untreated reference sample that was run on all gels (**L**). Numbers 1-6 represent individual samples in each treatment group. See Appendix V for representative Ponceau stains.

Western blots for fibronectin revealed that the protein had been fragmented into isoforms of various molecular weights. Statistical analysis of the 31kDa fragment yielded no effects of treatment or time (results not shown). However, analysis of the 45 and 50kDa bands (which were measured together - Figure 5.9) showed a main effect of time (ANOVA $p < 0.005$), where the protein content was much lower 21 days post-injury.

In fact, Placebo and Molsidomine groups were on average 104-fold and 274-fold lower, respectively, than their 5-day values. Mann-Whitney U tests confirmed that fibronectin content in the Molsidomine group was significantly lower than the Placebo group at the 21-day time point (indicated with a * in Figure 5.9).

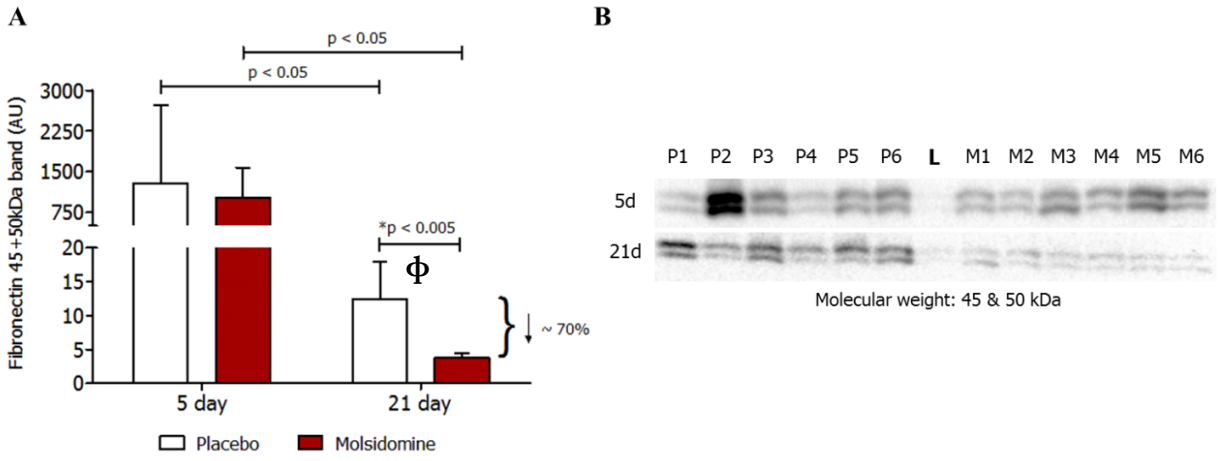

Figure 5.9 Quantification of total Fibronectin (45 & 50 kDa) protein content as an indicator of ECM formation in crush-injured rat gastrocnemius muscle. (A) Densitometry was performed on the blots using Bio-Rad Image Lab 4.0 software to yield semi-quantitative results. Data expressed as mean ± SD; n=6 per group. **Φ** = Molsidomine treatment effect. (B) Representative Western blots for Fibronectin on tissue lysates from 5 and 21 day post-injury time points. Each row represents 1 Western Blot. Ponceau S. staining was used as an internal loading control, after which samples were further normalised with an uninjured, untreated reference sample that was run on all gels (**L**). Numbers 1-6 represent individual samples in each treatment group. See Appendix V for representative Ponceau stains.

Chapter 6: Discussion

This thesis aimed to determine whether Molsidomine – a nitric oxide (NO) donor - would improve muscle regeneration after a damaging crush injury by modulating myogenesis, inflammation, and/or fibrosis. While muscle injury remains a common occurrence with debilitating symptoms (Smith *et al.*, 2008; Gharaibeh *et al.*, 2012), no globally effective therapies for regeneration have been developed. Current therapies target the related symptomatic complaints, by using the RICE principle, for example (Järvinen *et al.*, 2007; Alessandrino & Balconi, 2013). Here the results, however, show that acute Molsidomine treatment modulates both the immune cell MPO release and the fibrotic response without having a large effect on MRFs - all of which suggest a potential therapeutic role for NO in reducing the side-effects of injury during muscle recovery.

The first part of this study was to validate the delivery of NO to the injured muscle by Molsidomine. Quantification of NO is complicated by its high reactivity and short half-life, but the measurement of two products of NO metabolism, namely nitrite (NO_2) and nitrate (NO_3) is an effective indication of the presence of NO in a sample (Granger, 1996). We assayed tissue homogenates from our study groups 24 hours post-injury, approximately 1 hour after the second dose of either Placebo- or Molsidomine-infused jelly blocks. The resulting concentrations were below the lower detection limit. Further studies on the pharmacokinetics of Molsidomine and in particular its effect on NO metabolites in injured muscle are necessary.

There is a wealth of research on inflammatory parameters after a crush injury (Rubinstein *et al.*, 1998; Anderson, 2000; Darmani *et al.*, 2004 a & b; Sun *et al.*, 2010; Filippin *et al.*, 2011 a & b), as well as in other types of injuries such as muscle strains (Tomazoni *et al.*, 2012; Ramos *et al.*, 2012; de Paiva Carvalho *et al.*, 2013). In line with other studies using a crush injury model (Filippin *et al.*, 2011 a & b; Myburgh *et al.*, 2012), the injured tissue was characterised by a vast phagocytic cell infiltration into the injury site on 1, 3 and 5 days post-injury. By 5 days there was far less debris in the injured area indicating relatively rapid removal. Increased amounts of MPO in the tissue assist in the clearing of damaged tissue to prepare the site for regeneration (Smith *et al.*, 2008). It, therefore, plays an important role.

