THE ROLE OF NITRATES IN SKELETAL MUSCLE METABOLISM DURING CONTRACTION

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ABSTRACT

Inorganic nitrate (NO_3^-) is a chemical compound found naturally in some foods, mainly green leafy vegetables. Traditionally this anion was considered inert in the human body, but recently the consumption of dietary NO_3^- has been shown to result in a number of positive physiological effects. These effects include protection against ischaemic reperfusion injury and improvements in glucose regulation in diabetic animal models. Some studies have also shown a reduction in blood pressure and a decrease in oxygen consumption (VO_2) for a given sub-maximal exercise workload, i.e. an increase in exercise efficiency after both acute, and chronic NO_3^- ingestion

The increase in exercise efficiency after NO_3^- supplementation is an intriguing finding, although not all have corroborated this. Two mechanisms currently proposed for the increase in exercise efficiency are an increase in mitochondria efficiency via tighter coupling and/or an increase in contractile efficiency. A third potential mechanism is a shift towards greater carbohydrate oxidation during exercise, which is metabolically more efficient compared with fatty acid oxidation.

It has generally been assumed that the exercise efficiency effects of NO_3^- are via NO_3^- stepwise reduction to nitric oxide (NO) with the facilitation of oral bacteria. This is a reasonable assumption considering NO has been well established to be involved in many physiological processes. Indeed, the reduction in blood pressure with NO_3^- ingestion is abolished when the bacterial conversion of NO_3^- to NO_2^- in the mouth is eliminated with an antiseptic mouthwash. However, whether an antiseptic mouthwash prevents the effect of NO_3^- on exercise efficiency has yet to be examined. NO_3^- alone has been shown to increase force production and glucose uptake during contraction of isolated muscle albeit at supra-physiological dosages. Additionally, NO_2^- independent of NO has been known to have physiological effects. Based on these considerations the research of this thesis was designed in an attempt to separate out the effects of NO_3^- from NO_2^- and NO.

To assess if there were direct effects of NO_3^- on skeletal muscle glucose uptake at physiological levels of nitrate, the first study (Study 1) examined the potential effect of NO_3^-, as well as NO_2^- on glucose uptake during contraction of the extensor digitorium longus (EDL) mouse muscle *ex vivo*.

Given the conditions of the *ex vivo* preparation (hyperoxic), there is unlikely to be reduction of NO_3^- or NO_2^- to NO thus enabling the examination of NO_3^- and NO_2^- per se.

To confirm that NO was not formed from NO_3^- and NO_2^-, comparisons were made to the NOS inhibitor L-NMMA (100 μM) treatment, which attenuates the increase in skeletal muscle glucose uptake during contraction. If NO is generated, it would be expected the attenuation in glucose uptake with L-NMMA would at least be partially overcome. The concentration of NO_3^- and NO_2^- used were based on peak concentrations achieved in human studies after NO_3^- supplementation, this being 500 μM and 500 nM, respectively.

Our results showed both NO_3^- and NO_2^- significantly attenuated peak force during *ex vivo* contraction with no effect on the rate of fatigue. Despite this, neither NO_3^- nor NO_2^- had any effects on the increase in skeletal muscle glucose uptake with contraction. Therefore, relative to force, glucose uptake was significantly increased. L-NMMA attenuated the increase in skeletal muscle glucose uptake during contraction as our group has shown previously. When L-NMMA was combined with NO_3^- or NO_2^- initial force was normalised and the same as contraction alone, but skeletal muscle glucose uptake remained attenuated similarly to L-NMMA alone. This might suggest that there was little NO being produced from NO_3^- or NO_2^- since skeletal muscle glucose uptake remained attenuated by L-NMMA. Thus the reason for the reduction in force is unclear.

To further investigate the effects of NO_3^- effects on exercise efficiency in humans, Study 2 was designed to address whether NO_3^- or its metabolic by-products increased contractile efficiency, improve mitochondria function, and/or alter force kinetics in isolated mouse muscle. The first part of this study aimed to determine whether the acute application of NO_3^- (500 μM), NO_2^- (500 nM) or L-NMMA (100 μM) into an *ex vivo* preparation directly affects contraction efficiency and force kinetics during contraction. This was performed using bundles of muscle fibres from mouse EDL using a thermodynamic *ex vivo* model. In this model, total heat produced by muscle bundles is determined by measuring muscle temperature using a thermopile whilst simultaneously measuring total work during an isokinetic contraction. The sum of heat generated and work performed is defined as total enthalpy (Heat + Work). Thereafter contraction efficiency (work/enthalpy) can be determined. Additionally, recovery heat rate (post last contractile cycle) was examined, which is indicative of mitochondria oxidative phosphorylation. Force kinetics were also examined during isometric contraction. The second part of Study 2 investigated whether six days of NO_3^- feeding in mice affected contractile efficiency, recovery, and force kinetics using the same thermodynamic model.

