Evaluating the Effect of Nitric Oxide on Myoblast Proliferation, Migration and Differentiation

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biochemistry, School of Life Science of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, PMB, South Africa. The research was financially supported by **National Research Foundation and the Medical Research Council of South Africa.**

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Abstract

Wound healing is the process of restoring tissue integrity in response to injury. This process involves four major phases namely; haemostasis, inflammation, regeneration and remodelling. These phases are regulated by various growth factors and cytokines that are released at the site of injury to facilitate wound repair. Cells involved in wound healing include neutrophils, macrophages, lymphocytes, fibroblasts and stem cells. Satellite cells are mesenchymal stem cells which facilitate skeletal muscle regeneration through a process known as myogenesis. These cells are quiescently located underneath the sarcolemma of the muscle fiber and are activated upon injury to enter the growth phase of cell cycle. They then proliferate and migrate to the injury site to differentiate and fuse with existing fibers to form multinucleated muscle cells. Growth factors and signalling molecules, such as hepatocyte growth factor (HGF) and nitric oxide (NO), induce satellite cell activation by altering the expression of transcription factors such as paired box transcription factor 7 (Pax7), myogenic regulatory factor 5 (Myf5), myogenic differentiation antigen (MyoD) and Myogenin. The role of NO in the subsequent process of myoblast proliferation, migration and differentiation is however unclear. The present study therefore evaluated the effect of nitric oxide on myoblast proliferation, migration and terminal differentiation.

C2C12 myoblasts were cultured in standard growth media and subsequently plated for analysis in serum free media. Proliferation or differentiation was induced via the addition of either 2 ng/ml HGF or 2% horse serum respectively, while migration was stimulated using the standard *in vitro* wound healing assay. L-NAME (a NOS inhibitor; 100 µM and 200 µM) and SIN-1 (a NO donor; 10 µM or 25 µM) were utilized to modify NO levels *in vitro*, while NO levels were assessed using a nitric oxide colorimetric assay kit. Proliferation was assessed via cell counts, migration by assessing the percentage wound closure and differentiation determined by calculating myoblast alignment and subsequent fusion into multinucleated myotubes.

There was no significant change in nitric oxide generated by myoblasts during proliferation and migration studies. However, NO levels increased significantly in response to differentiation, L-NAME significantly prevented this NO increase at day 0 of differentiation. L-NAME also significantly decreased myoblast terminal differentiation by inhibiting myoblast alignment and fusion at day 5 of differentiation. L-NAME also significantly reduced the proliferative effect of HGF on myoblasts at 24 hours, and significantly reduced percentage wound closure at 16 hours post-injury.

NO release by C2C12 myoblast was observed to increase in response to SIN-1 in a dose dependent manner. NO levels significantly increased from 0.58 nmol in a control up to 1.35 nmol and 1.9 nmol at 1 hour in response to 10 μM and 25 μM SIN-1 respectively. These levels increased until they reached 2.5 nmol in response to 25 μM SIN-1 at 16 hours. SIN-1 showed no significant effect on myoblast proliferation, however, it significantly promoted myoblast migration in a dose dependent manner by increasing the percentage wound closure to 42% and 45% at 7 hours for 10 μM and 25 μM respectively compared to 38% of the control. SIN-1 also significantly stimulated myoblast fusion with myofiber area of 26% as compared to 18.6% of the control at day 5 of differentiation.

In conclusion, nitric oxide levels increase significantly during myoblast differentiation, but not during proliferation and migration. Despite this, inhibition of nitric oxide synthase significantly affects all these processes. In contrast NOS-independent elevation of NO (through incubation with SIN-1) significantly increased myoblast migration and fusion, but not proliferation. This suggests a central role for NO in regulating myogenesis; however, this role requires further investigation.

Acknowledgement

First and foremost, I would like to extend my heartfelt gratitude to my supervisors; Prof Carola Niesler, for all her guidance and supervision throughout this journey of two years. Thank you, Prof, that you never got tired of seeing my face whenever I knocked at your door, through your help and assistance you have contributed a great deal toward my future endeavours. To my Co-supervisor Prof Kathy Myburgh, thank you so much for all your support financially through your NRF SArchi Chair bursary and your valuable comment you made on my research. I wish I have ten thousand tongues to thank both of you for what you have done for me, I am proud to be a product of your supervision.

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Table of Contents

List of tables

List of figures

List of Appendices

Abbreviations

BH_4	Tetrabiopterin
CaM	Calmodulin
cGMP	Cyclic guanidine mono phosphate
DM	Differentiation media
DMEM	Dulbecco's modified eagle's medium
EGF	Epidermal growth factor
ECM	Extracellular matrix
FMN	Flavin mononucleotide
FBS	Fetal bovine serum
FAD	Flavin adenine dinucleotide
FGF	Fibroblast growth factor
GTP	Guanidine triphosphate
GSH	Glutathione
GM	Growth media
HGF	Hepatocyte growth factor
IL-1β	Interluekin-1 beta
IFN-γ	Interferon gamma
IGF-1	Insulin growth factor-1
LPS	Lipopolysaccharide
L-NAME	L-N-nitroarginine methyl ester
L-NNA	L-N-Nitroarginine
MMP	Matrix metalloprotease
MSC	Mesenchymal stem cell
MPC	Myogenic progenitor cell
MyoD	Myogenic differentiation
MyHC	Myosin heavy chain
MyF5	Myogenic regulator factor 5
MAPK	Mitogen activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
n/e/iNOS	Neuronal/ endothelial/ inducible NOS
PBS	Phosphate buffered saline
SIN-1	3-Morpholinosydnonimine
SNP	Sodium nitroprusside

SNAP	S-nitroso-N-acetylpenicillamine
SCM	Serum containing media
SFM	Serum free media
TNF-α	Tumor necrosis factor- alpha
TGF- α/β	Transforming growth factor- alpha/beta

Thesis output

Conference attended

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Sibisi N.C., Myburg K, Niesler C.U. Evaluating the role of nitric oxide on myoblast proliferation, migration and proliferation. Presented at the School of Life Science Postgraduate Research day, University of KwaZulu-Natal, Pietermaritzburg, South Africa 21 May 2018.

CHAPTER 1

1. Introduction

1.1 The basic mechanism of wound healing

Would healing is the process of generating new tissue following severe injury; in skeletal muscles it is mainly driven by satellite cells (Beldon, 2010). There are four overlapping classical stages of wound repair, including haemostasis, inflammation, new tissue regeneration and remodelling; these stages are regulated by growth factors, cytokines and reactive oxygen species such as nitric oxide (NO) (Gurtner et al., 2008).

1.1.1 Haemostasis

Following injury, platelets are attracted towards the wound site, where they initiate coagulation (Martin and Leibovich, 2005). The aggregation of platelets leads to the conversion of fibrinogen to fibrin; this reduces blood loss in the wound by forming a fibrin clot (Martin and Leibovich, 2005). During this stage, platelets release growth factors and cytokines such as transforming growth factor beta and alpha (TGF-β/α), platelet derived growth factor (PDGF), epidermal growth factor (EGF), tumour necrosis factor alpha (TNF-α) as well as reactive oxygen species such as NO stimulates generation of new tissue and deposition of extracellular matrix (Beldon, 2010).

1.1.2 Inflammation

The inflammatory phase of wound healing is associated with the activation of the innate immune system cells such as neutrophils as well as monocytes, which differentiate to macrophages (Oishi and Manabe, 2018). This phase is a crucial pre-requisite for repair and regeneration; it mediates restoration of normal tissue structure by clearing tissue debris as well as stimulating angiogenesis and other cell-based processes (Oishi and Manabe, 2018). Once the fibrin clot has been formed, immune cells such as neutrophils, macrophages and lymphocytes migrate towards the wound (Childress and Stechmiller, 2002). Neutrophils play a major role in repair by removing cell debris via phagocytosis and also prevent infection by bacteria (Young and McNaught, 2011). Macrophages further clear the wound and also produce cytokines, growth and angiogenesis factors (Martin and Leibovich, 2005). Macrophages also triggers activation of progenitor cells by releasing oxygen reactive species such as nitric oxide (NO) (Martin and Leibovich, 2005). As wound repair progresses the number of neutrophils decreases, whilst macrophage numbers increase; and these cells releases growth factors such as TGF-β, TGF-α and PDGF which stimulate proliferation and differentiation of myogenic and non-myogenic cells such as fibroblasts (Figure 1.1) (Childress and Stechmiller, 2002).

1.1.3 Regeneration

Parenchymal and stromal cells proliferate and differentiate to restore the integrity of the tissue (Figure 1.1) (Oishi and Manabe, 2018). During regeneration, factors released by fibroblasts, endothelial and epithelial cells stimulate new connective tissue generation and angiogenesis occurs to form new blood vessels (Young and McNaught, 2011, Childress and Stechmiller, 2002). In addition, fibroblasts lay down extracellular matrix proteins such as collagen, which gives strength to the tissue (Young and McNaught, 2011), and also differentiate to contractile myofibroblasts, which are involved in wound closure as well as remodelling (Young and McNaught, 2011).

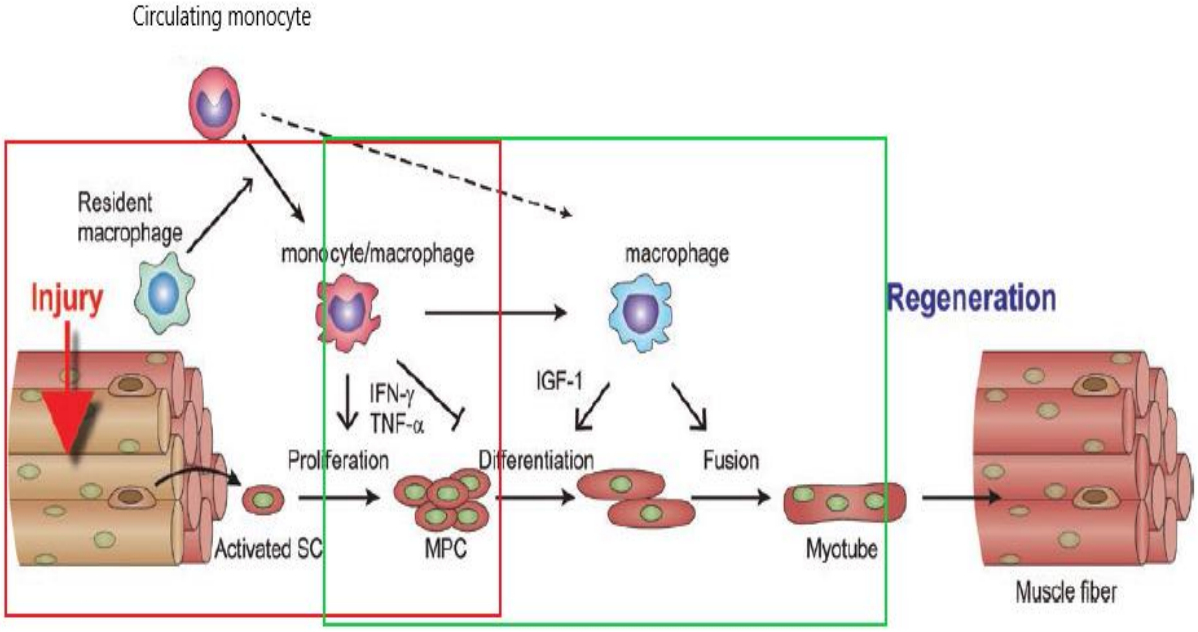


Figure 1.1: Skeletal muscle wound repair. The first two overlapping stages of wound repair are homeostasis and inflammation (red square). Macrophages release pro-inflammatory cytokines which stimulate the regenerative phase. New tissue regeneration (green square) ultimately occurs to restore damaged tissue and includes proliferation, differentiation, angiogenesis and wound closure. Remodelling is the final stage in wound repair where scar tissue matures (Oishi and Manabe, 2018).

1.1.4 Remodelling.

The remodelling phase occurs at 2-3 weeks post injury (Oishi and Manabe, 2018), this stage of wound healing can last for up to 1 year if scar tissue remains due to incomplete regeneration (Young and McNaught, 2011). At this point, maturation of the scar takes place which give strength to the wound (Young and McNaught, 2011). The myofibroblasts control the matrix remodelling in the wound by secreting matrix metalloproteases and modulating extracellular matrix deposition (Beldon, 2010). At this stage, tissue integrity is usually completely restored, and normal tissue functioning can resume.

1.2 Role of stem cells in wound repair

Stem cells are defined as unspecialised cells that can proliferate and differentiate to become specialized tissue (Crawford and Turner, 2008). Mesenchymal stem cells are thought to stimulate wound healing by secreting growth factors, which regulate extracellular matrix deposition during remodelling, and stimulate the migration of relevant cells toward the wound (Chen et al., 2008). MSC's also stimulate production of anti-inflammatory cytokines such as interleukin 4 and 10 (Maxson et al., 2012). This suppresses the pro-inflammatory cytokine profile, which would otherwise result in a chronic wound (Maxson et al., 2012).

2. Skeletal muscle

2.1 Activation of satellite cells and skeletal muscle repair

Skeletal muscle is the most dominant organ for movement, postural maintenance and energy metabolism, it has a high regenerative capacity as compared to other tissues/ organs (Oishi and Manabe, 2018). Skeletal muscle is mainly composed of a) epimysium which is the connective tissue surrounding the whole muscle, b) perimysium surrounding muscle fascicles and c) endomysium surround individual myofiber (Figure 1.2). Quiescent muscle stem cells (satellite cells) are resident cells in myofibers and tissue resident macrophages are localized near the capillary particularly in perimysium (Oishi and Manabe, 2018). Satellite cells are committed to the myogenic lineage and drive the muscle regeneration process (Shi and Garry, 2006). Therefore, repair and regeneration of skeletal muscle is highly dependent upon satellite cell activation and myogenesis (Tedesco et al., 2010). Satellite cells reside in a niche between the basal lamina and sarcolemma of muscle fiber (Tedesco et al., 2010). Following skeletal muscle damage, quiescent satellite cells are activated to myoblasts that proliferate and differentiate into myocytes and fuse to form multinucleated myotubes, which subsequently repair damaged muscle (Grounds, 2014).

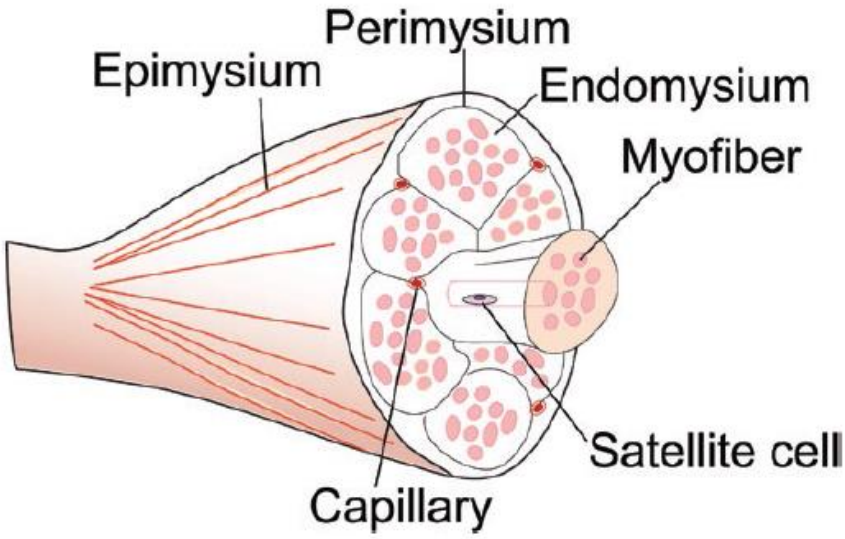


Figure 1.2: Skeletal muscle anatomy. Skeletal muscle is composed of different layers namely the epimysium (surrounding the muscle), perimysium (surrounding muscle fiber bundle), endomysium (surrounding myofiber). Capillaries and blood vessels also form part of the muscle. Satellite cells are quiescent residence cells found between the sarcolemma and basal lamina (Oishi and Manabe, 2018).

The activation of satellite cells is accompanied by the expression of transcription factors such as paired box transcription factor 7 (Pax7), myogenic regulatory factor 5 (Myf5) and myogenic differentiation antigen (MyoD) (Figure 1.3) (Grounds, 2014). Expression of transcription factors provides a molecular regulatory sequence that directs myogenesis (Shi and Garry, 2006). Pax7 is responsible for cell determination, while MyoD and Myf5 facilitate myoblast proliferation and differentiation. Activated satellite cells leave their niche and migrate towards the injury site, displaying an increased expression of MyoD, Myf5 (Le Grand and Rudnicki, 2007). As they prepare for differentiation, they downregulate Pax7 and upregulate myogenin and myosin heavy chain (MyHC) (Le Grand and Rudnicki, 2007). A small population of activated satellite cells can return to the quiescent state to maintain the satellite cell pool (Tedesco et al., 2010). Nitric oxide is known to mediate activation of these cells, however its role in regulating proliferation, migration and differentiation during skeletal regeneration is unclear (Anderson, 2000).