In human subjects, MPO is synthesised and released within the first hour after exercise-induced injury by both neutrophils and monocytes in circulation, indicating activation of these cells (van de Vyver *et al.*, 2015). Even after exhaustive exercise with no muscle damage, there is an acute response:

MPO activity is increased by about 40% in skeletal muscle (Belcastro *et al.*, 1996). Here, the injury resulted in significantly elevated muscle MPO content even 5 days after (Figure 5.3). However, an undesirable side-effect of excessive MPO activity is the associated secondary damage to the muscle that may occur in the days following the initial injury (Lapointe *et al.*, 2002; Tidball, 2008). Null mutation of MPO in mice resulted in a reduction of the neutrophil-mediated lysis of muscle cells *in vitro* and in an *in vitro* muscle/neutrophil co-culture (Nguyen *et al.*, 2005). Our study is, to our knowledge, the first to evaluate the effects of Molsidomine on skeletal muscle regenerative processes in an experimental crush injury. Nonetheless, the drug has been utilised in other models of muscle disruption.

In a rat model of hindlimb ischemia-reperfusion injury, Molsidomine reduced neutrophil infiltration upon reperfusion and decreased the number of necrotic fibers (Öztürk *et al.*, 2009). In a large pig study, L-arginine treatment – an NO precursor – significantly reduced the extent of necrosis, neutrophil accumulation and MPO activity due to ischemia-reperfusion injury in myocutaneous flaps (Cordeiro *et al.*, 1998). A study with the same model then found SIN-1 treatment to improve myocutaneous flap survival and to reduce the presence of MPO (Khiabani & Kerrigan, 2002). In the current crush injury model, Molsidomine slightly reduced the presence of MPO 5 days after injury by approximately 33% when compared to the Placebo group. In the ischemia-reperfusion studies just mentioned, exogenous NO also reduced neutrophil numbers in the injured limb, albeit at an earlier stage of injury. Taken together, the evidence suggests that Molsidomine treatment may reduce secondary damage associated with neutrophil invasion, although future studies quantifying the presence of neutrophils must still be done in the crush injury model.

There are a few studies that make use of the drug as a long-term therapy for skeletal muscle pathologies, and with promising results. In α-sarcoglycan null mice – a severe model of muscular dystrophy – Molsidomine treatment for 1 month amplified macrophage recruitment and the effective clearance of debris. After 4 months of treatment, it reduced the inflammatory condition, fiber swelling and the number of phagocytic leukocytes. It was associated with reduced fibrosis and preservation of muscle tissue (Zordan *et al.*, 2013). This illustrates a unique ability of Molsidomine to finely modulate the inflammatory response in dystrophic mice, which is comparable to its effects on MPO in our study, although there are obvious differences in model and timeline.

There is far less research focused on fibrosis following an impact injury, especially research into potential anti-fibrotic therapies. For these reasons, fibrotic parameters were robustly assessed.

Nonetheless, there may be cross-talk between myogenesis and fibrosis. Therefore, myogenesis was also examined by Western blots for MyoD and myogenin protein content, two prominent myogenic markers.

The main finding of our study was that Molsidomine significantly blunted the evidence of fibrotic scarring in the injured tissue 21 days after injury, despite administration of only two doses of the drug at 1 hour and 24 hours post-injury. In similar models of crush injury, fibrosis was generally evident 1, 2 and 4 weeks post-injury (Filippin *et al.*, 2011 a & b). These researchers have shown, using L-NAME, that the inhibition of NO early on (2 hours) after crush injury significantly increased the formation of fibrotic tissue at day 7 (Filippin *et al.*, 2011 a & b), which motivated the notion that endogenous NO is instrumental in the balance between scar formation and muscle repair. Our finding of a smaller amount of fibrosis complements and adds to their findings by showing the potential of additional, exogenous NO as a treatment. Even without quantification, reduced connective tissue is clearly evident in the histological sections (Fig 5.2, 5.3 & 5.7).

The current histological finding was supported by a lower TGF-β1 and fibronectin protein content ($p = 0.01$ and $p < 0.005$, respectively) in muscle homogenates after Molsidomine treatment, compared to Placebo groups at the same time point i.e. 3 weeks post-injury. In a rat model of a rotator cuff tear, TGF- β signalling was upregulated at 2 and even at 6 weeks post-injury, with substantial collagen deposition in the injured area (Liu *et al.*, 2014). In our model of a relatively severe injury, there is persisting fibrotic activity at the 21-day time point in the Placebo-treated rats, which suggests the muscle is still undergoing a wound healing response and effective remodelling is delayed.

From the literature, it is known that NO reduces TGF-β bioactivity, including suppression of mRNA in models where activity is elevated. This resulted in decreased collagen synthesis in mesangial cells cultured in diabetic conditions (Craven *et al.*, 1997). These findings were similar with both endogenously and exogenously generated NO. Here, the range of values for TGF-β1 content was small at 5 days post-injury and not different between the Placebo and Molsidomine groups. It is important to note that the synthesis of connective tissue is part of the initial response to an injury in order to provide structural stability to the area. This may explain why there were no significant differences at this time. In another study, also in cultured mesangial cells, NO was found to down-regulate connective tissue growth factor (CTGF), through decreased gene and protein expression (Keil *et al.*, 2002). CTGF has been implicated as a downstream mediator of profibrotic TGF-β signalling and is, therefore, an important role-player in the development of various forms of fibrosis

(Gupta *et al.*, 2000). Clearly, the literature is in support of the beneficial effect of an inhibition of TGF-β expression, but an explanation for a delayed response (at 21 days) is not clear. It has been suggested that these two growth factors (CTGF & TGF-β) play a combined role in the regulation of skeletal muscle matrix turnover (Heinemeier *et al.*, 2013), which would be a more long-lasting process, possibly with the involvement of fibroblasts. *In vitro*, SIN-1 (the active metabolite of Molsidomine) prevented fibroblast migration on fibronectin-coated plates (Sato *et al.*, 2001). This supports the hypothesis that Molsidomine not only reduced fibronectin protein content 3 weeks post-injury (Figure 5.9) but may have also prevented fibroblast migration to the injury site and excessive connective tissue synthesis by these cells.