The acute *ex vivo* application of NO_3^- (500 μM) reduced contraction efficiency, whereas NO_2^- (500 nM) had no effect on contraction efficiency compared with contraction alone. Both treatments had no effect on the rate of oxidative phosphorylation during contraction. L-NMMA also had no effect on efficiency of contraction or rate of oxidative phosphorylation in this model. Furthermore 6 days of NO_3^- feeding had no effect on contraction efficiency, or rate of oxidative phosphorylation compared to contraction alone. The reason for acute NO_3^- treatment reducing contractile efficiency is uncertain. As there was no alteration in rate of oxidative phosphorylation with any of the treatments indicates that the work performed per cross-bridge cycle is reduced by the acute NO_3^- treatment, rather than enthalpy being increased with NO_3^-. The most likely reason for this reduction in work is a reduction in filament movement per cross-bridge cycle. If there is an effect of NO_3^- on contractile efficiency this effect may be lost in this *ex vivo* model due to the lack of blood flow, or the hyperoxic environment, especially considering a hypoxic environment is required for the reduction of NO_2^- to NO. NO_3^- feeding did however significantly attenuate the normal increase in rate of force development with successive contraction cycles in bundles of muscle fibres with no alteration in peak force. This reason for this effect is unclear, however it may be due to an alteration in calcium handling as previous research has indicated such an effect with NO_3^- feeding in mice.

Lastly, as no studies to date have investigated the effects of NO_3^- ingestion on important aspects of exercise metabolism such as glucose kinetics or muscle glycogen utilisation during exercise in humans, a double blind, randomised cross over design human study (Study 3) was performed in recreationally active males. Beetroot juice (Beet; ~8mM NO_3^-) rich in NO_3^-, was given acutely (2.5hrs prior to exercise) with, and without a mouthwash. The mouthwash was given in attempt to separate the effects of NO_3^- from NO_2^-, and possibly NO on carbohydrate metabolism and exercise efficiency. Participants cycled for 1 hour on a cycle ergometer at a sub-maximal intensity (65% VO_2 peak) and results were compared with a placebo (NO_3^- depleted Beet).

Neither Beet, nor Beet with the addition of mouthwash significantly altered oxygen uptake during exercise compared with the placebo. This was despite there being a significant increase in NO_2^- after Beet ingestion similar to that obtained in other studies where an effect on efficiency has been demonstrated. Additionally, there was no effect of either treatment on glucose disappearance during exercise, respiratory exchange ratio (RER), or muscle glycogen utilisation. This suggests carbohydrate oxidation or glucose uptake was not altered during exercise with either treatment.

The lack of effect of Beet and Beet with mouthwash on exercise metabolism was further supported by no difference in plasma glucose, lactate, non-esterified free fatty acids (NEFA) and insulin between treatments during exercise. Muscle lactate, phosphocreatine (PCr), creatine (Cr) and adenosine triphosphate (ATP) contents were also similar at rest or after exercise between treatments implying no effect of Beet on exercise metabolism.

In summary, these studies found that neither NO_3^- ingestion in mice or humans, nor the application of NO_2^- *ex vivo* in mouse muscle had any significant enhancement on skeletal muscle efficiency during mouse *ex vivo* contraction, or during exercise in humans. On the contrary, the acute application of NO_3^- reduced contractile efficiency *ex vivo* in mouse muscle. This is likely to be a result of NO_3^- reducing the work performed per cross bridge cycle. The mouse *ex vivo* results suggest that glucose uptake relative to force is increased with NO_3^- and NO_2^-, however no such effect was found when healthy humans ingested NO_3^- (with or without mouth wash) acutely. On the other hand, the results of this thesis suggest that supplementation of NO_3^- in mice can alter cross-bridge kinetics possibly via calcium handling, without effecting peak force. Further research is required to clarify the effect of dietary nitrate on skeletal muscle metabolism during contraction/exercise.