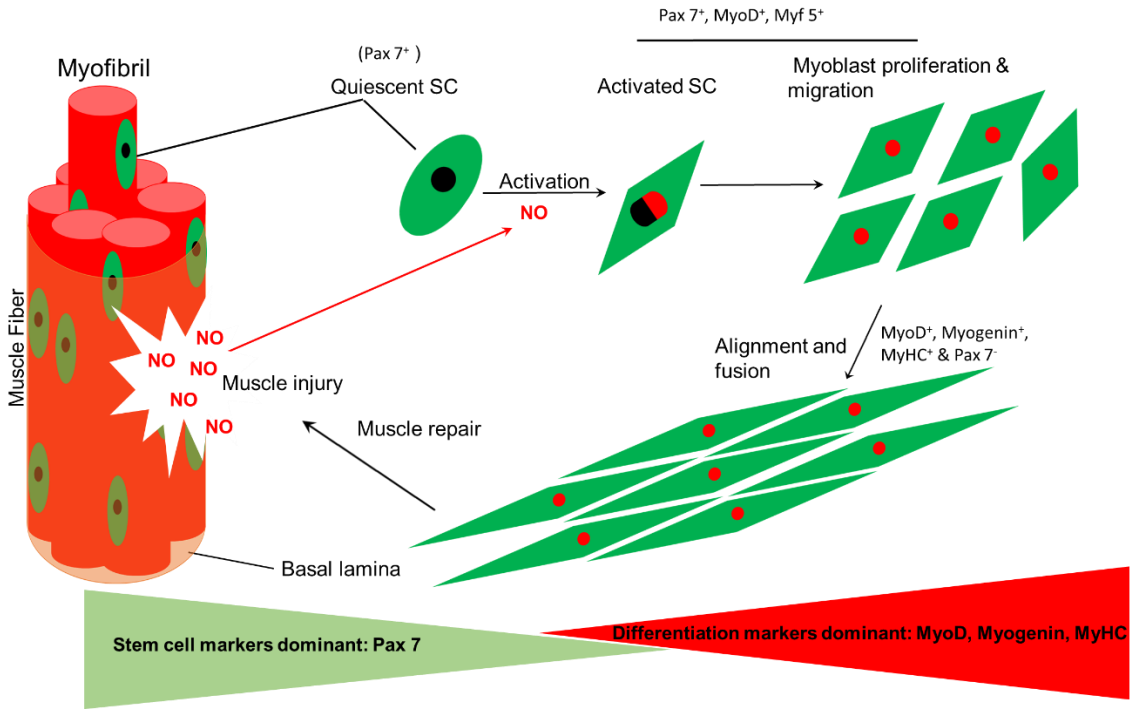


Figure 1.3: Myogenesis during skeletal muscle regeneration. Satellite cells (SC) are located between the sarcolemma and basal lamina of the muscle fiber. Satellite cells are activated to myoblasts by nitric oxide (NO), after which they proliferate and further differentiate to myocytes. The transcription factor Pax7 (Paired box 7) is expressed in both quiescent and activated SC, whilst Myf5 (Myogenic factor 5), MyoD (Myogenic differentiation factor) are expressed in proliferating myoblasts. MyoD, myogenin, myosin heave chain (MyHC) are expressed in cells committed to differentiation. Adapted from (Tedesco et al., 2010).

2.2 Myogenesis

Following activation, myoblasts proliferate and migrate to the wound where they align, differentiate and fuse to form multinucleated myofibers in a process referred to as myogenesis (Figure 1.3) (Allen and Rankin, 1990). The activation of satellite cells is regulated by growth factors such as fibroblast growth factor (FGF), insulin like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) as well as NO (Allen and Rankin, 1990). FGF has been shown to stimulate proliferation, while suppressing myoblast differentiation, whereas IGF can stimulate both proliferation and differentiation of myoblasts, depending on concentration (Allen and Rankin, 1990). Elevated TGF-β levels have been associated with myopathy and fibrosis *in vivo*; *in vitro*, this growth factor has been shown to promote proliferation and inhibit differentiation of myoblasts (Schabort et al., 2009). HGF on the other hand regulates differentiation and proliferation in a dose-dependent manner with higher concentrations of HGF stimulating differentiation and lower concentrations stimulating proliferation (Walker et al., 2015). The administration of exogenous HGF also has a dose dependent effect; high concentrations of HGF induce differentiation whilst low concentrations induce proliferation (Yamane et al., 2004).

Exogenous HGF increases migration of myoblast *in vitro* and has a significant role in controlling the early phase of muscle regeneration (Miller et al., 2000). HGF also stimulates the expression of transcription factors such as Pax7, MyoD, Myogenin and MyHC (Le Grand and Rudnicki, 2007).

2.2.1 Regulation of myogenesis by growth factors

TGF- *β* belongs to a family of growth factors which includes three isoforms (TGF- β1, TGF- β2 and TGF- β3) that regulate various cellular events including cell division, differentiation and tissue maturation (Delaney et al., 2017). TGF- β affects myoblast differentiation negatively, by stimulating MyoD degradation and reducing myogenin and MyoD during terminal differentiation, therefore fusion cannot occur (Schabort et al., 2009). Interestingly, TGF- β regulate myoblast proliferation by promoting cell division and promoting cell release from cell cycle arrest (Schabort et al., 2009, Sakaki-Yumoto et al., 2013). TGF- β1 plays a role in regulating muscle regeneration, it promotes connective tissue formation, influences fibroblast to excessively deposit ECM, and mediate fibroblast transition to myofibroblast, therefore TGF- β1 also promote wound healing as myofibroblast are contractile cell during wound healing (Delaney et al., 2017).

FGF refers to a large family of polypeptide growth factors that are present in many organisms from nematodes to humans, there are 23 FGF isoforms (Ornitz and Itoh, 2001). Depending on the isoforms, FGF can be secreted from the cell (e.g. FGF 9) or present at the cell surface and within extracellular matrix (e.g. FGF 1&2) (Ornitz and Itoh, 2001). FGF stimulates proliferation of activated myoblasts and fibroblasts, and promote angiogenesis (Delaney et al., 2017).

HGF also known as scatter factor (HGF/SF), is secreted by different cells including hepatocyte cells, mesenchymal cell, satellite cell and stromal cells (Nakamura and Mizuno, 2010). It plays an important role in epithelial cell proliferation, myogenesis and angiogenesis (Nakamura and Mizuno, 2010). HGF is produced as inactive pro-HGF and it is stored in the ECM in it inactive form; pro-HGF is activated by proteolytic cleavage by serine protease which cleave at Arg494 to Val495 residue (Forte et al., 2006). HGF binds to and signals via c-Met a tyrosine kinase receptor (Brand-Saberi et al., 1996). The c-Met receptor is expressed in quiescent satellite cells; the release of HGF and its binding to c-Met activates satellite cells and stimulates their migration as myoblasts (Tatsumi et al., 2002); absence of the c-Met receptor impairs this (Cornelison and Wold, 1997). The mechanical stretch of muscle fibers stimulates intracellular signal such as nitric oxide synthesis which activate MMP's and subsequent release of HGF from ECM protein (Le Grand and Rudnicki, 2007).

3. Nitric oxide.

Nitric oxide is a gaseous free radical endogenously generated by the activity of nitric oxide synthase (Han et al., 2012) and from nitrogen reactive species (Carpenter and Schoenfisch, 2012). Nitric oxide was first discovered in 1772 by Joseph Priestly; at that time, it was known as an atmospheric pollutant gas, but later identified to play a significant role in physiological processes (Furchgott, 1996). Nitric oxide is produced by both prokaryotes and eukaryotes; bacteria generate nitric oxide via reduction reactions in anaerobic condition, whereas mammals produce NO via an oxidation reaction (Lundberg and Weitzberg, 2010). Nitric oxide is generated by nitric oxide synthase in a number of cells including skeletal and cardiac muscle cells, epithelial cells, macrophages, endothelial cells as well as fibroblasts and hepatocytes (Filippin et al., 2009). NO is a very small molecule that can freely diffuse through cell membranes (Sakurai et al., 2013).

3.1 Nitric oxide synthase.

Nitric oxide synthase (NOS; E.C. 1.14.13.39) belongs to a family of enzymes catalysing oxidation reactions (MacMicking et al., 1997). The enzyme was identified in 1989 and its three isoforms were first cloned and purified in 1991 and 1994 (Alderton et al., 2001). The three different NOS isoforms are; neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) (Alderton et al, 2001). nNOS is mostly present in neuronal and skeletal muscle tissue, while iNOS is inducible in inflammatory cells such as macrophages, (Alderton et al., 2001). eNOS is predominantly present in vascular endothelial cells (Alderton et al., 2001). Nitric oxide synthase isoforms share some similarities in their structure and roles. All three isoforms have a similar structure consisting of an oxygenase domain at the amino terminal and a reductase domain at the carboxy terminal (MacMicking et al., 1997). Constitutively expressed isoforms (nNOS and eNOS) are characterized as calcium dependent and generate a low output of nitric oxide; their activity is triggered by an increase in calcium concentration within the cell (Nathan and Xie, 1994). In contrast iNOS activity is calcium independent, generating high nitric oxide levels; induced by cytokines such as interferon gamma (IFN-γ) and bacterial endotoxins such as lipopolysaccharide (LPS) (Nathan and Xie, 1994).

3.2 Structure, function and regulation of NOS

NOS is a homodimer consisting of five binding sites for cofactors such as tetrabiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM) and haem (Figure 1.4) (Förstermann and Sessa, 2011). Calmodulin, present within the reductase domain, is required to stimulate electron transfer and to sustain enzyme activity

(MacMicking et al., 1997). The binding site of calmodulin in constitutive NOS (nNOS and eNOS) is enhanced by an increase in intracellular calcium, which in turn mediates electron flow from NADPH to the haem binding site (Förstermann and Sessa, 2011). In addition, the haem binding site is important for electron transfer between domains from FAD to FMN and finally to the haem binding site (Förstermann and Sessa, 2011). NADPH and oxygen molecules are required as co-substrate to ensure that the enzyme is fully active (Witte and Barbul, 2002). All three NOS genes share identical genomic structure with slightly different sizes; nNOS is 161 kDa, iNOS is 131 kDa and eNOS is 133 kDa (Alderton et al., 2001). The oxygenase domain, located at the N-terminal region, consists of binding sites for L-arginine, BH_4 and haem, and is linked to the C-terminal via a calmodulin (CaM) binding site (Alderton et al., 2001). At the C-terminus, the reductase domain consists of binding sites for cofactors such as FAD, FMN and cosubstrate nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 1.4).

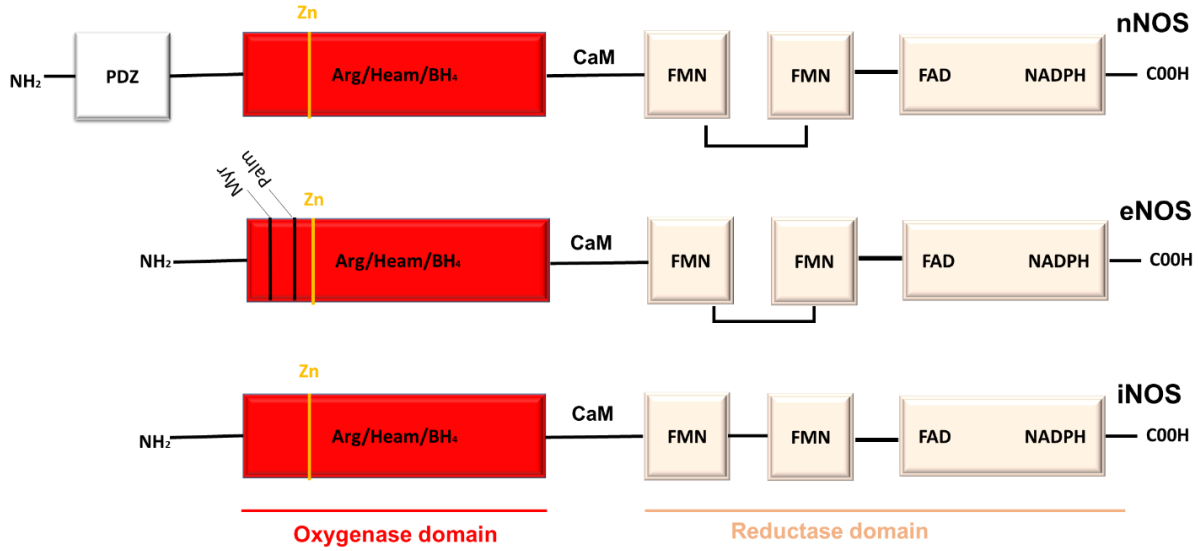


Figure 1.4: Structure of NOS isoforms. All NOS isoforms have an oxygenase domain (red) at the N-terminus and a reductase domain (yellow) at the C-terminus. nNOS has a PDZ motive, while eNOS contains myristoylation and palmitoylation sites at the N-terminus (Alderton et al., 2001).

The substrate (L-arginine) localizes its side chain to the active site of the enzyme, where the guanidine group interacts with the haem and becomes oxidized (Alderton et al., 2001). The zinc ion (present in all three NOS isoforms) in the oxygenase domain is tetrahedrally coordinated by cysteine residues which is important in ensuring that the dimer is stable (Alderton et al., 2001). Neuronal NOS (nNOS) has a sequence of amino acids at the N-terminus known as a PDZ motive, which locates the enzyme to the sarcolemma of skeletal muscle (Tengan et al., 2012). eNOS contains myristoylation and palmitoylation sites at the N-terminal region, which helps localize eNOS to the caveolae of endothelial cells (Tengan et

al., 2012). Inducible NOS is the shortest isoform and releases NO in a calcium independent manner. Vascular endothelial growth factor increases intracellular calcium levels, which stimulate the binding of calmodulin to eNOS and activates it (Papapetropoulos et al., 1997). However; several agonists including acetylcholine, ADP, histamine and norepinephrine are also known to trigger the increase in NO generation by endothelial cells (Tousoulis et al., 2012). Moreover; VEGF upregulates eNOS expression in human endothelial cells which in turn enhances NO generation via tyrosine kinase activation and increased intracellular calcium (Papapetropoulos et al., 1997). The generation of NO from vascular endothelial cells, stimulates cell activity of smooth muscle cells, platelet as well as immune cells (Carpenter and Schoenfisch, 2012).

3.3 Mechanism of nitric oxide production

In skeletal muscle, nitric oxide is endogenously generated by either neuronal or endothelial nitric oxide synthase (nNOS / eNOS), which catalyses the oxygen dependent electron oxidation of L-arginine to form NO and L-citrulline as co-product (Riccio and Schoenfisch, 2012, Lundberg and Weitzberg, 2010). In endothelial cells, nitric oxide generation is via constitutive nitric oxide synthase (eNOS and nNOS) (Tousoulis et al., 2012).

NO is generated from the substrate L-arginine, using NADPH as electron donor and oxygen (Aktan, 2004). This reaction involves a two-step oxidative conversion of L-arginine to L-citrulline and NO, where N-hydroxy-L-arginine is produced as an intermediate (Figure 1.5) (Aktan, 2004). L-Arginine is a key substrate for endogenous nitric oxide synthesis; it is a limiting factor of nitric oxide synthase activity in endothelial cells (Long et al., 2006). NO can also be generated from inorganic anions, nitrate and nitrite, which are mainly found in the diet (Lundberg and Weitzberg, 2010). However NO generated from nitrate reaction is increased by biological molecule such as vitamin C.

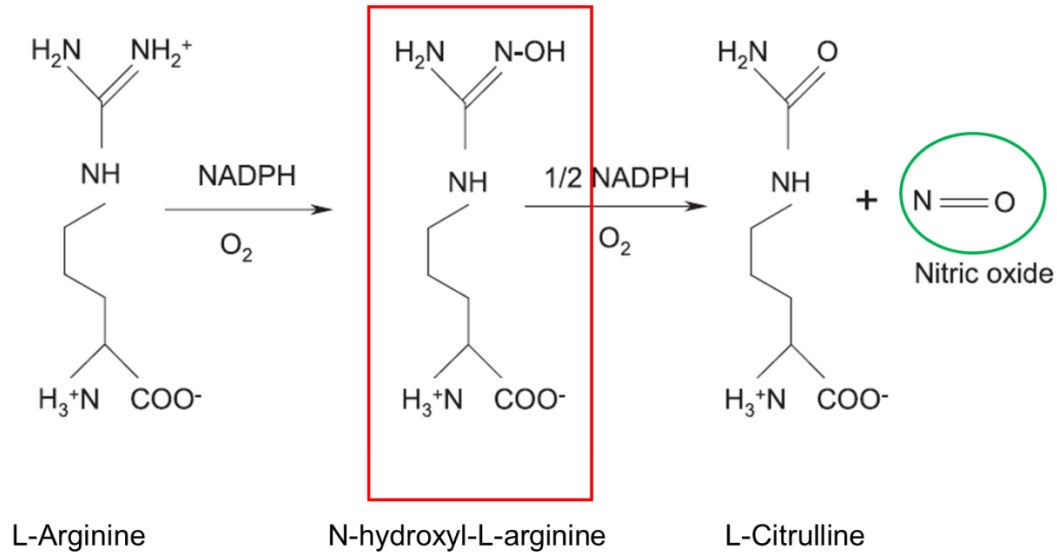


Figure 1.5: Schematic representation of NO production. NADPH and oxygen serve as electron donors to L-Arginine, to produce an intermediate N-hydroxyl-L-arginine which further react with NADPH in the presence of oxygen to produce the end products L-Citrulline and nitric oxide (Aktan, 2004).

3.3.1 Nitric oxide produced by macrophages

Macrophages play a significant role in immune and inflammatory processes by removing pathogens, tumour and apoptotic cells. The contribution of macrophages to the immune response is in part via the production of NO, as well as pro-inflammatory cytokines such as TNF-α and interleukin-1β (IL-1β) (Kawakami et al., 2016). iNOS catalyses NO production in macrophages, following exposure to cytokines such as interferon-γ (INF-γ), TNF-α, IL-1β and bacterial product such as lipopolysaccharide (LPS) (Chi et al., 2003). Exposing macrophages to LPS or IFN-γ stimulates NO production via nuclear factor kappa Bator (NF-k_B) or Janus kinase signal transducer and activation of transcription protein 1 (Jak-STAT-1) pathways (Aktan, 2004). LPS binding protein (LBP) directs LPS to bind the membrane receptor CD14, which activates inhibitor of kappaB kinase (IKK) which in turn activates NF-k_B to translocate to the nucleus to induce iNOS gene expression (Figure 1.6). IFN-γ on the other hand binds to its membrane receptor and activates Jak-1/Jak-2, which in turn phosphorylate STAT1 to translocate to the nucleus and induces interferon regulatory factor-1 (IRF-1) which stimulates iNOS expression as shown in Figure 1.6 (Aktan, 2004). These pathways upregulate the expression of iNOS gene expression, resulting in higher nitric oxide release by cells.