MyoD is expressed by activated satellite cells that are proliferating (Ceafalan *et al.*, 2014). Thus, more MyoD expression should be reflective of a better regenerative capacity. Rodent muscle cryo-lesions that were treated with low-level laser therapy (LLLT), showed significantly increased levels of MyoD and myogenin mRNA 4 days post-injury (Assis *et al.*, 2013), indicating greater myogenesis. An unexpected result from our study was that Molsidomine treatment blunted the peak in satellite cell proliferation 5 days after injury (Figure 5.4). However, it is possible that Molsidomine caused either an earlier or a later peak in MyoD expression, but this cannot be confirmed with our experimental timeline. When our MPO results are considered (Figure 5.3), the reduced magnitude of immune-cell mediated inflammation associated with Molsidomine treatment may have indirectly reduced satellite cell proliferation and MyoD expression. It was mentioned earlier that Molsidomine may have reduced the extent of the secondary damage. However, myogenin is a marker for terminal differentiation of satellite cells (Ceafalan *et al.*, 2014) and our results show that Molsidomine did not influence the normal myogenin expression profile. This implies that the myogenic response was not hindered in any way by Molsidomine treatment, but rather that it continued in an appropriate manner. MyoD and myogenin are transcription factors, that are expressed only in satellite cell nuclei. Immunohistochemical assessment of MyoD+ and myogenin+ nuclei would be useful for better interpretation.

Although satellite cells are adult progenitors dedicated to the skeletal muscle niche, under extreme disturbance of the niche they can transdifferentiate into myofibroblasts, or fibroadipogenic progenitors (FAPs). C2C12 cells cultured for 8 hours with TGF-β1 decreased their expression of both MyoD and myogenin and differentiated into profibrotic myofibroblastic cells (Li *et al.*, 2004). Our result of less fibrosis with Molsidomine treatment may thus, at least in part, be the result of a down-

regulated TGF-β-dependent switch to a fibroblastic cell type. In *mdx* mice – another model of muscular dystrophy - Molsidomine improved fibrotic parameters by reducing FAP numbers and subsequently reducing adipose tissue deposition, as well as reducing collagen expression (Cordani *et al.*, 2014). Importantly, this study recognised a mechanism for Molsidomine's action, where it down-regulated peroxisome proliferator-activated receptors gamma (*Pparγ1*) through induction of miR-27b, which reduced adipogenesis in dystrophic muscle. The latter finding is valuable, as it may be likely that NO acts through a similar pathway to modulate fibrotic signalling. By integrating our results with the known literature, specifically the *in vitro* studies, it is clear that NO elicits effects on all of our measured parameters – namely myogenesis, inflammation, and fibrosis – individually rather that the first effect having a knock-on effect on the subsequent processes. We have summarised the identified targets of Molsidomine in Figure 6.1.