DECLARATION

I, Scott Sheng-Yi Betteridge, declare that the PhD thesis entitled “The role of nitrates in skeletal muscle metabolism during contraction” is not more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnote. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated in the Preface, this thesis is my own work.

Signature:  Date: 13/04/2016

PREFACE

All work carried out in the preparation of this thesis was my own apart from that acknowledged below:

1) In Chapter 2, the surgery on mice to excise the muscles was performed by Dr. Mary Zhang, Victoria University, Melbourne, Victoria, Australia.
2) In Chapter 3, the surgery on mice to excise the muscles was performed by A/Prof. Chris Barclay, Griffith University, Gold Coast, Queensland Australia.
3) In Chapter 4, the muscle biopsies were conducted by Dr. Andrew Garnham and Dr. Mitch Anderson. Danielle Hiam assisted in performing the Western blot analysis.

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LIST OF ABBREVIATIONS

ΔH_{Net}	Total heat and work generated (Enthalpy)
ΔH_T	Total enthalpy
[^{14}C] Mannitol	D-[^{14}C] Mannitol
[^{3}H] 2-DG	[1,2-^{3}H] 2-deoxy-glucose
[6,6-^{2}H]	Deuterated Glucose
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
$Ba(OH)_2$	Barium hydroxide
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cGMP	Cycle guanosine monophosphate
Deta NONOate	Diethylenetriamine NONOate
DTT	Dithiothreitol
εNet	Net efficiency
EDL	Extensor digitorium longus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycerol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
GCR	Glucose clearance rate
GLUT4	Glucose transporter 4
HCl	Hydrochloric acid
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
KOH	Potassium hydroxide
L-NMMA	L-N^{G}-monomethylarginine
L_0	Optimal muscle length where tetanic force is maximum
$MgSO_4$	Magnesium sulphate
NaCl	Sodium Chloride

NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADHPH	Reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium Hydroxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_3^-	Nitrate ion
NO_2^-	Nitrite ion
O_2	Oxygen
Q_A	Activation heat
Q_{CB}	Heat generated by cross-bridges
Q_M	Heat generated by muscle
Ra	Rate of glucose appearance
Rd	Rate of glucose disappearance
RER	Respiratory exchange ratio
SDS	Sodium dodecyl sulphate
SDS-Page	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% Tween-20
TEMED	N,N,N',N'-tetramethylethleneduamine
VO_2	Volume of oxygen
VCO_2	Volume of carbon dioxide
VO_2 peak	Peak oxygen consumption
Vs	Versus
W_T	Total work
W	Work
$ZnSO_4$	Zinc sulphate

CHAPTER ONE:

LITERATURE REVIEW

1.1 INTRODUCTION

Nitric oxide (NO) has been well established as a key-signalling molecule in a number of physiological pathways including metabolic homeostasis [44,50,121,162]. The well-established means of NO generation is via the various isoforms of the enzyme NO synthase (NOS) [1]. However, of increasing interest is that inorganic nitrate (NO_3^-) and nitrite (NO_2^-), previously thought to be inert by products of NO oxidation [96], can be reduced to NO, especially under hypoxic and/or acidic environments [116]. Interestingly in some [6,7,103,104,165,175], but not all [20,27,92,175] studies, increasing NO_3^- and NO_2^- in the body via the ingestion of NO_3^- increases exercise efficiency (~3-4%) and reduces metabolic imbalance during exercise along with other beneficial physiological effects such as a reduction in resting blood pressure [91,102,142]. The mechanism(s) by which NO_3^- ingestion alters exercise efficiency and metabolism is not clear, and assumed to be via its reduction to NO [7,104]. This may be a valid assumption, considering that NO is known to be a key signalling molecule, however, no studies to date have directly examined this.

This literature review will be broken up into three main parts, these sections being Nitric oxide, NO and exercise, and NO_3^- and exercise. The first section will commence with a brief description of what NO is, its role as a signalling molecule, and the two physiological pathways to generate NO. The next section will examine the role of NO in exercise. The first part of this section will examine the role of NO in exercise metabolism, commencing with a brief summary of general exercise metabolism. The second part of this section will look at the role of NO on exercise efficiency. This part will begin by defining exercise efficiency, briefly covering the varying methods to measure exercise/contractile efficiency before looking at the effect of NO on exercise/contractile efficiency. The last section will review the literature regarding the effects of NO_3^- on exercise. This section will firstly cover the effects of NO_3^- ingestion on exercise efficiency. Following this, the literature examining metabolic effects in the context of muscle efficiency will be reviewed highlighting where current research is lacking regarding the effects of NO_3^- on exercise metabolism.