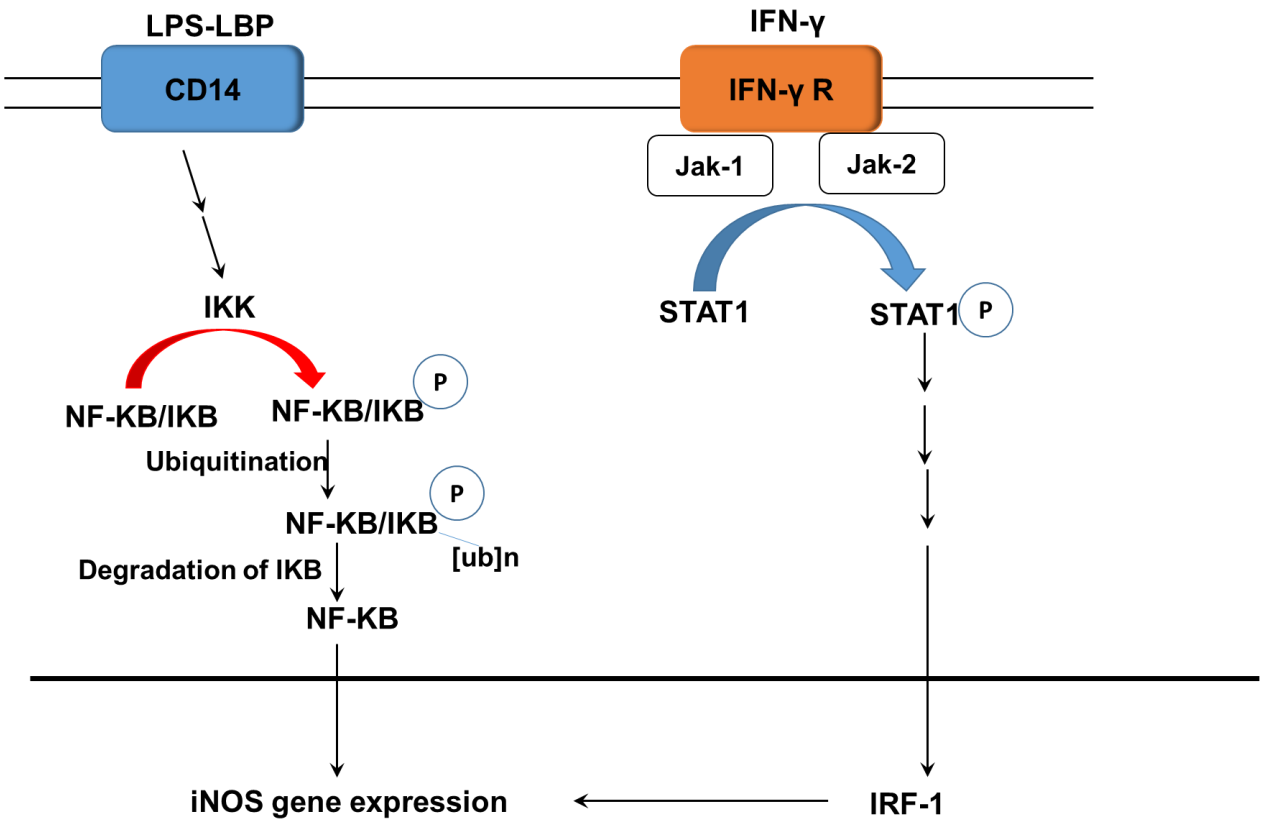


Figure 1.6: iNOS gene expression in macrophages. Lipopolysaccharide binding protein (LPS-LBP), binds to CD14 membrane receptor, to stimulate phosphorylation of NF-k_B/Ik_B. Interferon gamma (IFN-γ) binds to IFN-γ receptor to stimulate STAT1 phosphorylation. These pathways result in an increase in iNOS gene expression in the nucleus (Aktan, 2004).

3.4 Role of Nitric Oxide in wound healing.

Nitric oxide is generated during wound healing by an increase in NOS expression, stimulating conversion of L-Arginine to L-citrulline and NO (Rizk et al., 2004). NOS expression is higher during the early stages of wound healing, with NO generated at this stage mainly produced by pro-inflammatory cells like macrophages (e.g. in response to LPS exposure) and neutrophils present at wound site (Rizk et al., 2004). In addition, other cells including fibroblasts, keratinocytes, endothelial cells also stimulate NO generation, but at lower levels (Rizk et al., 2004).

NO has been shown to be a cytotoxic agent during the inflammatory stage of wound healing which helps to remove pathogenic cells (Childress and Stechmiller, 2002). High levels of NO are generated during the inflammatory stage by high iNOS expression in activated macrophages (Childress and Stechmiller, 2002). Nitric oxide metabolites, nitrate and nitrite, were found to be high in the wound fluid during this stage (Childress and Stechmiller, 2002). As wound healing progresses iNOS expression decreases; the mechanism of this down regulation at later stages of wound healing is not clearly understood (Isenberg et al., 2005).

However, expression of all NOS isoforms gradually decrease during wound healing; this allows other phases of wound healing that do not require high levels of NO, to take place (Isenberg et al., 2005). NO is known as a molecular messenger that orchestrates cell to cell and cell to matrix interactions during wound healing (Isenberg et al., 2005). The role of NO in wound healing has been further studied in the NOS knockout rat, where delayed wound closure is observed (Childress and Stechmiller, 2002). Inhibition of NOS activity reduces the process of collagen deposition and production as well as wound contraction, therefore delaying the process of wound healing (Isenberg et al., 2005).

Nitric oxide reacts with molecular oxygen resulting in the formation of free radical species, which have been shown to regulate wound healing (Rizk et al., 2004). At the molecular level, NO transduces signals via guanylate cyclase to activate the cGMP pathway; whereas at cellular level NO is cytostatic to many cell types (Rizk et al., 2004). NO induces apoptosis of pathogenic cells in three different ways; firstly, by alteration of plasma membrane integrity, damaging transport proteins and ion channels (Childress and Stechmiller, 2002), Secondly NO inhibits essential mitochondrial enzymes such as cytochrome oxidase, hence inhibiting respiration; lastly NO interacts with ribonucleotide diphosphate reductase, an enzyme involved in DNA synthesis and it's inhibition reduces DNA synthesis process (Childress and Stechmiller, 2002).

4. Role of NO in skeletal muscle repair.

Although skeletal muscle expresses both nNOS and eNOS, nNOS remains the predominant isoform expressed for NO release by this tissue. Nitric oxide is known to be involved in mediating the process of satellite cell activation in response to muscle injury (Anderson, 2000). Satellite cell activation is estimated to be within 1 minute of muscle injury; this is suspected to be via NO signalling (Anderson, 2000). *In vivo* studies using NOS knockout mice have shown that when the NOS gene is knocked out in muscle of a mouse, satellite cell activation is reduced (Anderson, 2000). The study, carried out *in vivo,* demonstrated that inhibition of NOS activity has a negative effect on myogenesis and skeletal muscle repair (Anderson, 2000). Nitric oxide stimulates changes in satellite cell adhesion, morphology and expression of membrane proteins like c-Met and m-Cadherin (Anderson, 2000). Nitric oxide can interact with m-Cadherin to stimulate the loss of satellite cell adhesion during activation, promoting their myogenic ability (Anderson, 2000). The activation process occurs step wise, muscle injury triggers the release of NO followed by release of growth factors such as HGF, which signal to activate satellite cells (Filippin et al., 2011). However, NO is not only involved

on satellite cell activation, but also plays a role in blood vessel dilation and angiogenesis, leading to a reduction in blood pressure (Tengan et al., 2012).

4.1. Proliferation

Proliferation of activated satellite cells during skeletal muscle injury is crucial to ensure that there are enough cells to repair damaged muscle and also to maintain the satellite cell pool (Soltow et al., 2010). Therefore, the activated satellite cell population proliferates to provide an adequate number of myonuclei for skeletal muscle maintenance and regeneration (Ulibarri et al., 1999). Myoblast proliferation is triggered by various factors such as shear stress, muscle stretch and contractile activity (Soltow et al., 2010). Nitric oxide exerts a significant effect on proliferation of both normal and tumour cells (Villalobo, 2006). NO has been shown to have a dose dependent effect on proliferation, low endogenous concentrations stimulates proliferation whilst high endogenous concentrations inhibit proliferation of fibroblasts, myoblasts and keratinocytes (Villalobo, 2006). Nitric oxide stimulates myoblast proliferation via the cGMP pathway, where it activates guanylyl cyclase to release cGMP. It also stimulates maintenance of the satellite cells pool to ensure that they are not exhausted during muscle damage repair (De Palma and Clementi, 2012). Nitric oxide also stimulates proliferation by s-nitroslylation of skeletal muscle protein (Figure 1.7). In skeletal muscle nNOS form a signalling complex with caveolin-3 and interacts with Src kinase and p21Ras (Figure 1.7) (Monteiro and Ogata, 2017). Nitric oxide is produced and nitrosylate GSH and signalling protein Src kinase and p21Ras, which then transduce signals via the Raf/ERK/ MAPK pathway, this leads to myoblast proliferation (Figure 1.7) (Monteiro and Ogata, 2017).

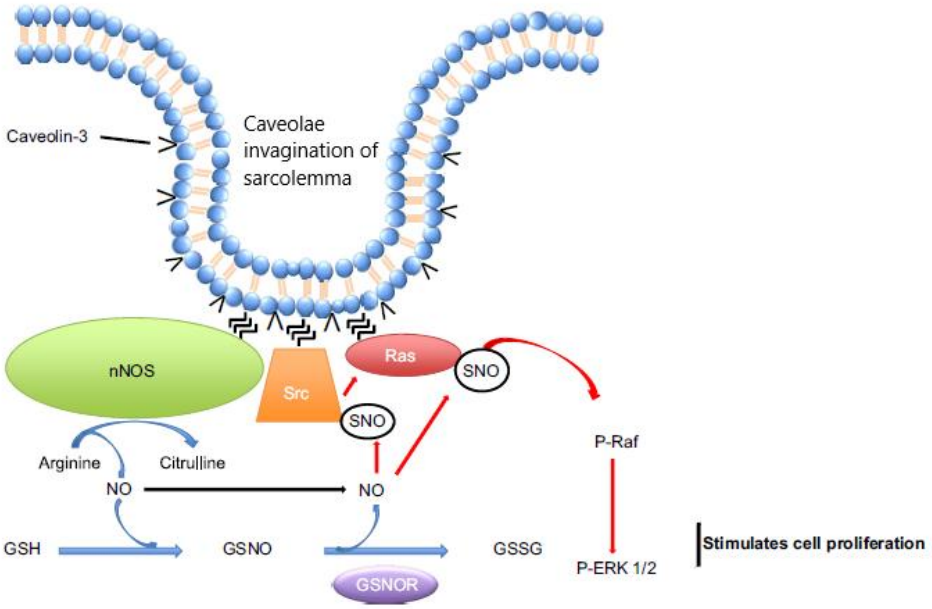


Figure 1.7: Mechanism of NO induced proliferation. Caveolin-3 (in the sarcolemma) interacts with neuronal nitric oxide synthase (nNOS) to release nitric oxide (NO) from arginine. Nitric oxide nitrosylate GSH (glutathione) to GSNO, nitric oxide also nitrosylate Src and Ras. Raf is phosphorylated by nitrosylated Ras and further phosphorylate ERK1/2 to ultimately stimulate myoblast proliferation (Monteiro and Ogata, 2017).

Following myoblast stretch, NO production increases which in turn enhances the release of HGF which promotes both myoblast activation and proliferation (Tatsumi et al., 2002). Myoblast proliferation following mechanical stretch of skeletal muscle is regulated by NF-K_B activation which in turn stimulates NOS expression (Soltow et al., 2010). To demonstrate that NO plays a crucial role on myoblast proliferation, previous studies have shown that inhibition of NO synthesis and knockout of the nNOS gene decreases proliferation (Villalobo, 2006). Whilst addition of nitric oxide donors such as sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) have been shown to stimulate myoblast proliferation (Ulibarri et al., 1999).

4.2. Migration

Activated satellite cells migrate to the injury site and proliferate to initiate regeneration; this is mediated by an inflammatory secretome released by activated macrophages (Ko et al., 2016). The exact mechanism whereby NO regulates myoblast migration is not clearly understood (Tatsumi et al., 2006). NO does not have a direct effect on migration, however it stimulates activation of signalling molecule, such as HGF and MMP, which directly affect migration (Filippin et al., 2009). NO has been shown to regulate focal proteins like talin and vinculin, which are crucial for myoblast migration (Zhang et al., 2004). These proteins also

interact with nNOS which is expressed in high levels in response to injury (Zhang et al., 2004).

4.3. Differentiation

Myogenic differentiation requires the fusion of mononucleated myoblasts with existing myofibers to form multinucleated myotubes, which further develop to become skeletal muscle fibers during muscle development and regeneration (Tengan et al., 2012). Nitric oxide stimulates myotube formation and is known to increase the fusion index (Long et al., 2006). Fusion involves myoblast, alignment relative to each other, arrangement of actin cytoskeleton at the contact sites and fusion with existing fiber to form myotubes (Hindi et al., 2013). NOS activity increases during myoblast fusion which is associated with an increase in calcium influx (Lee et al., 1994). Increases in calcium ion release by the endoplasmic reticulum formed complex with calmodulin to activate nNOS, hence NO is generated; NO activates soluble guanylyl cyclase to synthesize cGMP. Activated cGMP activates PKG to phosphorylate Raf 1 and MAPK to ultimately increase fusion (Figure 1.8) (Villalobo, 2006). Previous studies have provided evidence that shows that inhibition of NOS activity, via non-specific inhibitor L-NAME, impedes the formation of myotubes (Long et al., 2006). In addition, when differentiating cells are treated with L-Arginine, the fusion index increases (Long et al., 2006).

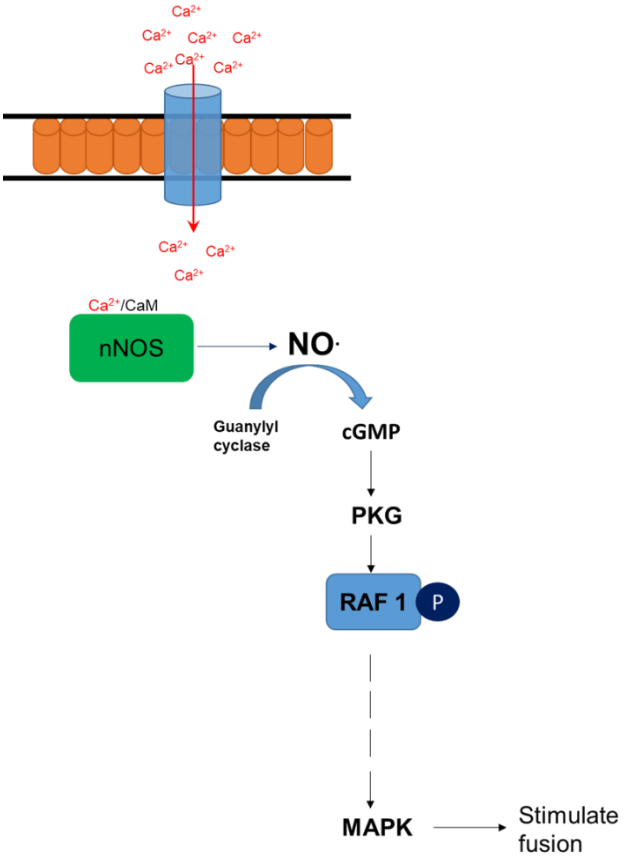


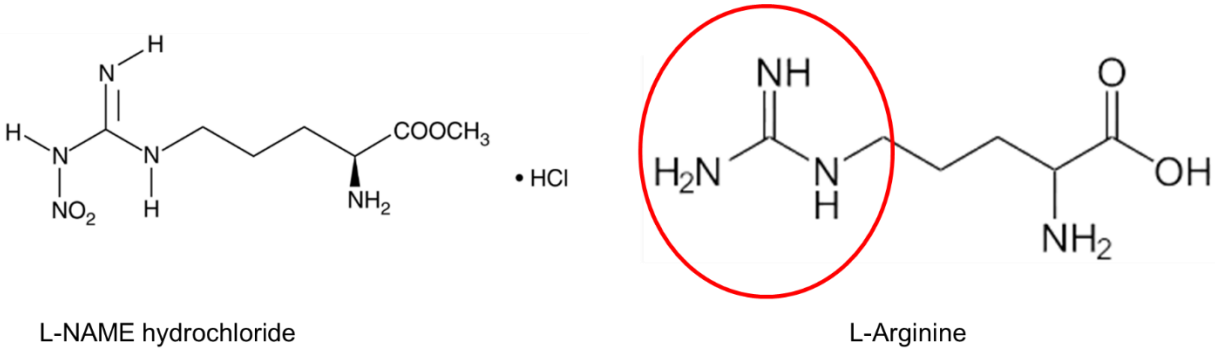
Figure 1.8: Nitric oxide stimulate myoblast fusion via MAP kinase pathway. Increase in intracellular calcium (Ca^{2+}) stimulate activation of nNOS and more NO is release which activate cGMP via guanylyl cyclase activity. cGMP activates PKG which phosphorylate Raf 1 and stimulate fusion in downstream pathway (Hindi et al., 2013, Villalobo, 2006).

4.4. NOS Inhibitors

Nitric oxide synthase isoforms can be inhibited in four different ways. Firstly; NOS inhibitors can interact with the L-arginine binding site, which then prevents binding of substrate, hence reducing NO production (Víteček et al., 2012). Secondly, NOS inhibitors can mimic the tetrahydrobiopterin cofactor, which is essential for the oxidation of the substrate. Thirdly NOS inhibitors can bind directly to the heme binding site, thus preventing the formation of the active form of the enzyme (Víteček et al., 2012). Lastly, NOS inhibitors bind with calmodulin and flavine cofactors; this prevents the transfer of electrons from one domain to another (Víteček et al., 2012).