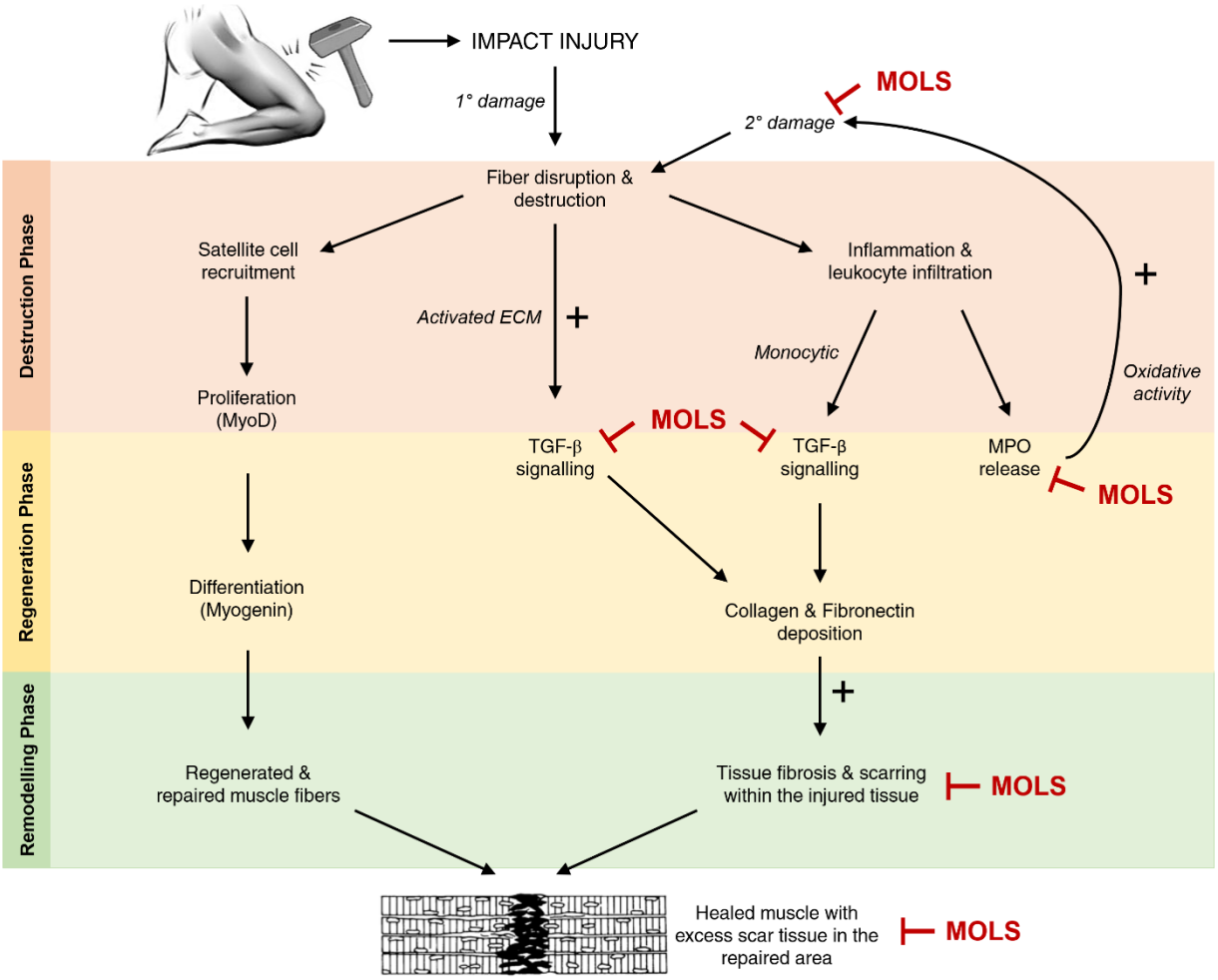

Figure 6.1 Summary of Molsidomine's effects after an acute impact injury. This diagram depicts the normal progression of skeletal muscle healing after an impact injury in a way that is relevant to the results of this thesis. The proposed

actions of Molsidomine (MOLS) are shown in red text, and indicate the potential targets of the drug as were elucidated by our study.

In various other models, SIN-1 has been used as a therapeutic agent based on its ability to improve blood flow. Systemic delivery of SIN-1 reduced chronic pain in a rat model of post-ischemic, neuropathic pain. The treatment effect was greater at 2 days post-ischemia than at 7 days (Xanthos & Coderre, 2008). In a similar model, even topically applied NO-donors increased microvascular perfusion to the hind-paw, as measured using the Doppler technique (Laferrière *et al.*, 2014), and in an earlier study, 6-hour preincubation with SIN-1 protected endothelial cells against cellular injury by TNF-α and increased their viability (Polte *et al.*, 1997). This could imply that SIN-1 might protect the capillaries during the destruction phase of injury, resulting in more perfusion to the damaged area and ultimately, better repair. It has also been shown that NO prevents apoptosis in some cells through the inactivation of active caspases and protects the mitochondrial membrane against depolarization (Zech *et al.*, 2003). It seems then, that future studies should investigate further the protective effects that Molsidomine might elicit on the microcirculation after an injury.

In conclusion, we report here for the first time that NO reduces the fibrotic profile after an impact injury to skeletal muscle, whilst also modulating the inflammatory response and maintaining a proper myogenic response in treated rats. The mechanism by which NO reduced fibrosis was through a reduction of TGF-β1 signalling and a subsequent decrease in collagen deposition, as well as reduced fibronectin levels, 3 weeks after injury. Moreover, we have identified a novel role for the NO-donor, Molsidomine, which has previously been reported to benefit dystrophic muscles.

Application

Our study indicates that Molsidomine may be suitable for the acute treatment of impact injuries in otherwise healthy muscle. Molsidomine has a good safety and tolerability profile in humans (Messin *et al.*, 2006) and therefore, clinical trials of Molsidomine in the context of acutely injured humans subjects may be possible in the near future. In a review of sports-related injuries in humans, 21 days post-injury was described as the time point at which either effective remodelling or fibrosis is underway (Jarvinen *et al.*, 2005) and the current study provides evidence within that time frame. Furthermore, muscle TGF-β1 and fibronectin mRNA were upregulated as early as 6 hours after a moderate eccentric kicking-exercise protocol in humans (Heinemeier *et al.*, 2013), suggesting that even in human subjects, early treatment is warranted.

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Appendices

Appendix I: HEMATOXYLIN & EOSIN STAINING

H & E staining reagents

Mayer's hematoxylin: Commercially available Mayer's Hematoxylin (Sigma-Aldrich, Cat. #: MHS80, USA) was filtered through Whatman's number 2 filter paper before each stain and placed in two Coplin jars in the auto-staining apparatus. The stock was kept at room temperature.

Eosin: Commercially available Alcoholic Eosin (Leica Biosystems, Cat. #: 3801600E, UK) was further diluted in 95% Ethanol and placed in a Coplin jar in the auto-staining apparatus. The stock was kept at room temperature.

Scott's tap water (blueing agent): 1% Magnesium Sulfate and 0.07% Sodium Bicarbonate were dissolved in 900mL of tap water, the solution was titrated to a pH of 8, using HCl, and made up to 1000mL. The fresh working solution was then placed in a Coplin jar (fresh every time) in the auto-staining apparatus. The stock was kept at room temperature.

Xylene (clearing agent): Commercially available Xylene (Merck, Cat. #: 1086619190, South Africa) was kept at room temperature in a covered container and used as required.

Ethanol: 100% Ethanol and 95% Ethanol in dH_2O were kept at room temperature and used for dehydration steps in Coplin jars in the auto-staining apparatus.