1.2 NITRIC OXIDE

1.2.1 What is Nitric oxide?

In 1980, Furchgott and Zawadzki [59] discovered a substance released from the blood vessels that induced vasodilation. This molecule was initially termed endothelium-derived relaxing factor (EDRF) and was later (1987) identified as nitric oxide (NO) [83]. The discovery of NO revolutionised research in physiology and pharmacology over the last two decades of the previous century. This was highlighted by Professors Robert Furchgott, Louis Ignarro and Ferid Murad being awarded the Nobel Prize in Physiology or Medicine in 1998 for their contributions in discovering the role of endogenous NO production as a signalling molecule in the cardiovascular system.

The signalling pathway of NO on smooth muscle in vasodilation has been shown to be via soluble guanylyl cyclase (sGC) and cyclic guanosine mono phosphate (cGMP) [82]. Aside from NO's role in vasodilation, NO has been discovered to be an important mediator in a number of other physiological processes regulating metabolic homeostasis [103]. These processes include muscle contractility, myocyte differentiation, glucose regulation at rest and during exercise, calcium homeostasis, and mitochondria respiration and biogenesis [44,50,121,162]. A number of these processes are independent of cGMP, for example NO's role in the regulation of skeletal muscle glucose uptake during exercise appears to be independent of cGMP, likely through S-nitrosylation [123]. The role of NO in exercise metabolism, more specifically glucose uptake will be discussed in further detail later.

1.2.2 Generation of Nitric Oxide

1.2.2.1 Enzymatic pathway of Nitric oxide synthesis

The enzymatic formation of NO is via the metabolism of L-Arginine to L-Citrulline via varying isoforms of the enzyme NO synthase (NOS), illustrated in Figure 1.1. This reaction requires oxygen (O_2), and a number of co-factors including (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) [1].

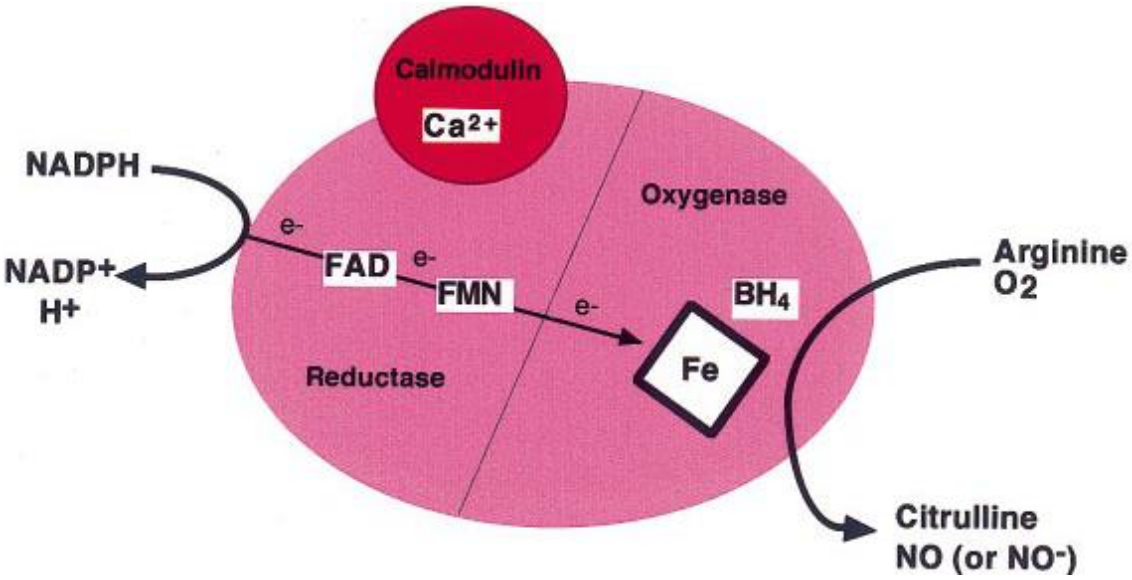

Figure 1.1: Overall reaction catalysed by NOS enzymes: NADPH donates electron (e-) at the reductase domain of the enzyme where they proceed via FAD and FMN to the oxygenase domain of the enzyme. Electrons then interact with the haem iron and BH4 at the active site to catalyse the reaction of arginine with oxygen to form citrulline and NO. Figure from Alderton et al. [1]