L-arginine derivative and analogues have been widely used as inhibitors of NOS; these analogues are similar to L-arginine in their structure with differences at the terminal guanadino nitrogen as shown in Figure 1.9 (Víteček et al., 2012). L-NAME is commonly use as NOS inhibitor because it has no limitation on solubility under aqueous conditions (Víteček et al., 2012). It binds competitively, but non- selectively, with the enzyme and this binding

can be reversed by increasing substrate concentration to out-compete the binding of inhibitor (Kopincová et al., 2012). Previous research on inhibition of NO production by L-NAME (100 µM / 200 µM) indirectly downregulates MMP-2 expression, which is crucial for ECM degradation (Yu et al., 2005). There is a significant decrease on NO generation by cultured cells in the presence of L-NAME (Yu et al., 2005). L-NAME has a short half-life ranging between 12-30 minute depending on the tissue, cell or organ; it is also hydrolysed to L-NNA (L-N-Nitroarginine) (Víteček et al., 2012).



L-NAME hydrochloride L-Arginine

Figure 1.9: Comparison of L-NAME and L-Arginine structures. L-NAME is an e L-arginine derivative with an altered side chain, L-NAME does not have a guanidine group whereas L-arginine does (circled) (https://www.biovision.com/l-name-hydrochloride.html).

5. Summary and Aims

Satellite cells are quiescent myogenic precursors found between the basal lamina and sarcolemma of the muscle fiber. These cells are activated to myoblasts in response to skeletal muscle injury in order to facilitate skeletal muscle regeneration. Nitric oxide is known to mediate the activation of these cells, however its role in mediating myoblast proliferation, migration and differentiation is less clear. Therefore, the current study aimed to:

a) Determine nitric oxide levels under different experimental conditions simulating myogenesis.
b) Determine the role of nitric oxide inhibition on myoblast proliferation, migration and differentiation.
c) Evaluate the effect of the nitric oxide donor 3-Morpholinosydnonimine (SIN-1) on myoblast proliferation, migration and differentiation.

Chapter 2: Evaluating the Effect of L-NAME on Myoblast Proliferation, Migration, Alignment and Fusion.

Abstract

Satellite cells are mitotically quiescent myogenic precursor cells that reside in a niche between the basal lamina and sarcolemma of the muscle fiber. They are activated in response to skeletal muscle injury and facilitate muscle regeneration. Nitric oxide is a free radical that is known to mediate satellite cell activation to myoblasts; NO production is facilitated by nitric oxide synthase. The role of NO as a potential mediator of myoblast proliferation, migration or differentiation is unclear. The current study therefore aimed to firstly establish the level of nitric oxide released by myoblasts during proliferation, migration and differentiation. Secondly, it aimed to assess the effect of L-NAME (a NOS inhibitor) on these processes. C2C12 myoblast were cultured in standard growth media and subsequently plated for analysis in serum free media. Proliferation or differentiation was induced via the addition of either 2 ng/ml HGF or 2% horse serum respectively, while migration was analysed using the standard *in vitro* wound healing assay. L-NAME was utilized at a concentration of 100 μM and 200 μM, while NO levels were assessed using a nitric oxide colorimetric assay kit. Proliferation was assessed via cell counts and migration was determined by assessing the percentage wound closure. Differentiation was determined by assessing myoblast alignment and subsequent fusion into multinucleated myotubes. HGF (2 ng/ml) significantly stimulated myoblast proliferation, however levels of NO were only found to be 0.58 nmol at 1-hour post-HGF stimulated. Similarly, NO following myoblast wounding were 0.31 nmol at 1 hour and increased to 0.56 nmol over 16 hours. In response to differentiation cues, NO levels rose sharply to 6 nmol; these levels dropped as differentiation progressed over four days. Addition of L-NAME (200 μM) only resulted in a small but significant decrease in proliferation (43%) and migration (10%). Addition of L-NAME (200 μM) to differentiating cells significantly reduced myoblast alignment and fusion by 18% and 6% at day five of differentiation. Results suggest that nitric oxide plays a significant role during myoblast differentiation, making NO crucial for skeletal muscle regeneration.

Key words: Myoblast, Myogenesis, Nitric oxide, NOS, L-NAME

2.1 Introduction

Nitric oxide (NO) was discovered in 1772 by Joseph Priestly, as a colourless gas with a short half-life estimated to be 6-10 second *in vivo* (Yetik-Anacak and Catravas, 2006). Ever since it's discovery, researchers have explored various roles of NO in physiological processes such as muscle relaxation, wound healing and vascular tone. For instance, smooth muscle relaxation is mainly dependent on endothelial cells, which secrete diffusible and labile substances to enhance relaxation (Furchgott, 1996). Robert Furchgott firstly described this relaxing factor as endothelium derived relaxing factor (EDRF), which was then identified to be nitric oxide (Furchgott, 1996). Nitric oxide is unstable, highly diffusible, highly reactive and activates smooth muscle relaxation by activating guanylyl cyclase to stimulate cGMP pathway (Moncada and Higgs, 2006). Nitric oxide is produced via the activity of nitric oxide synthase (Han et al., 2012), which catalyse oxidation of L-arginine to produce nitric oxide and L-citrulline as coproduct (Moncada and Higgs, 2006). NOS (E.C. 1.14.13.39) is categorised into three isoforms, which are named according to the tissue in which they were originally identified (Moncada and Higgs, 2006).

The first NOS isoform, eNOS (endothelial nitric oxide synthase) was identified in endothelial cells, the source of nitric oxide is nitrogen of the guanidine group of L-arginine; eNOS is the main isoform that drive the production of nitric oxide in endothelial cells (Palmer et al., 1987). The second isoform, a calcium-independent NOS can be induced in macrophages via bacterial toxic and cytokines and is known as inducible nitric oxide synthase (iNOS) (Moncada and Higgs, 1993). The third isoform (isolated and located in the central nervous system) and was named neuronal nitric oxide synthase (nNOS) (Moncada and Higgs, 1993). Both eNOS and nNOS are constitutively active, but their activity is regulated by calcium ion levels, whilst iNOS activity is calcium independent, but inducible.

Nitric oxide plays a central role in the maintenance of vascular tone to regulate blood vessel vasodilation and is also able to reduce blood pressure by stimulating vessel dilation (Moncada, 1999). NO also plays a role in immunology and inflammation by stimulating cellular defences and destroying pathogens (Moncada and Higgs, 1993). In the nervous system, nitric oxide activates guanylate cyclase which in turn stimulates activation of cGMP pathway (Moncada, 1999). Furthermore, NO modulate smooth muscle relaxation, proliferation and it plays a vital role in wound healing (Moncada and Higgs, 1993).

In skeletal muscle wound healing involves several cells including platelets, inflammatory cells, fibroblasts, epithelial cells and satellite cells (skeletal muscle stem cells) (Schwentker et al., 2002). Skeletal muscle contraction during exercise is associated with generation of free radicals such as nitrogen species (e.g. NO) (Patwell et al., 2004). In skeletal muscle NO

is generated because two NOS isoform, namely nNOS and eNOS are expressed in equal amounts in the sarcolemma (Frost et al., 2004). Muscle exercise and sheer stress enhance the expression of eNOS in skeletal muscle, while nNOS expression is upregulated during myotube fusion stage (Stamler and Meissner, 2001). Muscle destruction *in vivo* causes the calcium channel to be opened and release calcium, which increases the activity of nNOS (Xu et al., 1998)

Nitric oxide is known to mediate satellite cell activation in two ways; in response to shear stress and muscle fiber injury, it triggers various intracellular signalling molecule such as NO to activate MMP 2/9, which degrade ECM proteins, resulting in the release of HGF; HGF binds to the c-met receptor leading to changes in gene expression (Le Grand and Rudnicki, 2007). NO also stimulate expression of follistatin which antagonizes myostatin expressed by quiescent satellite cells; this contribute to satellite cell exit from the quiescent state to the growth phase (Le Grand and Rudnicki, 2007). Therefore, activation of satellite cells relies on NO production (Tatsumi et al., 2009)

In this study C2C12 myoblasts were used to investigate the role of nitric oxide in myogenesis. The study aimed to firstly establish the level of nitric oxide released by myoblasts during proliferation, migration and differentiation. Secondly, it aimed to assess the effect L-NAME (a NOS inhibitor) on these processes.

2.2 Material and Methods.

2.2.1 Cell culture.

C2C12 myoblasts (ATCC, USA, cat. CRL-1772^{TM}) were cultured in growth media (GM) containing Dulbecco's Modified Eagle's Medium (DMEM, Lonza cat. BE12-604F), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Biowest cat. 5181G-500), 2% (v/v) Penicillin-Streptomycin (P/S Lonza Biowhittaker cat. DE17-602E). The cells were cultured in T75 ventilated culture flasks (WhiteSci Nest cat. 70800) and allowed to reach 70% confluence at 37^{o}C, 5% CO_2. Cells were then subcultured by washing with phosphate buffered saline (PBS), followed by incubation with 2 ml trypsin (Lonza cat. BE17-161F) for 10 minutes at 37^{o}C to detach adherent cells. Growth media (2 ml) was then added to neutralize trypsin and cells passaged into new flasks or multiwell plates as appropriate. For differentiation studies, DMEM was supplemented with 2% horse serum (Biowest, cat SO91G-500) to make differentiation media. Proliferation and migration studies were carried in the absence of serum (serum free media; SFM). For nitric oxide experiments cells were cultured in phenol red free DMEM (Lonza cat. BE12-917F). All cell culture work was carried out in a Biological safety cabinet (level 2) to maintain sterile conditions and cultures incubated at 37^{o}C and 5% CO_2.

2.2.2 Nitric oxide inhibitor (L-NAME)

Non-specific nitric oxide synthase inhibitor L-N-nitroarginine methyl ester hydrochloride (L-NAME, cat. 2356-100, Bio Vision) was reconstituted in distilled water stock concentration (100 mM). Cells were treated with the L-NAME inhibitor at a final concentration of 100 µM or 200 µM (Long et al., 2006).

2.2.3 Nitric oxide assay.

The level of NO was quantified using a nitric oxide calorimetric assay kit (cat. K262-200, Bio Vision). The assay quantifies NO production in a simple two-step process; the first step being the conversion of nitrate to nitrite using nitrate reductase. The second step uses Griess reagent to convert nitrite to a deep purple azo compound; the amount of azochromophore indirectly reflects the amount of nitric oxide in a sample. A standard curve of absorbance (540 nm) against nitrite concentration was generated to extrapolate the amount of nitric oxide (NO) in a sample. The standard nitrate working solution (1 mM) was prepared by mixing 5 µl of Nitrate standard (100 mM) with 495 µl assay buffer. Standards were prepared according to manufactures instructions as outlined below (Table 1). The absorbance was measured at 540 nm in a 96 well plate using a VERSA max microplate reader, the absorbance of the generated nitrite was plotted to generate standard curve (Figure 2.1).

Table 2.1: Sample preparation for nitrite standard curve

Concentration(nmol/well)	**0**	**2**	**4**	**6**	**8**	**10**	Incubation time
Standard nitrate solution (µl)	0	2	4	6	8	10	-
Nitrate reductase (µl)	-	5	5	5	5	5	
Enzyme cofactor (µl)	-	5	5	5	5	5	1 hour
Enhance (µl)	-	5	5	5	5	5	10 Min
Griess reagent R1	-	50	50	50	50	50	10 Min
R2	-	50	50	50	50	50	
Assay buffer (µl)	115	-	-	-	-	-	-

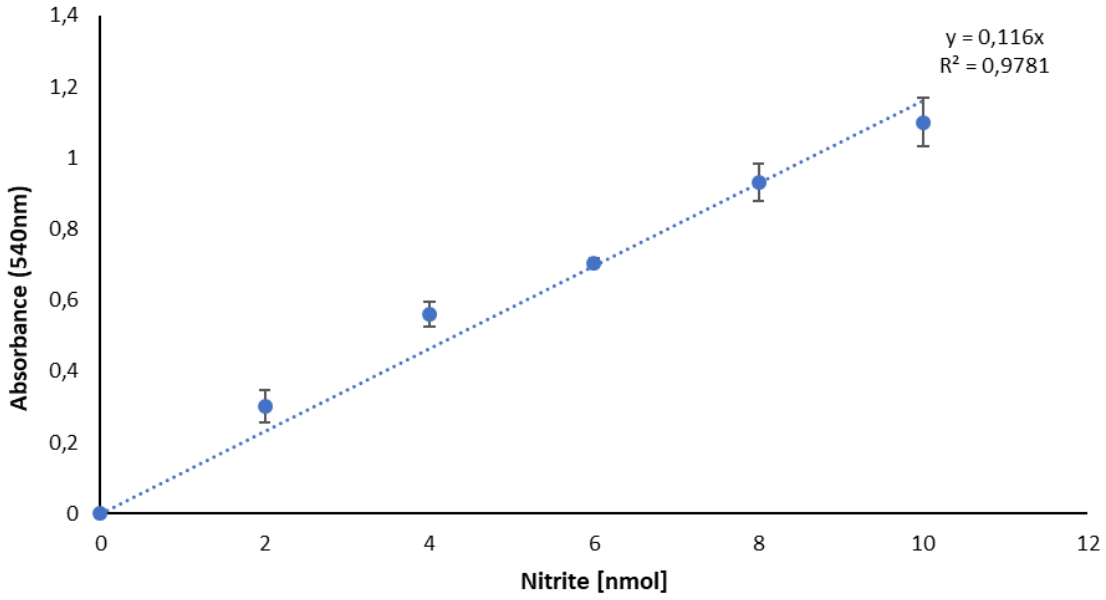


Figure 2.1: Standard curve of absorbance versus nitrite concentration (nmol). The absorbance of nitrite was measured at 540 nm using a 96-well plate. The standard curve was used to extrapolate the amount of nitric oxide generated by cells. The standard solution was prepared in triplicate. Data represent mean ±SEM values, n=3.

2.2.4 Hepatocyte growth factor (HGF)

Human HGF (PeproTech, cat. 100-39H-5UG) was reconstituted in PBS stock concentration (10 mg/ml) and was used at a final concentration of 2 ng/ml to stimulate myoblast proliferation (Walker et al., 2015). The cells were treated with HGF for 24 hours incubated at 37^{o}C, 5%CO_2.

2.2.5 Crystal violet assay

Crystal violet intercalates between bases of DNA of viable cells, 0.2% (w/v) crystal violet was dissolved in methanol. Following 24 hours of myoblast proliferation used media was discard, cells were washed with PBS, followed by staining cells with crystal violet for 10 minutes and washed with water and the plates were air dried at room temperature for overnight.

2.2.6 Analysis of NO release and cell number under proliferative condition.

C2C12 cells (20x10^3 cell/well) were plated in growth media in a 24-well plate and allowed to adhere for 3 hours. Cells were then washed with PBS and incubated with serum free media in the presence or absence of 100 µM/ 200 µM (L-NAME), 2 ng/ml (HGF), or 2 ng/ml (HGF) +100 µM /200 µM (L-NAME). Conditioned media (85 µl) was harvested at 1 hour and 24 hours and NO released quantified as described in Section 2.2.3. Control cells were stained with crystal violet (Section 2.2.5) for 10 minutes at this point t=0 hour to determine baseline cell number. After 24 hours, cells were washed with PBS, stained with crystal violet (Section

2.2.5) for 10 minutes, washed with water and the plates left to air dry at room temperature. Images (five field of view per well) were taken using 4x objective lens of inverted Olympus CKX41 light microscopy with a Motic camera and cell number was determined using ImageJ.

2.2.7 Analysis of NO release and migration during the in vitro wound healing assay.

C2C12 cells ($120x10^3$ cells/well) were seeded in a 12-well plate and growth media was added. The cells were cultured to 90% confluence and scratched with a pipette tip to stimulate a wound (Goetsch and Niesler, 2011). The media was then removed, and the cells washed with phosphate buffered saline (PBS). Cells were then incubated with serum free media (SFM) as a control or SFM treated with 100 µM or 200 µM L-NAME. Conditioned media was harvested at 1 hour, 7 hours and 16 hours post wounding and NO levels quantified using the nitric oxide colorimetric assay kit (as described in Section 2.2.3). The migration of C2C12 cells was monitored by taking images of wound closure at 0, 7 and 16 hours using 4x objective lens of an inverted Olympus CKX41 light microscopy with an attached Motic camera. The percentage wound closure was determined at 7 and 16 hours using image analysis (ImageJ macro) to measure the wound area in each image. Percentage wound closure was determined using the formula below.

$$\%\text{Wound closure} = \frac{\text{Area of wound at 0hr} - \text{Area of wound at Xhour}}{\text{Area of wound at 0hour}}$$

2.2.8 Analysis of NO released and alignment & fusion under differentiation condition

Myoblasts ($60x10^3$ cells/well) were cultured in growth media in a 24 well plate. At 80% confluence, the cells were washed with PBS and switched to differentiation media. Cells were treated with differentiation media in the presence or absence of L-NAME (100 µM or 200 µM) over a period of five days; differentiation media in the absence of cells was used as a negative control. Differentiation media was changed every 48 hours; L-NAME was added at each media change. Conditioned media was harvested at day 0, day 1, day 2 and day 3 of differentiation. Nitric oxide released during these days of differentiation was measured immediately after harvesting condition media using the nitric oxide colorimetric assay kit as outlined in section 2.2.3. At day 5, cells were fixed with 70% ethanol for 10 minutes and stained with 6% Ladd's stain for 15 minutes (McColl et al., 2016). Images (5 fields of view per well) were taken using Olympus CKX41 inverted light microscope and Motic camera using 10x objective for alignment analysis. The images were first analysed using ImageJ macro and then further analysed using CT Fire which analyse cell orientation to determine alignment index (Venter and Niesler, 2018). For fusion analysis images were taken using a

20x objective. The images showing fusion were analysed using ImageJ macro to determine myofiber area (percentage).

2.2.9 Statistical analysis

All data generated were determined to be normally distributed; results were analysed using a parametric paired, two-tailed student T-test and values of $p< 0.05$ were considered to be statistically significant compared to the control. Experiment represented either 3 (n=3) or 6 biological repeats (n=6). All data were represented as mean ±SEM.