H & E staining protocol

1. Remove frozen tissue sections from the freezer and allow to thaw at room temperature for 5 minutes.
2. Place slides into the auto-staining apparatus and set the timer to 120 seconds per reagent/jar.
3. Staining proceeds automatically as follows:
    a) Tap water
    b) Two changes of Mayer's hematoxylin
    c) Warm tap water (26°C)
    d) Scott's tap water
    e) Tap water
    f) 5% Eosin in 95% Ethanol
    g) Tap water
    h) 95% Ethanol
    i) 100% Ethanol
    j) Final clearance in Xylene (leave in container for 5 minutes)
    k) Mount in a resinous mounting medium

Appendix II: MASSON'S TRICHROME STAINING

Masson's staining reagents

Bouin's solution (fixation agent): 75mL picric acid (saturated), 25mL Formaldehyde (37-40%) and 5mL Glacial acetic acid were mixed well to make a working solution, which was stored in a fume hood at room temperature.

Weigert's iron hematoxylin solution: Stock Solution A consisted of 1g Weigert's hematoxylin in 100mL of 95% Ethanol. Stock Solution B consisted of 4mL of 29% Ferric chloride in dH_2O, 95mL dH_2O and 1mL of concentrated Hydrochloric acid. Equal parts of Stock A and Stock B were mixed together for the working hematoxylin solution, which was stored at room temperature for a maximum of 3 months.

Biebrich scarlet-acid fuchsin solution: 90mL of 1% aqueous Biebrich scarlet was mixed with 10mL of 1% aqueous Acid fuchsin and 1mL glacial Acetic acid. The solution was stored at room temperature.

Phosphomolybdic-Phosphotungstic acid solution: 25mL of 5% Phosphomolybdic acid was mixed with 25mL of 5% Phosphotungstic acid and the stock stored at room temperature.

***Aniline blue solution*:** 2.5g of Aniline blue powder was mixed with 2mL of glacial acetic acid and 100mL of dH_2O. The solution was stored at room temperature.

1% Acetic acid solution: 1mL of glacial acetic acid was mixed in 99mL of dH_2O and stored at room temperature.

Xylene (clearing agent): Commercially available Xylene (Merck, Cat. #: 1086619190, South Africa) was kept at room temperature in a covered container and used as required.

Masson's staining protocol

1. Fix tissue in Bouin's solution for 1 hour at 56°C to improve staining quality.
2. Rinse running tap water for 5-10 minutes to remove the yellow colour.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
4. Rinse in running warm (26°C) tap water for 10 minutes.
5. Wash in dH_2O for 1 minute.
6. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes.
7. Wash in dH_2O for 1 minute.
8. Differentiate in Phosphomolybdic-Phosphotungstic acid solution for 10-15 minutes or until collagen is not red.
9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes.
10. Rinse briefly in dH_2O and differentiate in 1% acetic acid solution for 2-5 minutes.
11. Wash in dH_2O for 1 minute.
12. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
13. Mount with resinous mounting medium.

Appendix III: TISSUE LYSATE PREPARATION AND SDS-PAGE

Tissue Lysate Preparation

Approximately 40mg (Western blotting) or 120mg (Nitrite/Nitrate assay) of injured muscle tissue was sectioned and collected using a cryostat (Leica CM1860 UV, Leica Biosystems Nussloch GmbH, Germany) at -25℃. Samples were kept in 2.0mL reaction vials (Eppendorf, Germany) and 550µL of Lysis Buffer (Western blotting) or 1.1mL of 1% phosphate-buffered saline, or PBS, in dH_2O (Nitrite/Nitrate assay) was added to each tube. Samples were then homogenised on ice using a handheld homogenizer (Polytron PT2100, Kinematica AG, Switzerland) at 30 000rpm. After each sample had been homogenised, the homogenizer was rinsed twice in dH_2O and once in 1% PBS in dH_2O in order to remove any residual tissue from the blades, and any excess droplets were shaken off into a glass beaker. Homogenised samples were then centrifuged at 10 000g for three minutes (Spectrafuge 24D, Labnet Int., Inc., USA), after which the supernatant was removed and placed on ice for analysis or stored at -80°C until later analysis.

Measurement of Sample Protein Concentration

For Western blotting, 30µg of protein per sample was loaded into the wells, thus all samples had to be diluted so that equal amounts of protein were loaded. A protein standard calibration curve was generated using bovine serum albumin (BSA) (Bovine Serum Albumin Fraction V, Roche, USA) in lysis buffer, ranging from 0mg/mL to 6mg/mL. For the Nitrite/Nitrate assay, samples were all equalised and diluted down to the sample with the lowest protein concentration and a protein standard calibration curve was generated using Bovine serum albumin (BSA) (Bovine Serum Albumin Fraction V, Roche, USA) in 1% PBS in dH_2O, also ranging from 0mg/mL to 6mg/mL.

For both protocols, protein concentrations were measured using a commercial Bicinchoninic acid (BCA) kit (Pierce ® BCA protein assay, Thermo Fischer Scientific, USA). The assay was performed on a 96-well plate (PS microplate, Greiner Bio-One, USA) with 200µL BCA added to 10µL of the sample or standards. All samples and standards were loaded in duplicate. The absorbance of BCA was measured at 595nm in a plate reader (EL-500 Universal Microplate reader, Bio-Tek Instruments Inc, USA). Concentrations of the samples were calculated by KC Junior software, using the standard calibration curve on a Dell desktop computer (Dell, USA).

SDS-PAGE and Western Blot Reagents

RIPA buffer: 50mM Tris HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% vol/vol nonidet p40, 0.25% weight/vol Sodium deoxycholate in dH_2O. Kept at 4°C.