L-Arginine is a constituent of many dietary proteins; the most common dietary sources of L-Arginine are poultry, fish, nuts, and dairy products [168,178]. L-arginine is also synthesised endogenously in the kidneys from L-citrulline as well in the liver, although the L-arginine formed by the liver is consumed in the urea cycle [22].

Three major isoforms of the NOS protein have been identified, named after the cells, or systems which they were first purified and characterised [162]; these being endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms of NOS [57]. Endothelial and neuronal NOS are regarded as low-output isoforms and are constitutively expressed with activity being regulated by calcium (Ca^{2+}) and calmodulin [57]. Inducible NOS is a high-output isoform whose activity is predominantly, if not completely Ca^{2+} independent and whose expression is induced by cytokines [57]. Inducible NOS is involved in the generation of NO which is one of the main effectors in the innate immune function [110]. Endothelial NOS is important in vasodilation among other functions [57]. Neuronal NOS was first characterised in rat and porcine cerebellum [117,153] and since, has been shown to be widely distributed in specific neurons in the central and peripheral nervous system playing an important role in neuronal function [177]. Additionally, nNOS, more specifically nNOSμ splice variant has been identified as the predominant isoform in skeletal muscle in humans [119,134] and mice [106].

Many studies that have investigated the role of NO in physiological processes have used NOS inhibition such as L-NG-monomethyl L-arginine (L-NMMA), which is a competitive inhibitor of NOS, resulting in the reduction of endogenous NO production.

1.2.2.2 Non-enzymatic pathway

The inorganic anions NO_3^- and NO_2^- are naturally high in certain foods especially green leafy vegetables [172]. They can also be found in processed meats such as bacon preservatives [172]. Both anions are also products of NO oxidation, therefore have been used as a marker of NO synthase (NOS) activity i.e. endogenous production of NO [96]. However, these two anions are now also recognised as a source of NO [116].

NO_3^- is readily absorbed across the gastrointestinal tract resulting in a rapid elevation in plasma NO_3^- concentration with peak plasma concentrations observed within 60 minutes of ingestion [112]. Up to 25% of ingested NO_3^- is actively taken up by the salivary glands from the circulation via the proposed co transporter sialin (SLC17A5) [143], and concentrated 10-20 times before being secreted in the saliva [116,161]. Once in the oral cavity commensal bacteria convert NO_3^- to NO_2^- via nitrate reductase enzymes [171]. Subsequently, NO_2^- is swallowed and absorbed in the gastrointestinal tract leading to an elevation in plasma NO_2^- concentration peaking ~2.5 hours post ingestion [161]. NO_2^- is subsequently distributed rapidly throughout all major organs and tissues [32,38]. Currently the process in which NO_2^- crosses biological membranes remains unclear. However, it has been suggested that NO_2^- may cross the lipid bilayer of the cell via acidification to nitrous acid (HNO_2), which can freely diffuse across the lipid bilayer [152].

The reduction of NO_2 to NO is particularly predominant under hypoxic conditions where NOS isoforms are likely to be impaired [178]. One important mediator proposed for this reduction is deoxyhaemoglobin [45]. Vitturi et al. [169] proposed a model where haemoglobin deoxygenation regulates NO_2^- metabolism by red blood cells (RBC) allowing for a greater reduction of NO_2^- to NO along the arterial-venous oxygen gradient. NO can then freely diffuse out of the RBC as illustrated in Figure 1.2. Using human RBC their data suggest that the exportation of NO_2^- out of the red blood cell via the transport protein AE-1 is inhibited by deoxyhaemoglobin, thereby keeping intracellular NO_2^- concentrations high. This allows for greater reduction of NO_2^- to NO as oxygen desaturation increases.

Therefore, the generation of NO via the step-wise reduction of NO_3^- is now seen as a complementary pathway that can maintain the synthesis of NO in the body where enzymatic generation may be compromised due to hypoxia [112] as exists in the exercising muscle [104].