2.3 Results

2.3.1 Baseline NO levels during proliferation

Nitric oxide levels were quantified under proliferative conditions in serum free media in the presence or absence of HGF (2 ng/ml), L-NAME (200 µM) or HGF+L-NAME (Figure 2.2). HGF is known to stimulate myoblast proliferation (Walker et al., 2015). Nitric oxide levels were significantly changed at 1 hour in response to HGF. NO levels at 1 hour was 0.58±0.032 nmol in response to HGF compared with 0.44±0.018 nmol NO in control conditioned media ($p<0.05$) (Figure 2.2). Despite the significant increase in response to HGF, levels of NO were low under all conditions ranging from approximately 0.4 – 0.6 nmol; these levels were similar to those observed in DMEM. There was no significant change in nitric oxide levels under any other conditions (Figure 2.2).

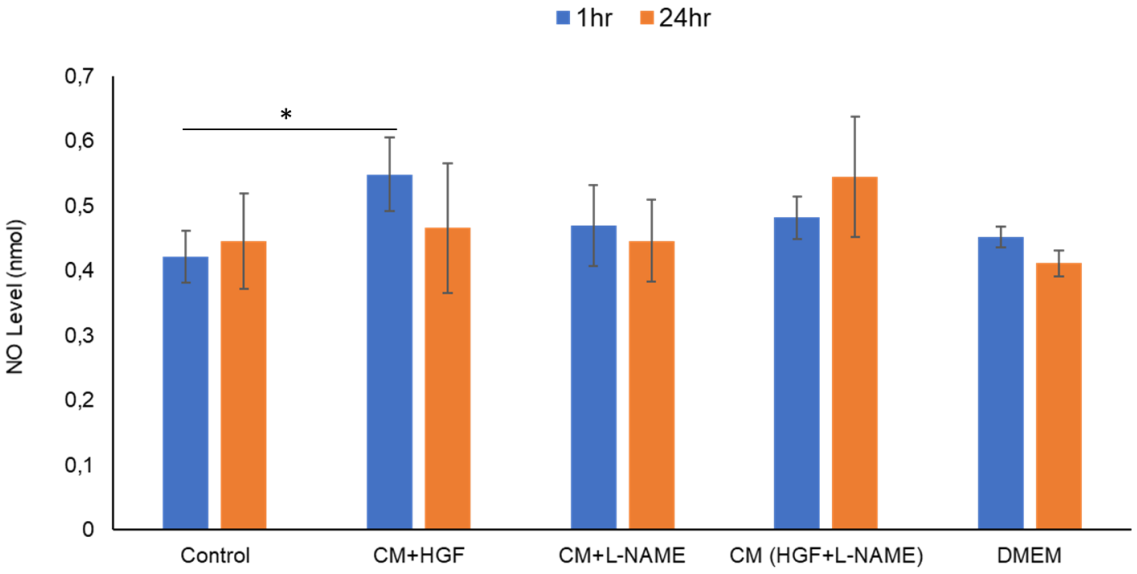


Figure 2.2: Nitric oxide levels released by proliferating C2C12 cells. Cells were cultured in SFM in the presence or absence of HGF (2 ng/ml), L-NAME (200 µM) or HGF+L-NAME. Conditioned media was harvested at 1 hour (blue bars) and 24 hours (orange bars) for NO quantification using the nitric oxide colorimetric kit assay. DMEM was used as a negative control. The experiment was done in 3 biological and technical repeats, each in duplicate (n=3). Data are presented as mean ±SEM *p<0.05.

2.3.2 Effect of nitric oxide inhibition on myoblast proliferation

To establish whether the pro-proliferative effect of HGF is mediated by nitric oxide, cells were cultured in the presence and absence of HGF and L-NAME (100 µM and 200 µM) (Figure 2.3A). In response to HGF there was a clear increase in cell number, which was reduced in the presence of L-NAME (Figure 2.3A). Myoblast cell numbers increased significantly to 195.8%±22.4 in the presence of HGF compared to control (128%±14.9, $p<0.05$; Figure 2.3B). L-NAME did not significantly affect baseline myoblast proliferation in the presence of HGF, as compared to control (SFM) (Figure 2.3B). The effect of HGF on myoblast proliferation was however, significantly reduced in the presence of both 100 µM and 200 µM L-NAME to 156.9%±17.2 and 152%±17.1 respectively ($p<0.05$; Figure 2.3B). The proliferative effect of HGF was however not completely abolished by L-NAME, suggesting that nitric oxide may not be the only mediator of HGF induced proliferation.

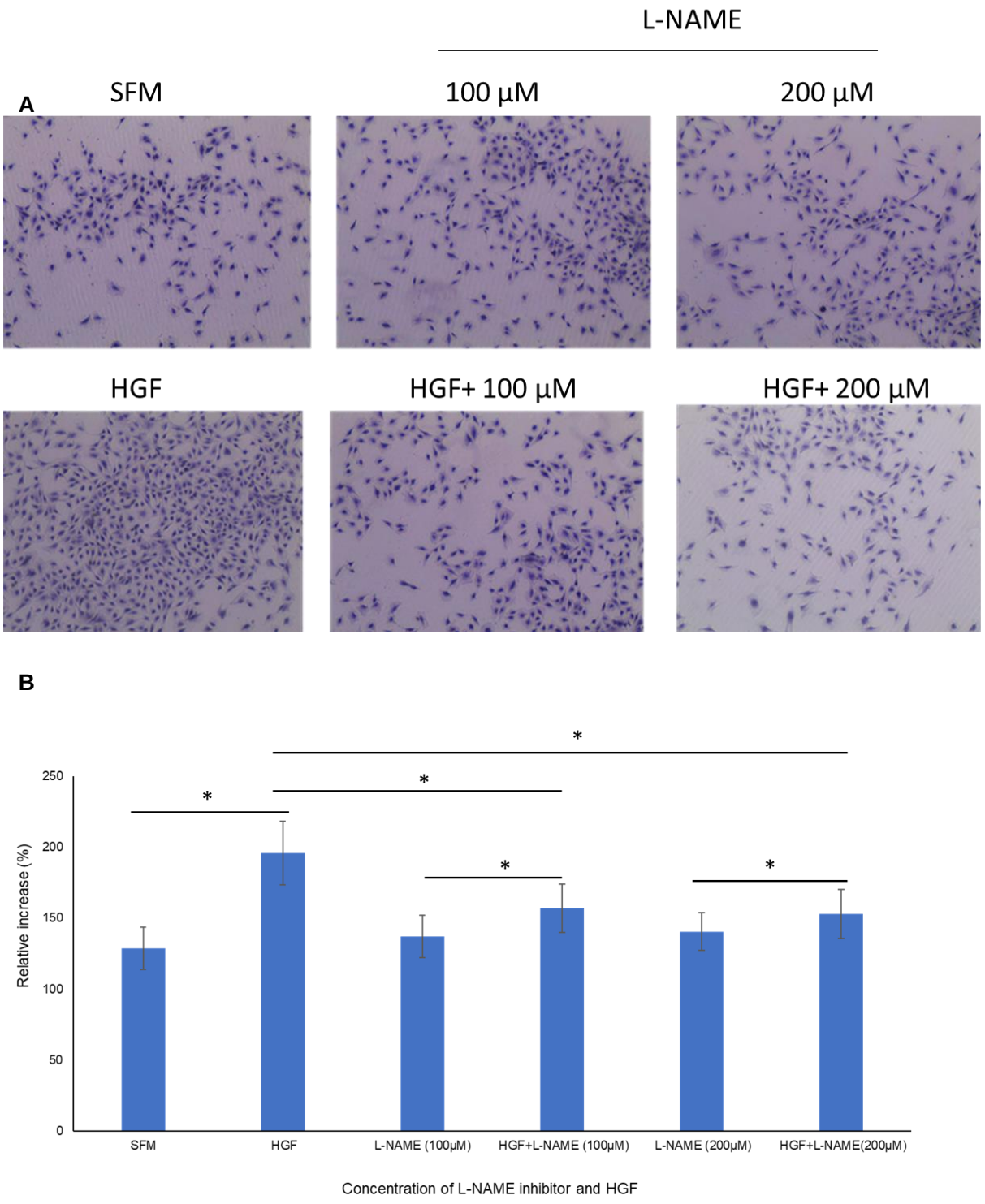


Figure 2.3: Effect of L-NAME on HGF-stimulated myoblast proliferation. Cells were treated in the presence & absence of L-NAME ± HGF for 24 hours and stained with crystal violet. Images were taken (**A**) and analysed using ImageJ macro to determine cell number expressed as relative increase (%) (**B**). The relative increase percentage was calculated by comparing cell numbers at 0 hour to cell number at 24 hours. The experiment was done in serum free media, in 6 biological and technical repeats (n=6) in quadruplet. Data are presented as mean ±SEM, *p<0.05.

2.3.3 Nitric oxide release during myoblast migration is minimal.

Nitric oxide release by myoblasts was further quantified during cellular migration (Figure 2.4). C2C12 cells were wounded with a pipette tip and then treated with or without L-NAME in SFM; conditioned media (85 µl) was harvested at 1 hour, 7 hours and 16 hours post-wounding. Nitric oxide levels remained low, ranging between 0.4 nmol – 0.6 nmol and there was no significant changes in the levels at all times (Figure 2.4).

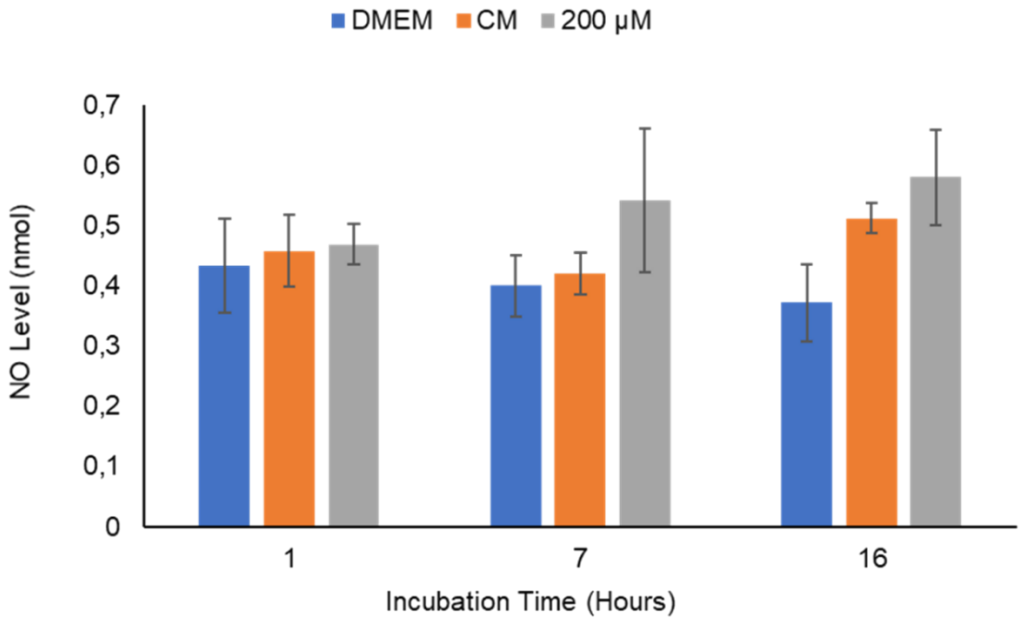


Figure 2.4: Nitric oxide levels following myoblast injury. Cells were culture in SFM ±200 µM L-NAME (orange & grey) and DMEM (in the absence of cell) was used as a negative control (blue). Conditioned media was harvested at 1 hours, 7 hours and 16 hours for NO quantification. The experiment was done in 3 biological and technical repeats (n=3) in duplicate. Data are presented as Mean ±SEM

2.3.4 Myoblast migration in the presence of L-NAME.

Despite the low nitric oxide levels quantified in conditioned media of migrating myoblasts, we wished to confirm the role of nitric oxide in myoblast migration after injury *in vitro*. The role of NO on myoblast migration was investigated at 7 and 16 hours respectively in the presence of L-NAME (Figure 2.5). Myoblasts were observed to migrate towards the injury site to close the wound; wound closure increased as incubation time increased both in the presence and absence of L-NAME (Figure 2.5A). Percentage wound closure was 28%±2.4 and 43%±5.1 under control condition at 7 and 16 hours respectively (Figure 2.5B). No significant effect of L-NAME on percentage wound closure was observed at 7 hours post-wounding. However, at 16 hours the percentage wound closure in response to L-NAME (200 µM) decreased significantly to 33%±4.8 (Figure 2.5B). L-NAME (100 µM) however had no significant effect on myoblast migration at either 7 hours and 16 hours.

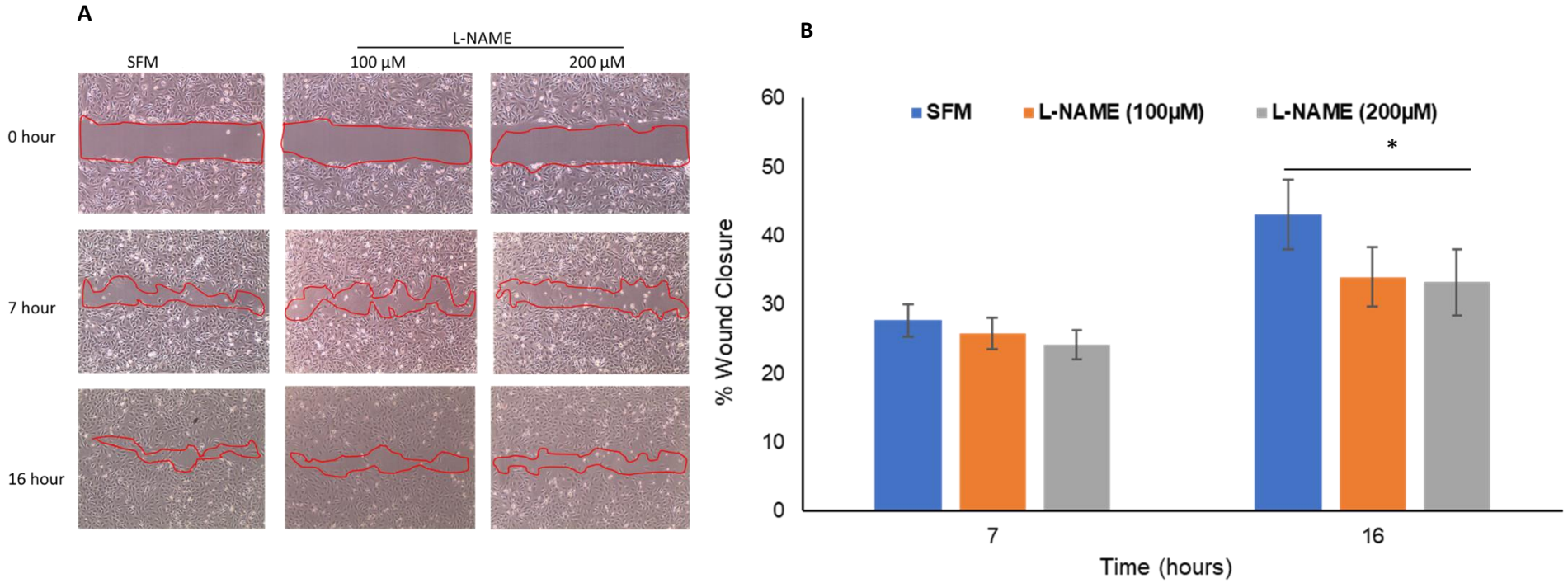


Figure 2.5: Myoblast migration in the presence of L-NAME inhibitor. Cells were scratched and treated with 100 µM (orange bar) and 200 µM (grey bar) L-NAME. Myoblast migration was monitored at 0, 7 and 16 hours using inverted light microscopy a with Motic camera (**A**). The percentage wound closure was calculated at each time point (**B**). The experiment was carried at 6 times biological and technical repeats (n=6) in triplicate. Data are presented as mean±SEM, *p<0.05.

2.3.5 Nitric oxide levels during myoblast differentiation.

Nitric oxide released by differentiating myoblasts was quantified over four days, in the presence or absence of L-NAME (200 µM) (Figure 2.6). Nitric oxide levels in differentiation media in the absence of cells was 0.54 nmol±0.09 at day 0 (DM; Figure 2.6). Immediately after induction of differentiation, nitric oxide increased to 6.3 nmol±0.74 (CM; Figure 2.6); L-NAME significantly prevented this increase at day 0 and maintained baseline levels at 0.78 nmol±0.13 (L-NAME; Figure 2.6). From day 1 to day 3, nitric oxide levels generated by myoblast in the absence of L-NAME, decreased slowly reaching baseline level by day 3 (0.35 nmol±0.087; $p<0.05$). During this time, the presence of L-NAME resulted in variable nitric oxide levels; This may be related to the fact that L-NAME was added on day 0 and day 2, not day 1 or day 3 (Figure 2.6).