Lysis buffer: RIPA buffer, supplemented with 0.01mg/mL phosphatase inhibitor (Roche, Cat. #: 04 906 837001), 0.02mg/mL protease inhibitor (Roche, Cat. #: 04 693 116001), 100ng/mL pepstatin (Sigma-Aldrich, Cat. #: P5318, USA). Used immediately or stored at -20°C.

Gels: Gels were hand-cast immediately before running the SDS-PAGE. The following reagent volumes were sufficient for the casting of two gels.

Table I SDS-PAGE gel preparation. Below, the reagent volumes used for stacking and separating gels.

Reagents	*Stacking Gel (4%)*	*Separating Gel (8%)*	*Separating Gel (12%)*
dH_2O	3mL	6.5mL	8mL
Tris (pH 6.8)	1.25mL	-	-
Tris (pH 8.8)	-	3.75mL	3.75mL
40% Acrylamide	670µL	4.5mL	3mL
10% SDS	25µL	150µL	150µL
10% APS	25µL	85µL	85µL
TEMED	5µL	12µL	12µL

Running Buffer (1%): 0.025M Tris, 0.192M Glycine and 0.1% SDS (w/v) were mixed into 1000mL of dH_2O and stored at room temperature.

Transfer Buffer (1%): 0.025M Tris and 0.192M Glycine were dissolved in dH_2O and 20% methanol added to make a 1% working solution before use. Kept at room temperature.

Tris-buffered saline in Tween (TBS-T: 1%): 50mM Tris, 150mM NaCl and 0,1% Tween-20® were mixed into 900mL of dH_2O and titrated to a pH of 8.3 using HCl. It was made up to 1000mL and stored at room temperature.

Ponceau S: 0.1g of Ponceau S. (Sigma-Aldrich, Cat. #: P3504, USA) was dissolved in 1mL of 100% (v/v) acetic acid and made up to 100mL with distilled water. The Ponceau S. stain was stored in a glass bottle at 4°C.

Ammonium Persulfate: APS (Sigma-Aldrich, Cat. #: A3678, USA) was made up to 10% (w/v) in dH_2O and stored at -20°C.

Stripping buffer: 0.2M Glycine, 0.1% SDS, 1% Tween-20® were mixed into 800mL of distilled water and titrated to a pH of 2.2 using HCl, then made up to 1000mL and stored at room temperature.

Membrane Stripping Protocol

Some membranes were re-probed for proteins at different molecular weights. Membranes were washed for 2 x 10 minute changes in 1% TBS-T and then incubated twice for 15 minutes in stripping buffer, with gentle shaking. Membranes were then vigorously rinsed in two changes of 1% TBS-T, followed by a 15 minute was with shaking. Membranes were then blocked in 5% fat-free milk (Parmalat SA (Pty) Ltd., South Africa) for one hour at room temperature and then treated with the appropriate primary and HRP-linked secondary antibodies.

Appendix IV: PRIMARY AND SECONDARY ANTIBODIES FOR WESTERN BLOTTING

Table II List of primary and secondary Western blot antibodies as well as the respective dilutions.

Primary Antibody	*Secondary Antibody*
Rabbit polyclonal anti-MyoD1 (Sigma-Aldrich, Cat. # SAB1410813, USA) 1:1000	Anti-rabbit HRP-linked secondary Ab (Cell Signaling, Cat. # 7074S) 1:15 000 in 5% fat-free milk in TBS-T
Rabbit polyclonal anti-Myogenin (Sigma-Aldrich, Cat. # SAB1305721, USA) 1:1000	Same as above
Rabbit polyclonal anti-Myeloperoxidase (Abcam, Cat. # ab45977, UK) 1:1000	Same as above
Rabbit polyclonal anti-TGFβ1 (Santa Cruz, Cat. # sc-146, USA) 1:1000	Same as above
Mouse monoclonal anti-Fibronectin (Santa Cruz, Cat. # sc-80982, USA) 1:1000	Anti-mouse HRP-linked secondary Ab (Cell Signaling, Cat. # 7076S) 1:20 000 in 5% fat-free milk in TBS-T