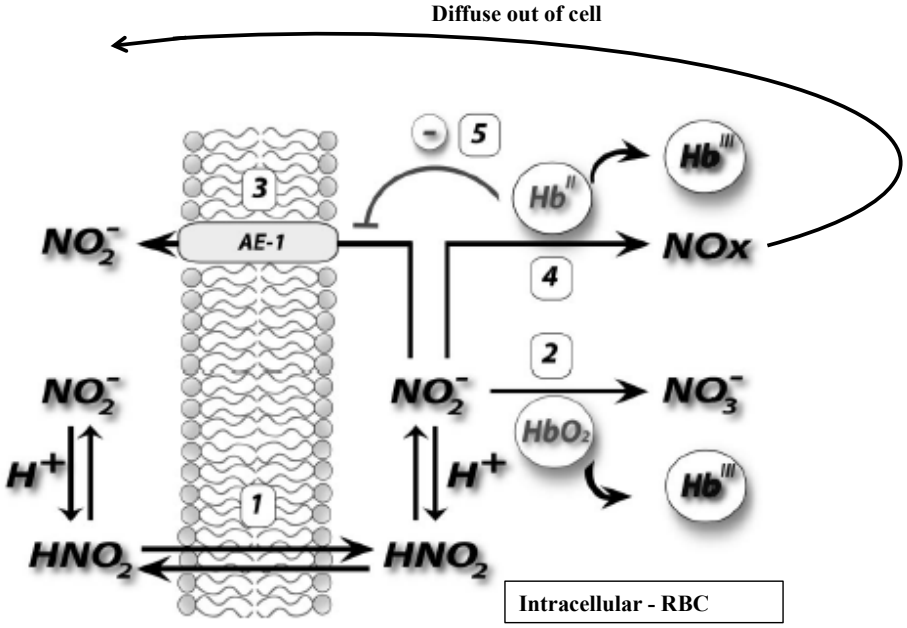

Figure 1.2 - Proposed mechanism Hb deoxygenation regulates nitrite metabolism by RBS's from Step (1) Nitrite enters the RBC down an electrochemical or concentration gradient via a channel and/or possibly as undissociated nitrous acid (HNO2). Step 2: In an oxygenated state nitrite oxyHb is oxidised to nitrate or (Step 3) Exported out of the RBC via anion exchanges (AE-1). As RBC desaturate nitrite intake accelerates due to nitrite reaction with deoxyHb resulting in active nitrogen species (NOx) that can produce NO, which can diffuse outside of the RBC (Step 4). Along with the increase consumption of nitrite, exportation of nitrite is inhibited via deoxyHB binding to AE1 (Step 5) thus maintaining intracellular nitrite concentrations. Adapted from Vitturi et al. [169].

1.3 NITRIC OXIDE AND EXERCISE

1.3.1 Metabolism during acute exercise

Whilst performing exercise, the energy contribution of the differing metabolic pathways to meet the metabolic demand is determined by the relative exercise intensity during the exercise bout [51]. Combining indirect calorimetry with isotope tracers has enabled the assessment of substrate utilisation during exercise [150,164]. At low exercise intensities (~25% VO_2) the predominant fuel is free fatty acids (FFA).

As exercise intensity increases the proportion of ATP generated from carbohydrate (CHO) oxidation increases as indicated by an increase in RER, in particular from the breakdown of muscle glycogen and plasma glucose as demonstrated by Romjin et al. [150]. As exercise intensities increase beyond 65% of VO_2 peak, there is a reduction in fat oxidation as indicated with by a reduction in muscle triglyceride and plasma FFA utilisation as shown in Fig. 1.3.