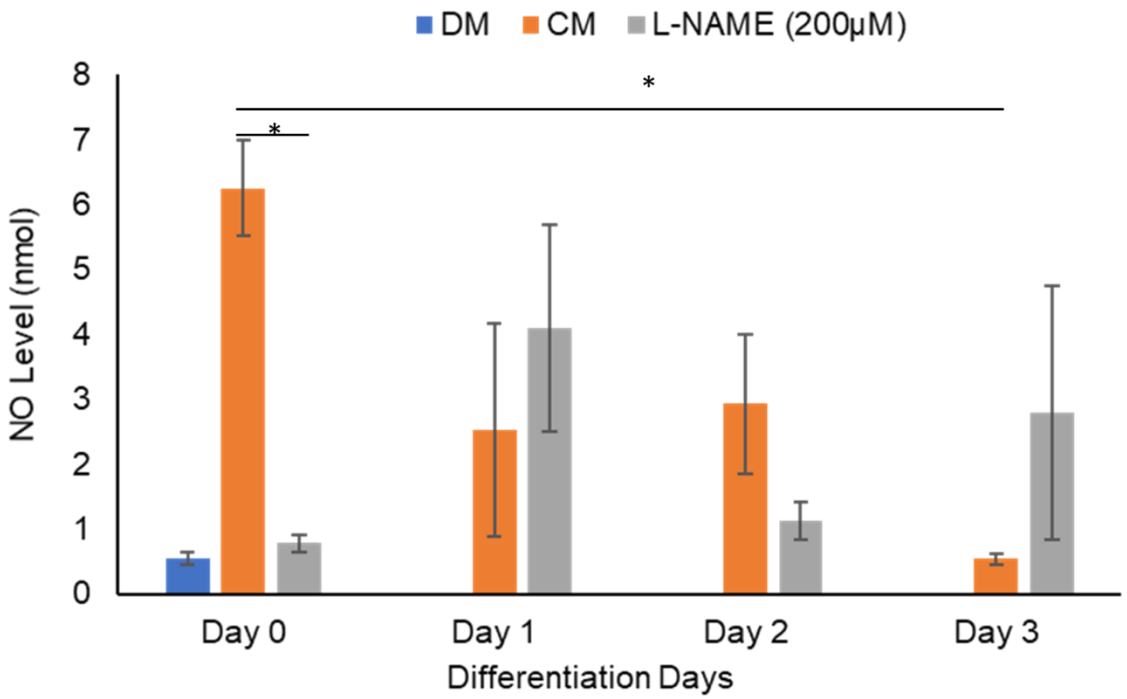


Figure 2.6: Nitric oxide released by differentiating C2C12 myoblasts. Myoblasts were cultured in growth media, then switched to differentiation media. Conditioned media of differentiating cells (CM, orange bar), differentiation media treated with 200 µM L-NAME (grey bar) and differentiation media without cells (DM, blue bar). Conditioned media was harvested at day 0, 1, 2 and 3. Nitric oxide levels were quantified using the nitric oxide kit assay. Differentiation media ±L-NAME was changed every 48 hours. The experiment was done in 3 biological and technical repeats (n=3) in duplicate. Data are presented as mean ±SEM, *$p<0.05$.

2.3.6. L-NAME reduces myoblast terminal differentiation.

During differentiation, myoblasts align with each other and fuse with the existing fiber to form multinucleated myotubes which mature to muscle fibers. In order to determine the role of NO in alignment and fusion, L-NAME was added, and differentiation analysed at day 5. (Figure

2.7). Cells that were cultured with differentiation media alone (control) had an alignment index of 0.312±0.05. Low concentration of L-NAME (100 µM) had no negative effect on myoblast alignment with an alignment index 0.316±0.04 (Figure 2.7A). Higher concentrations of L-NAME (200 µM) significantly decreased myoblast alignment to 0.262±0.07; $p<0.05$ (Figure 2.7A). The myofiber area also decreased in response to L-NAME as compared to the control (Figure 2.7B). The cells that were treated with differentiation media only had a myofiber area of 17.2%±2.7, whereas in response to 100 µM L-NAME they had 15.7%±1.5 a myofiber area (Figure 2.7B). In response to L-NAME (200 µM) the myofiber area was further decreased significantly to 11.9%±0.96 (Figure 2.7B; $p<0.05$).

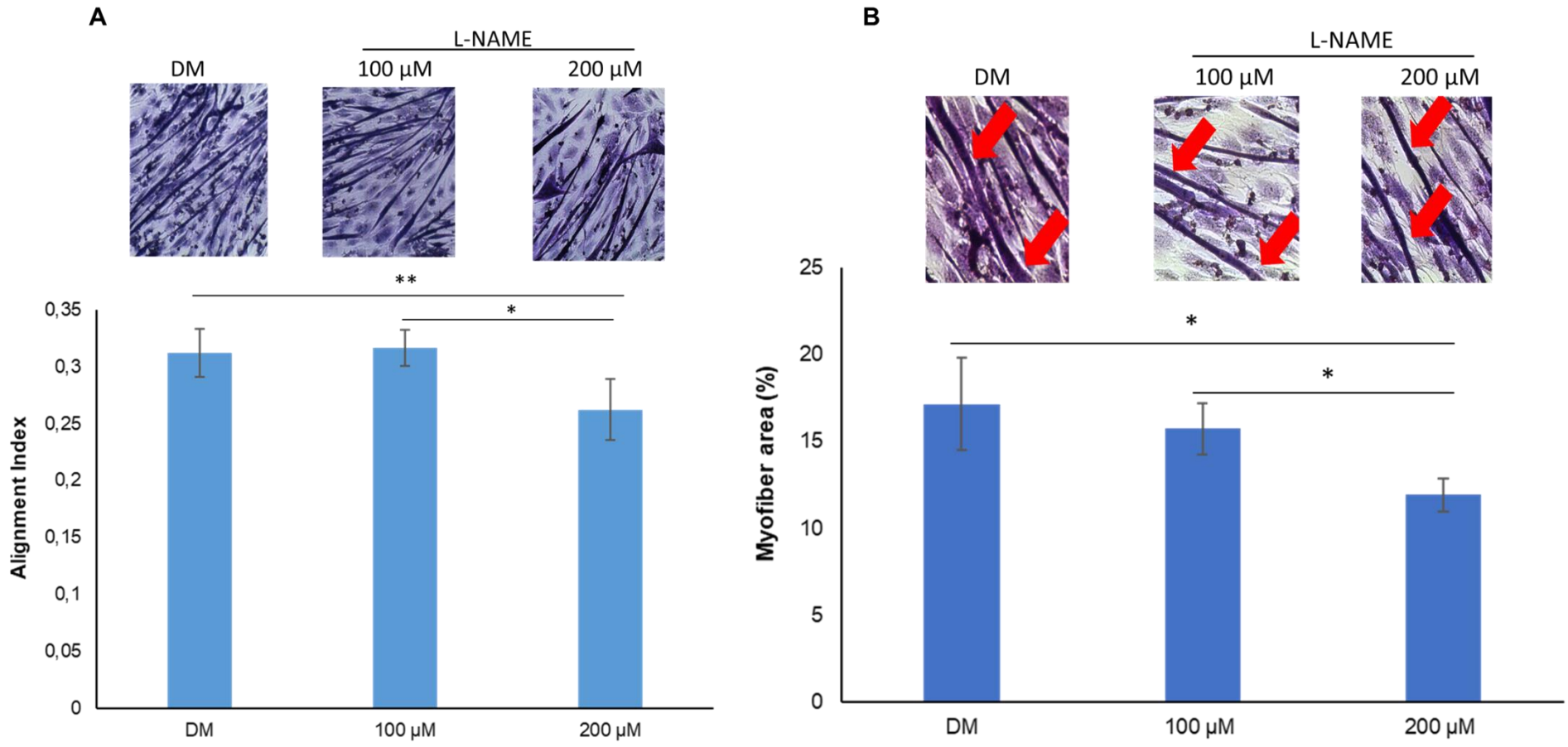


Figure 2. 7: Effect of L-NAME on myoblast alignment and fusion. Myoblast were differentiated for 5 days in differentiation media in the presence or absence of 100 µM and 200 µM L-NAME. Myoblast alignment was analysed at day 5 and CT fire and imageJ programs were used to process image and to determine alignment index, the image of myoblast cell stained with Ladd's stain (10X magnification) (**A**). Myoblast fusion was determined at day 5 by calculate fusion index, images of multinucleated myotubes shown by red arrows (20X magnification) (**B**). n=6 in quadruplet, data represent mean ± SEM, **p<0.005,*p<0.05.

2.4 Discussion.

Nitric oxide is known to mediate satellite cell activation (Tengan et al., 2012), however, its role in myogenic processes, such as proliferation, migration and differentiation, is unclear. In the present study we analysed the level of nitric oxide released by myoblasts during myogenesis and then determined the effect of NOS inhibition on these processes. HGF signalling via the tyrosine kinase receptor c-Met, is known to promote satellite cell activation and proliferation, which drives skeletal muscle repair (Gal-Levi et al., 1998). HGF was therefore utilized to promote proliferation under serum free conditions. In response to HGF, a small but significant increase in NO release was detected at 1-hour post HGF stimulation; NO levels were subsequently reduced to baseline by 24 hours. Addition of L-NAME prevented the significant increase in NO levels at 1 hour, but did not reduce levels to baseline. The reason for this is unclear, but may be due to a relative short half-life of L-NAME estimated to range between 12-30 minutes (Víteček et al., 2012). This means that by the time nitric oxide was quantified at 1 hour the cells were starting to recover the inhibition and express more NOS to stimulate nitric oxide release. The effect of inhibiting NO generation on myoblast proliferation was then investigated. Myoblast proliferation was significantly promoted in response to HGF (2 ng/ml); addition of L-NAME significantly decreased this effect. This demonstrates that HGF achieves its effect on proliferation, at least in part, via NO. However, L-NAME was not observed to decrease the proliferative effect of HGF to baseline seen in serum free media, suggesting that perhaps other mechanisms may also mediate HGF directed proliferation.

Generation of NO by migrating myoblasts was further quantified in response to *in vitro* injury; there was no significant change in nitric oxide levels, suggesting that little nitric oxide is released by migrating myoblasts. Previous studies have shown that NO levels increase after injury and they gradually decrease as the healing process proceeds *in vivo* (Han et al., 2012). At 16 hours, the level of nitric oxide in the conditioned media was increased slightly both in the presence and absence of L-NAME. This may be attributed to proliferation and instability of L-NAME. The generation of nitric oxide by myoblasts is endogenously stimulated by neuronal nitric oxide synthase (nNOS) which is located at the sarcolemma (Patwell et al., 2004). Low levels of NO might be due to the fact that myoblast outside their niche are unable to release sufficient NO; as nNOS requires calcium to be activated, levels may not have been optimal in culture.

In response to L-NAME, *in vitro* wound closure was not affected over 7 hours; this was expected given the lack of NO release. However, a significant effect on migration was observed at 16 hours in response to 200 µM L-NAME. These results correlate with the literature, which state that treatment of myoblasts with 200 µM reduce the migration of cells

toward the scratch (Soltow et al., 2010). The process of wound healing, which include, satellite cell activation, division, alignment and fusion to repair damage site depends on NO generation and release of HGF from extracellular matrix (Filippin et al., 2009). Therefore, the inhibition of NO synthesis makes it difficult for the process to be successfully achieved.

Interestingly, in response to differentiation stimuli, nitric oxide levels increase more than 10-fold within the first hour and subsequently decrease to baseline over 3 days. Addition of L-NAME prevented this increase in NO release These results suggest that NO may play a role in myoblast terminal differentiation. The role of nitric oxide on myoblast terminal differentiation was further evaluated, where myoblast alignment and fusion were analysed in response to L-NAME treatment. Inhibition of nitric oxide production by L-NAME resulted in a small, but significant, decrease in fusion and alignment. This further suggests that NO has a role during terminal differentiation. The reduction of fusion in response to L-NAME, suggests that the inhibition of NO release might have an inhibitory effect on expression of myogenic proteins such as MyHC, MyoD, and myogenin, this requires further investigation. The results obtained are congruent with the literature regarding the inhibitory effect of L-NAME on differentiation and fusion. Previous results have also demonstrated that inhibition of NO generation by L-NAME delayed differentiation and subsequently reduced fusion (Long et al., 2006). In addition, nitric oxide has been shown to stimulate fusion via L-arginine supplementation which is a substrate for NO generation (Long et al., 2006).

2.5 Conclusion.

In conclusion, we have established that nitric oxide generated by myoblast cells plays a significant role in myoblast proliferation and differentiation, thereby contributing to skeletal muscle regeneration. Significant nitric oxide release was not observed following injury indicating that NO release by myoblasts might not play a crucial role in myoblast migration *in vitro*. The release of NO by other cells such as macrophages may be more important. In the next chapter we utilize the nitric oxide donor SIN-1 (3-Morpholinosydnonimine) to determine the direct effect of NO on myoblast proliferation, migration and differentiation.

Chapter 3: Effect of 3-Morpholinosydnonimine (SIN-1, NO donor) on Myoblast Proliferation, Migration and Differentiation

Abstract

3-Morpholinosydnonimine (SIN-1) is a nitric oxide donor which spontaneously decomposes to released nitric oxide and superoxide anion radicals. This compound is spontaneously metabolized to release nitric oxide, independent to NOS activity. SIN-1 has been extensively used in *in vivo* studies and has been demonstrated to stimulate skeletal myoblast proliferation and fusion in rats. The present study aimed to analyse the effect of raised NO levels on myogenesis *in vitro*. Nitric oxide levels released in response to SIN-1 (10 µM or 25 µM) were first quantified over 24 hours using a nitric oxide colorimetric assay kit. Nitric oxide levels increased in a dose-dependent manner, reaching maximal levels at 16 hour post-treatment in response to 25 µM. C2C12 myoblasts were then cultured in the presence or absence of 10 µM and 25 µM SIN-1 to determine the effect of this NO donor on myoblast proliferation, migration and differentiation. Proliferation was analysed using the crystal violet assay, migration using the *in vitro* wound healing assay and differentiation by assessing myoblast fusion to multinucleated myotubes. SIN-1 demonstrated no significant effect on myoblast proliferation after 24 hours post-treatment, however it significantly stimulated myoblast migration in a dose-dependent manner, increasing percentage wound closure to 45% at 7 hours compared to 38% under control conditions. In addition, SIN-1 was found to significantly stimulate myoblast fusion. Increasing NO levels can therefore significantly affect myogenesis by stimulating myoblast migration and fusion, further supporting a role for nitric oxide plays during skeletal muscle regeneration.

Key words: L-Arginine; Myoblast; Nitric oxide; NOS; SIN-1

3.1 Introduction.

Endogenous NO generation can be facilitated by both NOS-dependent as well as NOS-independent mechanisms. NOS-dependent nitric oxide synthesis requires the amino acid L-arginine, which is a key substrate and a limiting factor for NOS activity (Long et al., 2006). This enzyme catalyses oxidation of L-arginine to L-citrulline and nitric oxide. L-Arginine is the source of nitrogen in nitric oxide generation by NOS, due to the guanadino group present in the side chain of this amino acid (Kohli et al., 2004). L-arginine supplementation therefore boosts the generation of endogenous nitric oxide (Long et al., 2006). Diabetic and hypertensive patients, that display impaired nitric oxide biosynthesis, can increase NO production via L-arginine supplementation, thereby stimulating vasodilation and reducing blood pressure (Alvares et al., 2012, Long et al., 2006). In addition, L-arginine supplementation has been shown to (by stimulating myoblast fusion) maintain skeletal muscle function and stimulate regenerative capacity; these effects are attributed to an increase in NO production (Long et al., 2006).

Nitric oxide donors are compounds that support nonenzymatic endogenous nitric oxide generation and are useful tools to further evaluate the fundamental role of NO in physiological process. These compounds are spontaneously metabolized to release nitric oxide, independent of NOS activity (Ignarro et al., 2002). In the 1980's, interest in the physiological role of nitric oxide grew rapidly, resulting in the development of many NO donors (Ignarro et al., 2002). Due to known short half-life of nitric oxide *in vivo*, compounds with capacity to release more NO are now extensively used in research to further understand NO functioning (Ignarro et al., 2002).

Sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) are nitric oxide donors that have demonstrated positive effects on myoblasts, increasing proliferation and stimulating early fusion *in vitro* (Ulibarri et al., 1999). Nitric oxide release from SNP is either enzymatic or nonenzymatic, in biological system it requires tissue or oxidizing agent (Wang et al., 2002). 3-Morpholinosydnonimine hydrochloride (SIN-1) was first synthesized in 1957 as a NO donor; it nonenzymatically decomposes in three steps to release nitric oxide and superoxide anion radical (Wang et al., 2002). SIN-1 has been extensively used both *in vivo* and *in vitro* studies (Singh et al., 1999). It firstly isomerizes into an open ring to form SIN-1A, which reduces oxygen by one electron transfer to yield superoxide and SIN-$1^{\cdot+}$ (radical), SIN-$1^{\cdot+}$ then spontaneously decomposes to form nitric oxide ($NO^{\cdot}$) and SIN-1C (Singh et al., 1999) (Figure 3.1). SIN-1 decomposition increases nitric oxide levels, which activate soluble guanylyl cyclase, which in turn converts GTP to cGMP resulting in various cellular response such as proliferation and fusion (Reden, 1990). Nitric oxide release by NO donors stimulate

a cascade of signalling mechanisms in skeletal muscle (Godfrey and Schwarte, 2010). SIN-1 by-products, superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$), are highly reactive radicals which react with each other to form peroxynitrite ($ONOO^-$), an oxidizing agent (Singh et al., 1999). In addition, superoxide dismutase stimulates dismutation of superoxide resulting in excessive nitric oxide bioavailability (Singh et al., 1999).

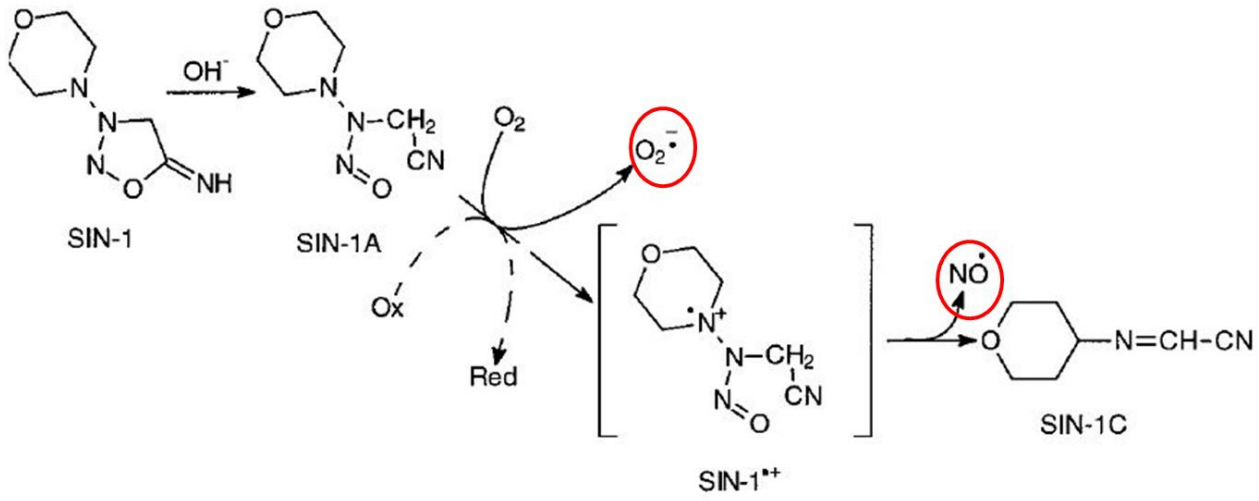


Figure 3.1: SIN-1 decomposition to yield superoxide and nitric oxide. SIN-1 isomerize to an open ring (SIN-1A) due to electron transfer from a hydroxyl radical. The oxidizing agent SIN-1A yields superoxide anion radical ($O_2^{\bullet-}$) and intermediate (SIN-$1^{\bullet+}$), which spontaneously decompose to release nitric oxide ($NO^{\bullet}$) and SIN-1C (Singh et al., 1999).