Appendix V: PONCEAU IMAGES FOR EACH SDS-PAGE

Myeloperoxidase Gels

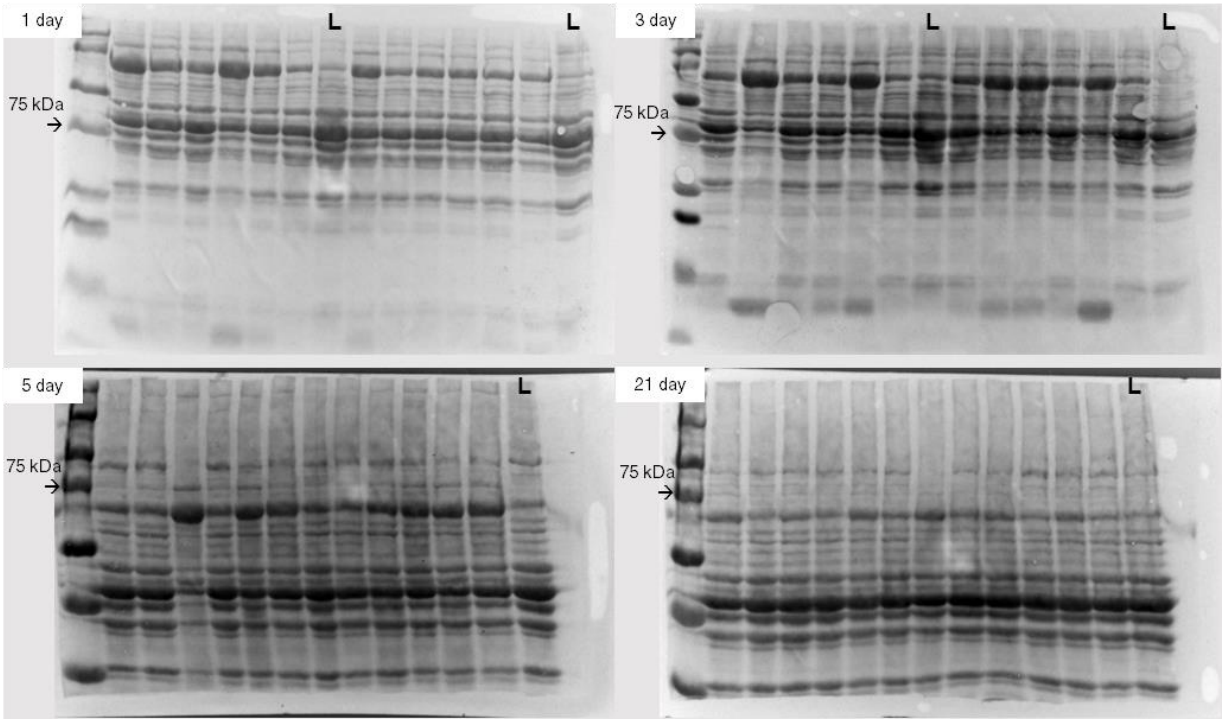

Figure I Ponceau stained gels prior to Western blotting for MPO. Gels used for MPO Western blots at 1, 3, 5 and 21-day time points. **L** – indicates the lane containing the loading control reference sample, which was run on every single gel. The protein ladder is in the left-most lane, where the 75 kDa protein standard is indicated.

MyoD Gels

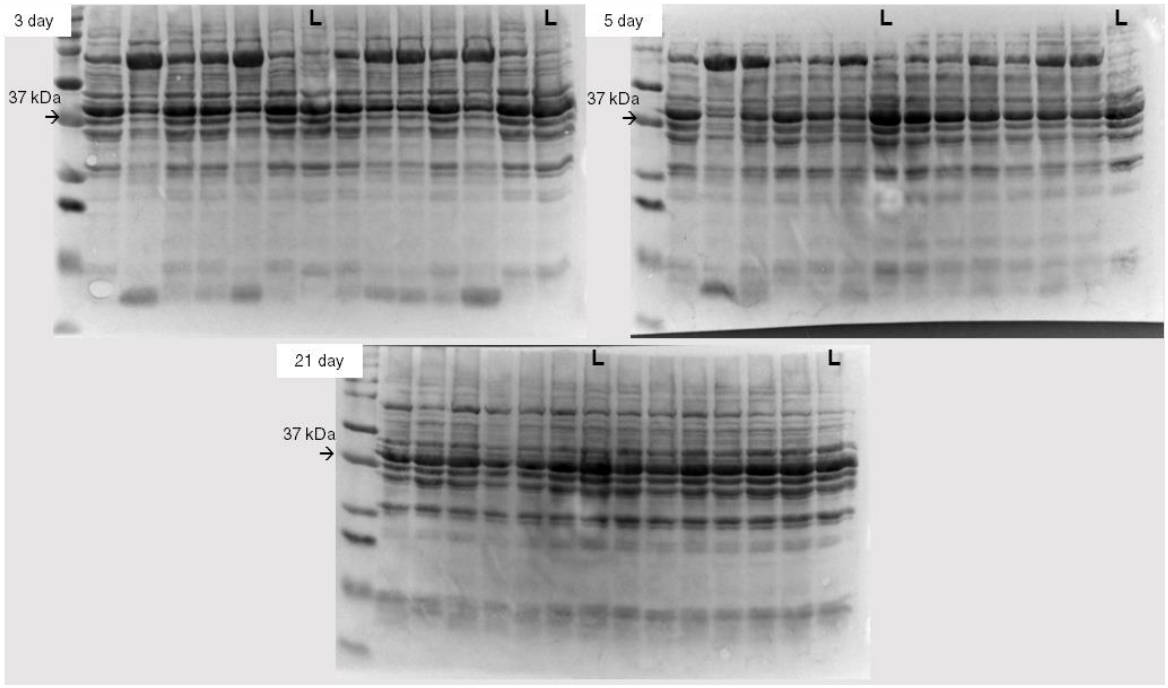

Figure II Ponceau stained gels prior to Western blotting for MyoD. Gels used for MyoD Western blots at 3, 5 and 21-day time points. **L** – indicates the lane containing the loading control reference sample, which was run on every single gel. The protein ladder is in the left-most lane, where the 37 kDa protein standard is indicated**.**