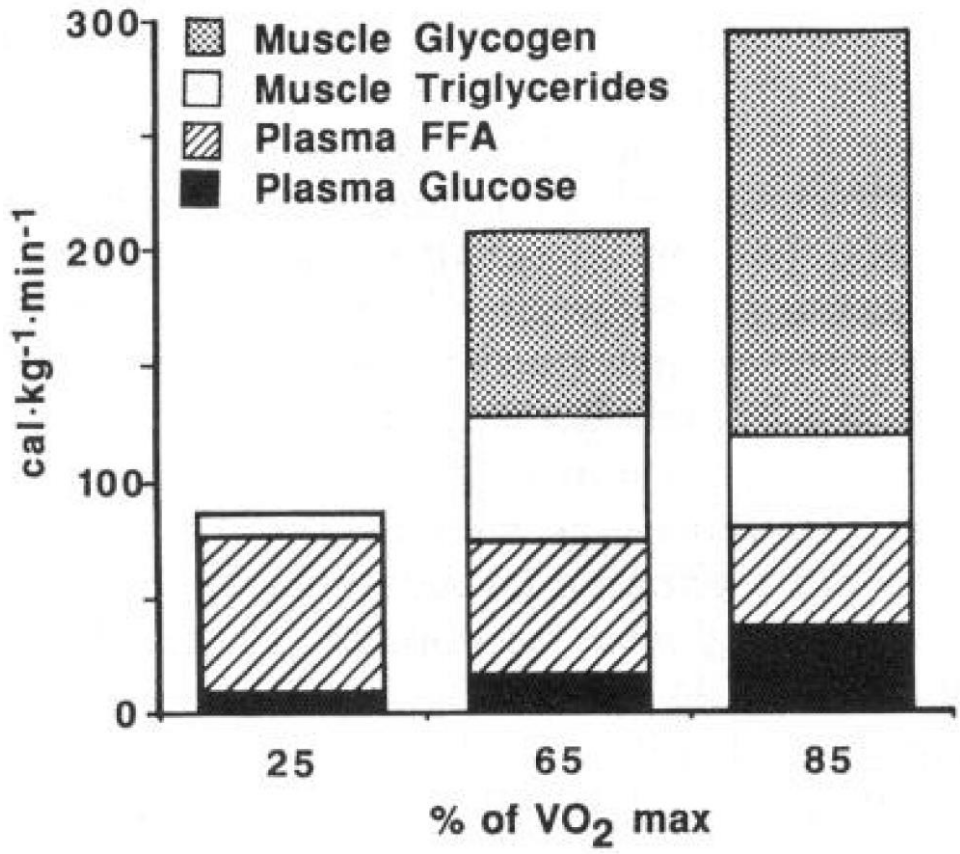

Figure 1.3 - Energy contribution from the varying fuel substrates at three relative intensities, 25, 65 and 85% of VO_2 max. Romjin et al. [150].

The proportion of substrate utilisation is also altered by exercise duration. At a moderate intensity (~65% of VO_2 max) there is steady increase in the proportion of lipid and glucose oxidation and a con-current decrease in energy derived from muscle glycogen as illustrated in Fig 1.4 [150].

The regulation of substrate utilisation is complex but some of the key regulatory proteins include AMPK, CaMK, and NOS [51,148]. Since the focus of this literature review is on NO and NO_3^-, the roles of these molecules will be discussed specifically in the following sections.