In the present study, SIN-1 was used to generate nitric oxide *in vitro* and analyse the effect of this radical on myogenesis, specifically proliferation, migration and fusion, in C2C12 myoblasts.

3.2. Materials and Methods

3.2.1 Cell culture

C2C12 myoblasts (ATCC, USA, cat. CRL-1772™) were cultured, as described in section 2.2.1, in growth media (GM) containing Dulbecco's Modified Eagle's Medium (DMEM, Lonza, cat. BE12-604F), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Biowest, cat. 5181G-500), 2% (v/v) Penicillin-Streptomycin (P/S, Lonza, Biowhittaker cat. DE17-602E).

3.2.2 Nitric oxide donor 3-Morpholinosydnonimine (SIN-1) preparation.

3-Morpholinosydnonimine, HCl (SIN-1) (NO donor, Merck, cat. 567028-20MG) is a nitric oxide donor which spontaneously decomposes to yield nitric oxide and other superoxide anion radicals. It was reconstituted in sterile distilled water (50 mM stock concentration); cells were treated with SIN-1 at a final concentration of 10 µM or 25 µM.

3.2.3 Nitric oxide levels release by myoblast in response to NO donor treatment

C2C12 myoblasts ($60x10^3$ cells/well) were plated in growth media in a 24-well plate, then incubated for 3 hours to promote adherence. Media was then removed, and cells were washed with PBS; cells were then incubated with serum free media (SFM) as a control or SFM supplemented with SIN-1 (NO donor, cat no: 567028-20MG, Merck) at a final concentration of either 10 µM or 25 µM. Nitric oxide levels were quantified at 1 hr, 7 hr, 16 hr and 24 hr using a nitric oxide calorimetric assay kit (previously described in Section 2.2.3).

3.2.4 Analysis of myoblast cell number in response to SIN-1 treatment.

C2C12 myoblasts ($20x10^3$ cell/well) were plated in growth media in a 24-well plate and allow to adhere for 3 hours. Cells were then washed with PBS and incubated with serum containing media (SCM; to stimulate proliferation) as a control and SCM supplemented with 10 µM or 25 µM SIN-1 for 24 hours. Baseline cell number was determined at t=0 hour following staining with crystal violet (Section 2.2.5). In addition, after 24 hours, treated cells were washed with PBS, stained with crystal violet and cell number assessed (Section 2.2.5).

3.2.5 Effect of SIN-1 on myoblast migration.

Myoblasts ($120x10^3$ cells/well) were seeded in a 12-well plate and growth media was added. The cells were cultured to 90% confluence and scratched with a pipette tip (Goetsch and Niesler, 2011). The media was then removed, and the cells were washed with phosphate buffered saline (PBS). Cells were then incubated with serum containing media (SCM) as a control and SCM supplemented with 10 µM or 25 µM SIN-1. The migration of C2C12 cells was monitored by taking pictures of wound closure at 0, 5 and 7 hours using 4x objective lens of an inverted Olympus CKX41 light microscopy with a motic camera. The percentage wound closure was determined at 5 and 7 hours using image analysis (ImageJ macro) to measure the wound area in each image. Percentage wound closure was determined using the formula below.

$$\%\text{Wound closure} = \frac{\text{Area of wound at 0hr} - \text{Area of wound at Xhour}}{\text{Area of wound at 0hour}} \times 100$$

3.2.6 Myoblast differentiation in the presence of SIN-1

Myoblasts ($60x10^3$ cells/well) were cultured in growth media in a 24 well plate. At 80% confluence, the cells were washed with PBS and switched to serum containing media (SCM) supplemented with or without SIN-1 (25 µM) for 5 days. The media (with or without SIN-1) was changed every 48 hours until day five of differentiation. One set of cells were stimulated with SIN-1 at day 0 only (single stimulation). A second set of cells were treated with SIN-1 every 48 hours (triple stimulation) until day five of differentiation. At day 5, cells were fixed with 70% ethanol for 10 minutes and stained with 6% Ladd's stain for 15 minutes (McColl et al., 2016). Images (5 fields of view per well) were taken using the Olympus CKX41 inverted light microscope. For fusion analysis, images were taken using a 20x objective lens. The images showing fusion were analysed using ImageJ macro to determine percentage myofiber area.

3.2.7 Statistical analysis

All data generated was determined to be normally distributed; results were analysed using a parametric paired, two-tailed student T-test and values of $p< 0.05$ were considered to be statistically significant compared to the control. Experiments represent 3-6 biological repeats (n=3-6). All data were represented as mean ±SEM.

3.3 Results

3.3.1 Nitric oxide levels in response to SIN-1 treatment

To determine the concentration of NO released in response to SIN-1 in the presence of myoblasts, myoblasts were plated and allowed to adhere in growth media for 3 hours, then treated for 24 hours with SFM in the absence or presence of SIN-1 (10 µM or 25 µM) (Figure 3.2). Nitric oxide levels significantly increased in response to SIN-1, as compared to control, in a time dependent manner (Figure 3.2). Under control conditions NO levels were 0.58 nmol±0.18, at 1 hour; levels were maintained between 0.3 and 0.6 nmol over 24 hours (Figure 3.2). In response to 10 µM SIN-1, NO levels increase significantly to 1.4 nm±0.009, 1.6 nm±0.10 and 1.58 nm±0.11at 1h, 7h and 16h respectively; by 24h levels returned to baseline (Figure 3.2; $p<0.05$). In response to 25 µM; nitric oxide was measured to be 1.9 nmol±0.19 at 1h post-treatment. Moreover, nitric oxide levels significantly increased to 2.4 nmol±0.15, 2.5 nmol±0.22, $p<0.05$ at 7h and 16h respectively. These levels had decreased to baseline by 24h.

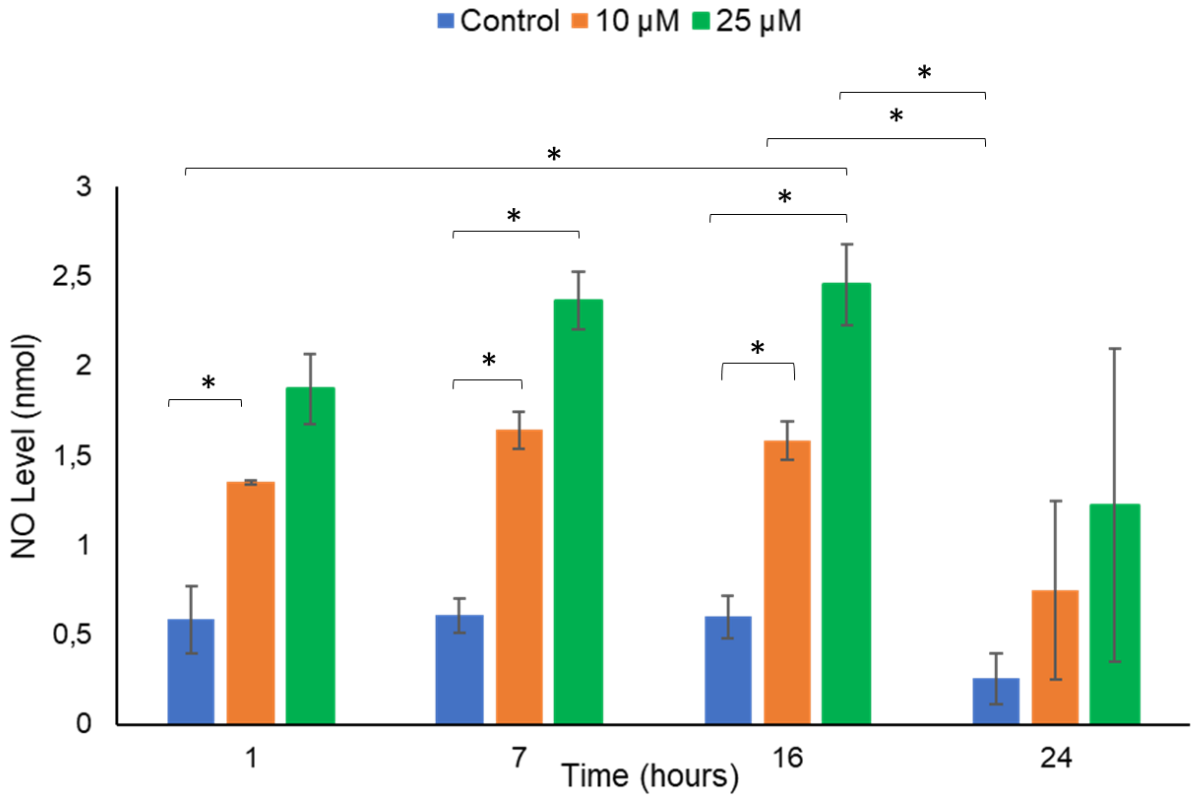


Figure 3.2: Nitric oxide levels post SIN-1 treatment. Myoblasts were treated with NO donor SIN-1 (10 µM and 25 µM) in serum free media (SFM). Conditioned media was harvested at 1, 7, 16 and 24 hours to quantified nitric oxide levels release by cells at these different time points. The experiment was done in 3 biological and technical repeats, each in duplicate (n=3). Data are presented as mean ±SEM *p<0.05.

3.3.2 Effect of 3-Morpholinosydnonimine (SIN-1) on myoblast proliferation.

To determine the effect of increased nitric oxide levels on proliferation, myoblasts were treated with 10 µM or 25 µM SIN-1 in serum containing media (SCM). After 24h, cells were stained with crystal violet to quantify relative cell numbers. Images of stained cells revealed that incubation with SIN-1 appeared to reduce cell number (Figure 3.3A). However, this effect was not significant, as incubation with SIN-1 (10 µM) resulted in relative cell numbers of 173%±7.6, whilst 25 µM resulted in 184%±8.9, compared to the control of 191%±9.3 (Figure 3.3B).

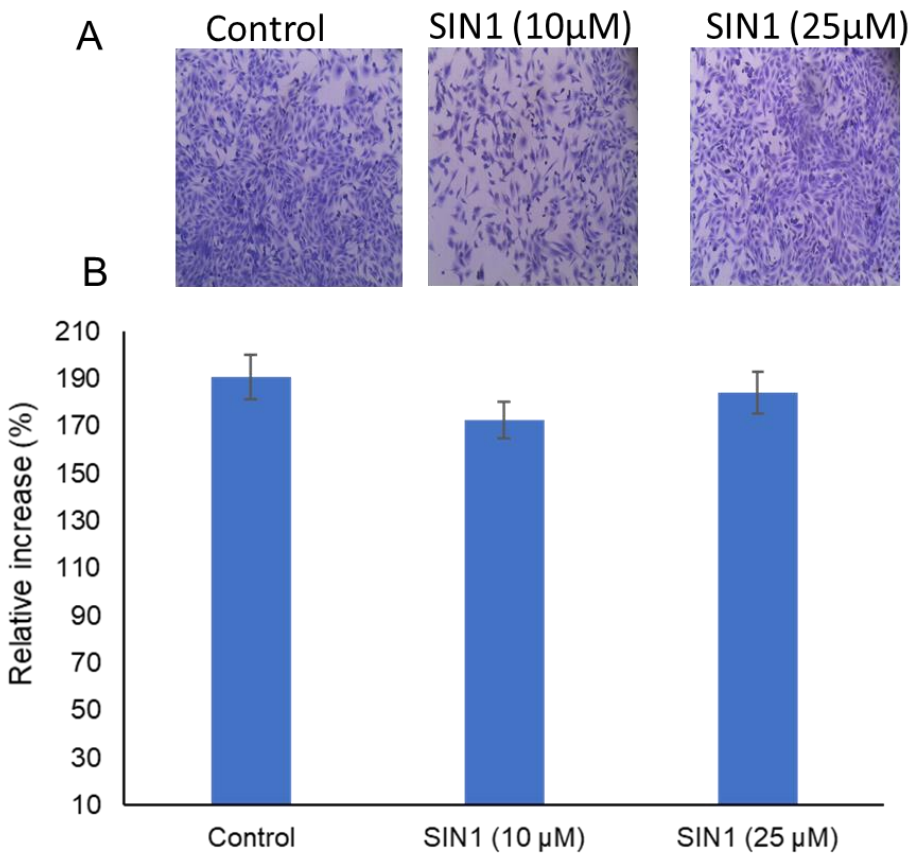


Figure 3.3: Effect of SIN-1 on myoblast proliferation. Cells were treated with SIN-1 for 24 hours and stained with crystal violet. Images were taken using an inverted light microscope with an attached Motic camera, using 4x objective lens (**A**) and analysed using ImageJ macro to determine cell number expressed as relative increase (%)(**B**). The relative increase was calculated by comparing cell number at 0 hour to cell number at 24 hours. The experiment was done serum containing media (SCM), 3 times in triplicate (n=3). Data are presented as mean ±SEM.

3.3.3 SIN-1 promotes myoblast migration.

To evaluate the effect of nitric oxide on myoblast migration, C2C12 myoblasts were treated with SIN-1 (10 µM and 25 µM) in serum containing media (SCM). Myoblast wound closure (monitored at 5 and 7 hours) was observed to increase in a dose-dependent manner (Figure 3.4 A). under control conditions, percentage wound closure was 24%±2 and 38%±2.2 at 5 and 7 hours respectively (Figure 3.4 B). However, in response to SIN-1 (10 µM), percentage wound closure significantly increased to 29%±1.9 ($p<0.05$) at 5 hours and 42%±0.84 at 7 hours. Higher concentrations of SIN-1 (25 µM) resulted in a further significant increase in percentage wound closure to 32%±1.7 ($p<0.005$) at 5 hours and 45%±2.6 ($p<0.05$) at 7 hours (Figure 3.4 B).

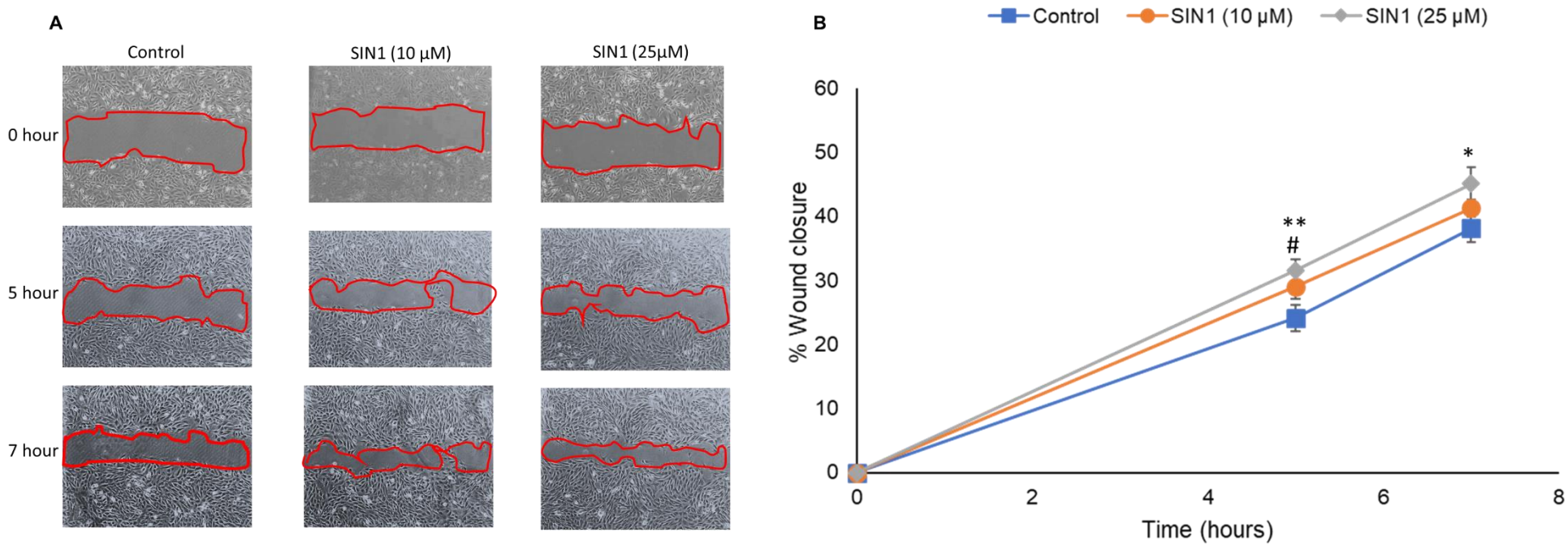


Figure 3.4: Myoblast migration in response to an in vitro scratch in the presence of SIN-1. The cells were scratched and treated with 10 µM (Orange line), 25 µM (Grey line) SIN-1 or SCM alone (Blue line) as a control, myoblast migration was monitored by taking pictures at 0, 5 and 7 hours using an inverted light microscope (4x objective lens) with a Motic camera (**A**). The percentage wound closure was calculated at each time point in the presence or absence of SIN-1 (**B**). The experiment was done in 5 biological and technical repeats (n=5) in triplicate. Data are presented as mean±SEM, #p<0.05 SCM vs SIN-1 10 µM at 5 hours, **p<0.005 SCM vs SIN-1 25 µM at 5 hours, *p<0.05 SCM vs SIN-1 25 µM at 7 hours.