Myogenin Gels

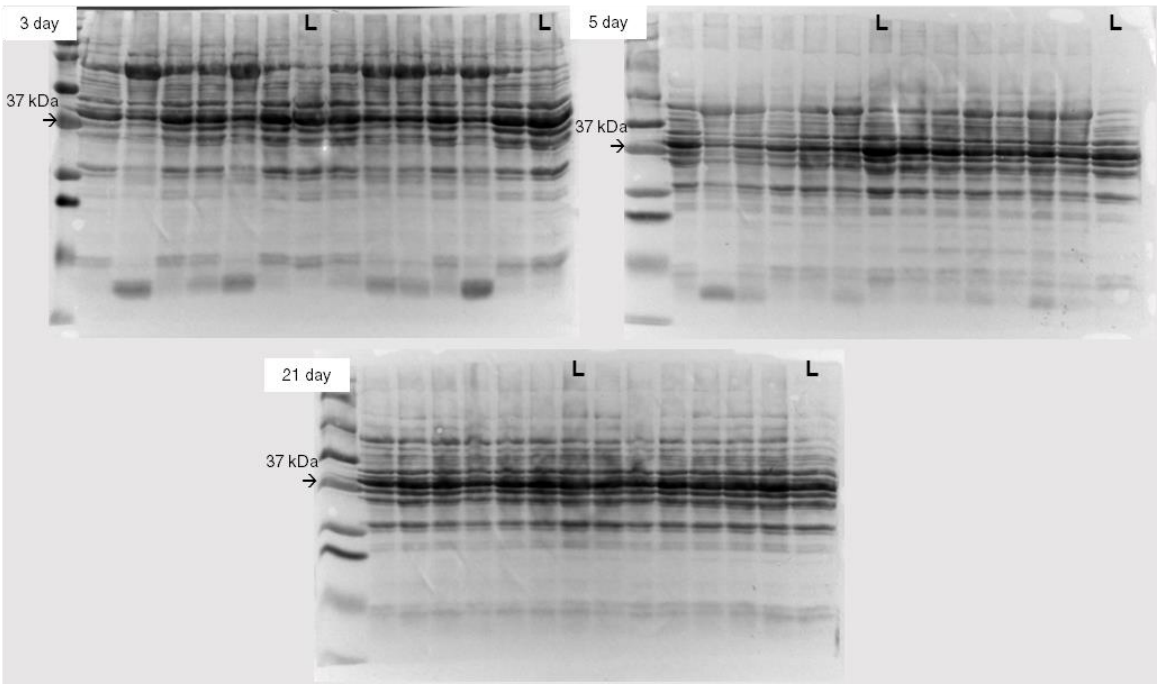

Figure III Ponceau stained gels prior to Western blotting for Myogenin. Gels used for Myogenin Western blots at 3, 5 and 21-day time points. **L** – indicates the lane containing the loading control reference sample, which was run on every single gel. The protein ladder is in the left-most lane, where the 37 kDa protein standard is indicated.

TGF-β Gels

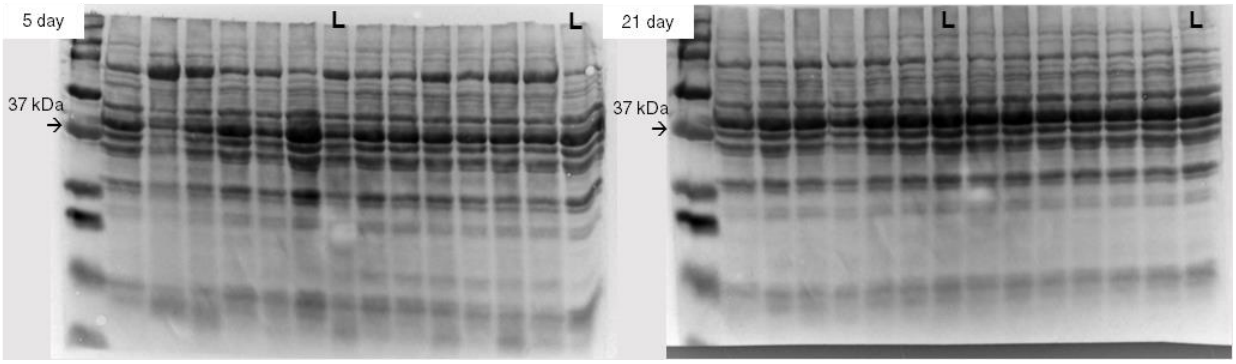

Figure IV Ponceau stained gels prior to Western blotting for TGF-β. Gels used for TGF-β Western blots at 5 and 21-day time points. **L** – indicates the lane containing the loading control reference sample, which was run on every single gel. The protein ladder is in the left-most lane, where the 37 kDa protein standard is indicated

Fibronectin Gels

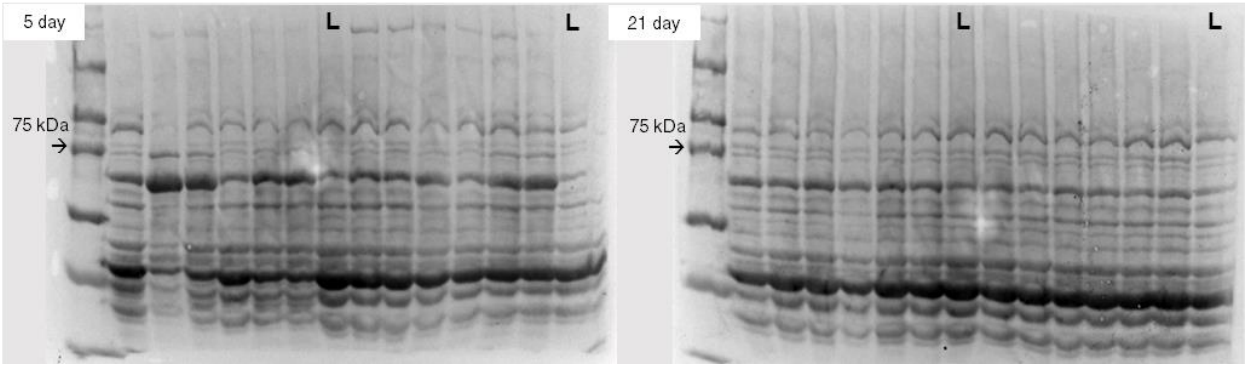

Figure V Ponceau stained gels prior to Western blotting for fibronectin. Gels used for fibronectin Western blots at 5 and 21-day time points. **L** – indicates the lane containing the loading control reference sample, which was run on every single gel. The protein ladder is in the left-most lane, where the 75 kDa protein standard is indicated.