3.3.4 SIN-1 stimulates myoblast fusion

The effect of nitric oxide was further evaluated on myoblast fusion at day five of differentiation. Cells were treated in the absence or presence of SIN-1 (25 µM). The cells treated with SIN-1 were either only exposed to the donor at day 0, or at day 0, 2 and 4 (at each media change). Images (taken at day 5) depict that there is more fusion in cells treated multiple times with SIN-1 as compared to a single SIN-1 dose and control (Figure 3.5 A). Myofiber area in the absence of SIN-1 was 18.6%±3.4 whilst in response to SIN-1 (single) stimulation fusion index increased to 20.3%±3.0 (Figure 3.5B). Myofiber area significantly increased to 26%±1.8, $p<0.05$ in response to multiple SIN-1 (25 µM) treatment compared to both control and single SIN-1 addition (Figure 3.5). The increase in myofiber area shows the increase in fusion and myotube formation.

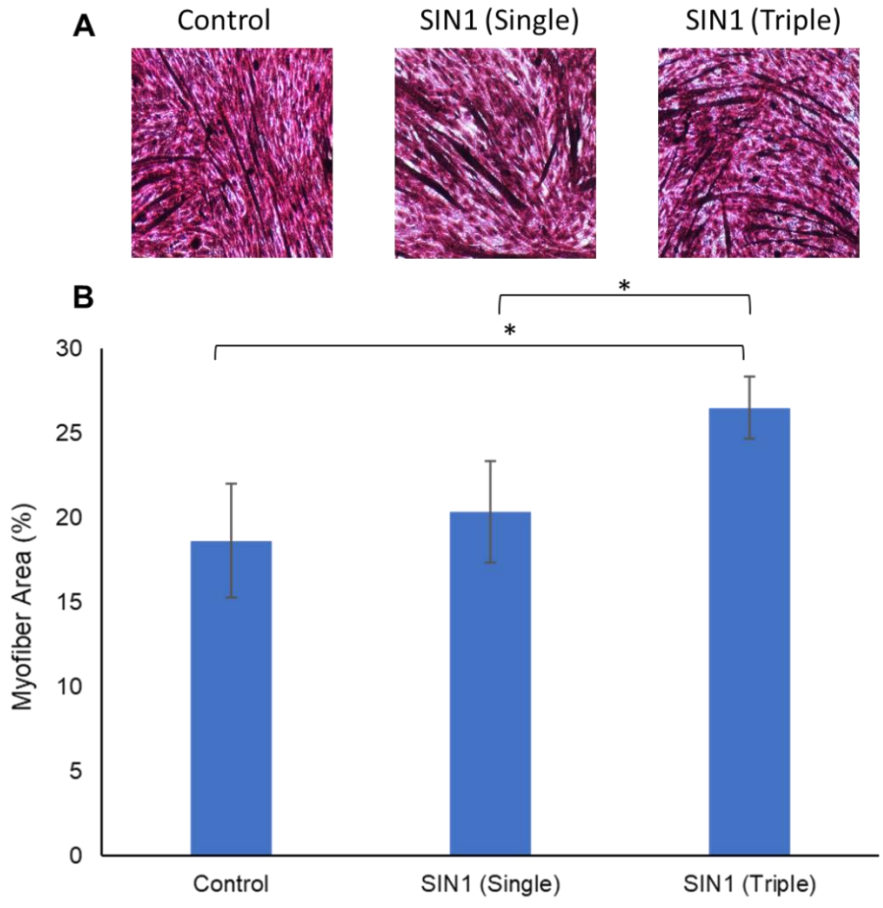


Figure 3.5: Myoblast fusion in response to SIN-1. Myoblasts were cultured for 5 days in the presence or absence of 25 µM SIN-1 (single addition or multiple addition); control refers to cells cultured in SCM alone. Images of multinucleated myotubes were taken at 20x magnification (A). Myoblast fusion was determined at day 5 by calculating fusion index using ImageJ (B), n=4 in quadruplet, data represent mean ± SEM, *p<0.05.

3.4 Discussion

In this chapter, we investigated the ability of elevated NO levels to directly influence myogenesis. The NO donor, SIN-1, was used to achieve elevated NO levels in myoblast cultures. Nitric oxide levels increased over 16 hours in response to SIN-1 and returned to baseline by 24 hours post-incubation. This established that SIN-1 successfully yielded nitric oxide, quantified using Griess reagent kits. Maximum nitric oxide levels were achieved at 16 hours following SIN-1 addition; however, by 24 hours, NO concentrations had returned to control levels. The decrease in NO level at 24 hours is most likely due to the short half-life of both the donor and NO itself. Although most studies using SIN-1 have been carried out *in vivo*, our results concur with previous studies, which have shown that SIN-1 administration enhances nitric oxide production in rats (Xu et al., 2001).

Having seen that SIN-1 increases nitric oxide production in C2C12 myoblast cultures, we were eager to understand its role on experimental processes simulating myogenesis. When the cells were treated with SIN-1 under proliferative condition, there was no significant effect of the elevated NO levels on myoblast proliferation. This is in contrast with the results of the previous chapter, which indicated that HGF-stimulated proliferation is mediated at least in part by NO. This could suggest that HGF-stimulated proliferation utilises pathways in concert with NO. However, the increase in NO concentration in response to HGF was far lower than that caused by SIN-1. Therefore, it is possible that at higher NO levels, this free radical is detrimental to proliferation; this is supported by the negative impact that SIN-1 (although non-significant) had on proliferation. The negative effect of SIN-1 on proliferation could also be mediated by superoxide anion release during decomposition of SIN-1 *in vitro* cultures. SIN-1 is known to reduce myoblast survival in dose dependant manner, where higher concentration of SIN-1 (32 µM) reduced cell survival to 0.1% (Lepore et al., 1999).

We further investigated the effect of SIN-1 on myoblast migration in response to an *in vitro* scratch. SIN-1 significantly promoted myoblast migration and increased the percentage wound closure at 5 and 7 hours in a dose-dependent manner. Migration is a crucial step during myogenesis, ensuring wound healing and skeletal muscle regeneration in response to injury. From these results we can deduce that SIN-1 released NO does support the migration of these muscle precursor cells.

Elevated nitric oxide by SIN-1 were also found to play a significant role in myoblast terminal differentiation. In response to SIN-1, myofiber area significantly increased to 26% at day 5 of differentiation. Addition of SIN-1 at day 0, and every after subsequent 48 hours, stimulated myoblast fusion, suggesting that nitric oxide is essential to initiate and maintain terminal differentiation. The cells that were exposed once (at day 0) with SIN-1 did not display

significant increases in their myofiber area. Results obtained suggests that formation of myotube is significantly enhanced by a consistently increased level of nitric oxide. Previous studies *in vivo* have shown that Molsidomine modulates the myogenic differentiation of embryonic progenitor cells by enhancing expression of differentiation markers (Tirone et al., 2016). In addition, administration of nitric oxide donors in animal models significantly improves muscular regeneration and hence muscular dystrophy diseases is reduced (Tirone et al., 2016).

SIN-1 significantly affected myogenesis by stimulating myoblast migration and fusion; this shows that nitric oxide plays an important role during skeletal muscle regeneration and repair. Nitric oxide release by SIN-1 is independent to NOS activity and it achieves its effect via the cGMP pathway. These results suggest that nitric oxide plays a fundamental physiological role in myoblast migration and fusion and therefore skeletal muscle regeneration.

3.5 Conclusion

In conclusion, SIN-1, a nitric oxide donor, positively affects myogenesis by significantly increasing myoblast migration and wound closure over 7 hours period; this effect is dose dependent. SIN-1 further promotes myoblast fusion by increasing myotube formation at day 5 of differentiation. However, SIN-1 had no significant effect on myoblast proliferation. Dose dependent effect of NO on processes underlying myogenesis do therefore require investigation. However, the current studies have provided evidence that this free radical is a central regulator of myogenesis.

Chapter 4: General discussion and recommendation

Skeletal muscle wound healing and regeneration, involves several cell types including resident skeletal muscle stem cells and non-muscle cell populations, which all ensure that this process occurs successfully (Shi and Garry, 2006). Satellite cells, known as the engine of skeletal muscle wound healing and regeneration exist in a quiescent state and must be activated to initiate myogenesis (Wang and Rudnicki, 2012). In our study we utilize myoblasts in experiments simulating myogenesis process in order to understand various factors and mechanisms involved during this process in the *in vitro* setting. We have an understanding that *in vitro* studies cannot be compared direct with *in vivo* studies, but we can utilize *in vitro* studies to advance *in vivo* hence the whole sum process may be understood well.

In the present study we have utilized activated satellite cells (myoblasts) to evaluate and further understand the role of nitric oxide in myogenesis; we specifically investigated proliferation, migration and differentiation. Our results demonstrated that L-NAME (a NOS inhibitor), reduces myoblast proliferation, migration and terminal differentiation. Furthermore, application of SIN-1, an NO donor, results in a significant increase in myoblast migration and terminal differentiation, but not proliferation. Therefore, the free radical NO plays an essential role in myogenesis.

Nitric oxide quantification in biological samples is essential in providing information concerning nitric oxide bioavailability and metabolism (Bryan and Grisham, 2007). Nitric oxide levels released by cells cultured in the presence or absence of L-NAME or SIN-1 were quantified; this helped us to determine whether C2C12 myoblasts generate NO in NOS-dependent or independent manner. Baseline NO levels were very low (0.4-0.6 nmol) under proliferation or migration conditions. However, in response to SIN-1 treatment or differentiation cues we observed a significant 2-10-fold increase in nitric oxide generation with levels ranging between 1.3-2.5 nmol (SIN-1) or 6 nmol (in response to differentiation). NO levels were evaluated at 1, 7, 16 and 24 hours; short time spans were chosen due to the inherent instability of this radical. Previous studies utilized a real time device to monitor actual NO levels release in cell culture, this method accurately quantifies actual NO level dose and its duration, the device traps all NO particles released and determines how long they last in a culture medium (He and Frost, 2016).

We were then interested in evaluating the role of NO on myoblast proliferation, migration and differentiation, in order to understand the role of nitric oxide in myogenesis. HGF was used to stimulate myoblast proliferation under serum free media conditions; L-NAME significantly reduced HGF-stimulated proliferation. However, when myoblasts were treated

with SIN-1, we obtained results that were contrary to our expectation. We incubated the cells in SCM to overcome the toxic effect of superoxide anion generated from SIN-1 decomposition, but did not obscure an increase in proliferation. This suggests that, although NO mediates (at least in part), HGF-stimulated myoblast proliferation, it does not mediate serum stimulated proliferation. Previous results by others showed that NO donors, (SNP and SNAP) increase myoblasts proliferation *in vivo* (Ulibarri et al., 1999); this contrast with our studies as the donor were administered in rats.

SIN-1 demonstrated a positive effect on myoblast migration and terminal differentiation. NO is therefore likely to be important for myoblast migration, a crucial step in myogenesis. However, as inhibition of NO synthesis by L-NAME was not shown to affect myoblast migration in the absence of other non-myogenic cells, we suggest that cells such as macrophages or fibroblasts may be responsible for the NO-directed regulation of myoblast migration. Myoblast movement toward the injury site precedes alignment and fusion and together these processes support regeneration. *In vivo* studies on NOS knockout mice and L-NAME administration have demonstrated a delay in myoblast migration, hence impaired muscle repair (Anderson, 2000). Our results are in agreement and suggest that NO released during SIN-1 decomposition diffuses rapidly to exert its effect on migration.

NOS inhibition by L-NAME results in a decrease in myoblast terminal differentiation, whilst SIN-1 increased terminal differentiation. This suggests a central role for NO in terminal differentiation. The increase in myoblast terminal differentiation in response to SIN-1 treatment is attributed to NO increase, which could subsequently stimulate MRF expression and regulate differentiation (Sabourin and Rudnicki, 2000). Inhibition of NO generation reduces the expression of these essential factors; hence myoblast terminal differentiation is reduced. Previous results have further demonstrated that NO deficiency and nNOS absence impairs muscle development and reduces the number of myonuclei in a fiber (De Palma and Clementi, 2012). Our results suggest that nitric oxide is an important signalling molecule and it plays a significant role in promoting differentiation. It must be remembered that nitric oxide has several limitations: a relatively short half-life, high reactivity and dual effect toxicity at high concentration and stimulator at low concentration. To contract at least some of these effects, SIN-1 could be coupled with a scavenger that would scavenger superoxide anion radicals to reduce toxicity. In addition, superoxide release during SIN-1 decomposition could also be quantified. In summary, we have established that inhibiting NOS using L-NAME has a significant effect on myoblast proliferation, migration and differentiation. We have also shown that the NO donor SIN-1 promotes myoblast migration and terminal differentiation. Together these results further underscore the potential importance of NO as a myogenic regulator.

Appendix 1: Conference attendance

1) **1st Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST)**

Evaluating the Role of Nitric Oxide on Myoblast Proliferation, Migration and Differentiation

Ntethelelo Sibisi, Prof Carola Niesler, Prof Kathy Myburgh

The current study is aiming to establish the level of nitric oxide released by myoblasts during proliferation, migration and differentiation. And also, to assess the effect of L-NAME (a NOS inhibitor) on these processes, in order to understand the role of nitric oxide on myogenesis process. C2C12 myoblast were cultured in standard growth media and subsequently plated for analysis in DMEM. Proliferation or differentiation was induced via the addition of either 2 ng/ml HGF or 2% horse serum respectively, while migration was analysed using the standard in vitro wound healing assay. L-NAME concentration used was 100 µM or 200 µM. NO levels were assessed using a colorimetric assay. Proliferation was assessed via cell counts; migration by calculating the percentage wound closure and differentiation by myoblast alignment and fusion index analysis. HGF stimulated myoblast proliferation, however levels of NO were only found to be 0.58 nmol at 1h post-HGF stimulated. Similarly, NO following myoblast wounding were 0.31 nmol at 1h and increased to 0.56 nmol over 16h. In response to differentiation cues, NO levels increased to 6 nmol; then dropped as differentiation progressed over five days. Addition of L-NAME (200 µM) resulted in a significant decrease (43% and 10%) in proliferation and migration respectively. Myoblast alignment and fusion was significantly reduced to 18% and 6% respectively at day five of differentiation in response to L-NAME (200 µM). Results suggest that nitric oxide play a significant role during myoblast myogenesis, making NO crucial for skeletal muscle regeneration.

2) South African Society of Biochemistry and Molecular Biology – Federation of African Society of Biochemistry and Molecular Biology (SASBMB-FASBMB).

EVALUATING THE ROLE OF NITRIC OXDE ON MYOBLAST PROLIFERATION, MIGRATION AND DIFFERENTIATION.

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Satellite cells are quiescent myogenic precursor cells, present between the basal lamina and sarcolemma of skeletal muscle. They are activated in response to skeletal muscle injury for muscle regeneration. Nitric oxide is a gaseous free radical that is known to stimulate satellite cell activation to myoblasts; NO production is facilitated by nitric oxide synthase. The role of NO as a potential mediator of myoblast proliferation, migration or differentiation is unclear. The current study therefore aimed to firstly establish the level of nitric oxide released by myoblasts during their proliferation, migration and differentiation. Secondly, it aimed to assess the effect L-NAME (a NOS inhibitor) on these processes. C2C12 cells were cultured in standard growth media and subsequently plated for analysis in serum free media. Proliferation or differentiation was induced via the addition of either 2 ng/ml HGF or 2% horse serum respectively, while migration was analysed using the standard in vitro wound healing assay. L-NAME was utilized at a concentration of 100 µM and 200 µM. NO levels were assessed using a colorimetric assay. Proliferation was assessed via cell counts, while migration was determined by assessing the percentage wound closure. Differentiation was determined by assessing myoblast alignment and subsequent fusion into multinucleated myotubes. HGF (2 ng/ml) stimulated myoblast proliferation, however levels of NO were only found to be 0.58 nmol at 1-hour post-HGF stimulated. Similarly, NO following myoblast wounding were 0.31 nmol at 1h and increased to 0.56 nmol over 16 h. In response to differentiation cues NO levels rose sharply to 6 nmol; these levels dropped as differentiation progressed over five days. Addition of L-NAME (200 µM) only leads to minor, but significant decrease in proliferation (43%) and migration (10%). Addition of L-NAME (200 µM) to differentiating cells significantly reduced myoblast alignment and fusion by 18% and 6% at day five of differentiation. Results suggest that nitric oxide play a significant role during myoblast differentiation, making NO crucial for skeletal muscle regeneration.

3) School of Life Science Postgraduate Research Day

EVALUATING THE ROLE OF NITRIC OXDE ON MYOBLAST PROLIFERATION, MIGRATION AND DIFFERENTIATION.

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Satellite cells are quiescent myogenic precursor cells present between the basal lamina and sarcolemma of skeletal muscle cells. They are activated to myoblasts during muscle damage to facilitate regeneration and repair processes. Nitric oxide is a gaseous free radical produced by nitric oxide synthase; it is known to stimulate satellite cell activation to myoblasts. However, the role of NO as a potential mediator of myogenesis is unclear. The current study therefore aimed to establish the level of nitric oxide released by myoblasts during proliferation, migration and differentiation. And to assess the effect of $_{L}$-NAME (a NOS inhibitor) on these processes. C2C12 cells were cultured in standard growth media and subsequently plated for analysis in DMEM. Proliferation or differentiation was induced via the addition of either 2 ng/ml HGF or 2% horse serum respectively, while migration was analyzed using the standard *in vitro* wound healing assay. $_{L}$-NAME concentration used was 100 µM or 200 µM. NO levels were assessed using a colorimetric assay. Proliferation was assessed via cell counts; migration by calculating the percentage wound closure and differentiation by myoblast alignment and fusion index analysis. HGF stimulated myoblast proliferation, however levels of NO were only found to be 0.58 nmol at 1h post-HGF stimulated. Similarly, NO following myoblast wounding were 0.31 nmol at 1h and increased to 0.56 nmol over 16h. In response to differentiation cues, NO levels increased to 6 nmol; then dropped as differentiation progressed over five days. Addition of $_{L}$-NAME (200 µM) resulted in a significant decrease (43% and 10%) in proliferation and migration respectively. Myoblast alignment and fusion was significantly reduced to 18% and 6% respectively at day five of differentiation in response to $_{L}$-NAME (200 µM). Results suggest that nitric oxide play a significant role during myoblast myogenesis, making NO crucial for skeletal muscle regeneration.

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