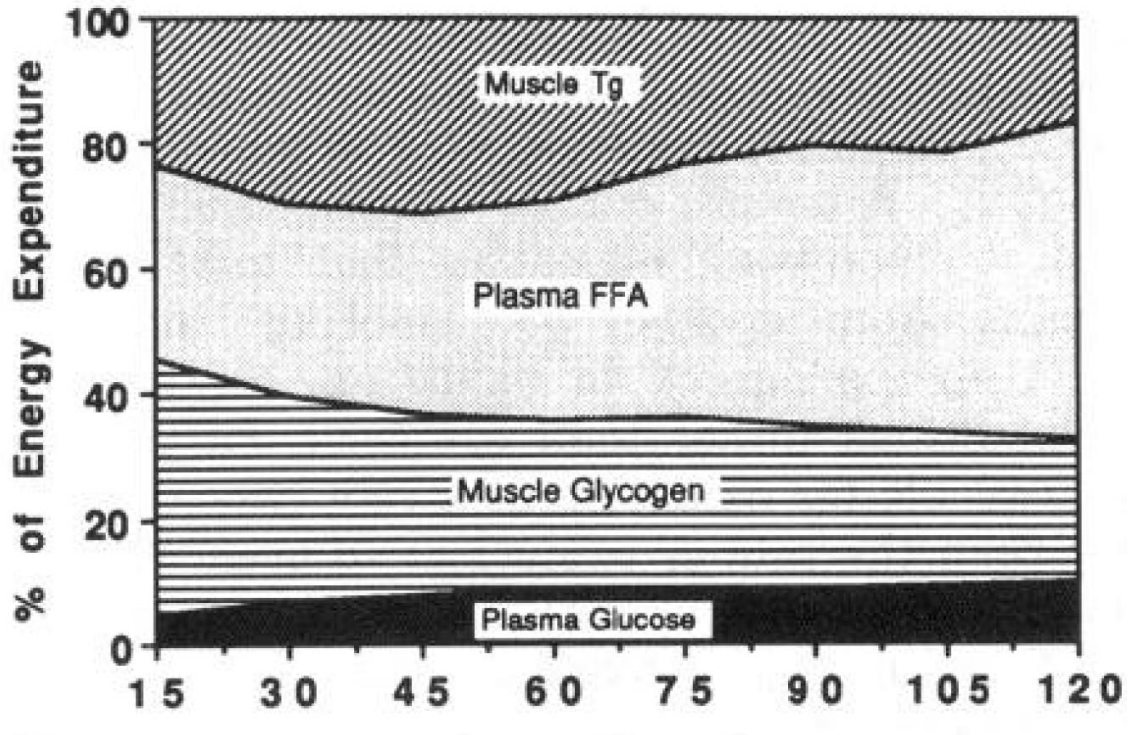

Figure 1.4 – The proportion of energy expenditure (%) from the varying metabolic substrates. Romjin et al. [150].

1.3.2 Nitric oxide and metabolism during exercise

The role of NO in exercise metabolism has been examined in regards to free fatty acid (FFA) oxidation and more extensively in glucose metabolism. One of the few studies that has investigated the role of NO in FFA oxidation found that with low intensity exercise there was slight, but non-significant increase in FFA uptake when NOS was inhibited with L-NMMA [69]. Most research to date has focused on NOS role in glucose metabolism, more specifically its role in the increase in glucose uptake during exercise.

Insulin [40] and skeletal muscle contraction [148] have both been well established to result in GLUT4 translocating to the cell membrane, one of the main proteins to facilitate glucose uptake into the muscle [33], although this occurs via different pathways [122,123]. For example in type 2 diabetic individuals, where insulin resistance is present, blood glucose concentrations are restored to normal levels during moderate intensity exercise (70% of work maximum) [133].

There is evidence that the generation of NO in skeletal muscle plays a key role in skeletal muscle glucose uptake during exercise [95,109,121,123] although not all studies support this [52,69,73,86]. The role of NO in glucose uptake during exercise has been established via the use of NOS inhibitors in *ex vivo* [123,124] animal studies, as well as *in vivo* [24,95] human studies. Possible reasons for the lack of effect of NOS inhibition in some studies [52,69,73,86] on glucose uptake during exercise include the time when glucose uptake/transport was measured, and the intensity that contraction/exercise is performed as outlined by McConell et al [122]. In some studies, glucose transport/uptake was measured over 20 minutes after contraction, or exercise had ceased [52,73]. With such a delay, glucose uptake at this stage is more indicative of post exercise/contraction stimulated glucose uptake. On the contrary, where an effect of NOS inhibition is found, glucose uptake is measured during exercise and contraction [95,109,121,123]. The varying intensities may also explain some of the differences in results between studies. In the study by Inyard et al. [86] little effect on glucose uptake was shown with NOS inhibition when the low intensity contraction protocol was used in rats. However, in the higher intensity contraction protocol NOS inhibition resulted in a ~50% attenuation in glucose uptake, although not significant. Further supporting such a proposal, Silveira et al [159] demonstrated NO is only released from primary muscles cells at higher intensities, suggesting NO role in exercise/contraction stimulated glucose uptake is more significant at higher intensities.

Since the attenuation in glucose uptake during contraction is evident in an *ex vivo* model, where blood flow is not present, this indicates the role of NO in glucose uptake during contraction/exercise is independent of blood flow [122]. Indeed, when L-NMMA is infused across the exercising muscle in humans, it results in a reduction in glucose uptake without affecting blood flow in the working leg in healthy [24], and type II diabetic individuals [95]. The signalling pathway by which NO promotes the translocation of GLUT4 during exercise/muscle contraction is not clear, but appears independent of cGMP/AMPK pathway as has been shown via the inhibition of enzymes upstream and downstream of these pathways [123]. Processes put forward that may be involved in the up-regulation of glucose uptake via NO include post-translational modification of proteins via S-nitrosylation, S-glutathionylation and tyrosine [122]. However, further elucidation of how NO regulates glucose uptake during contraction is required.

Neuronal NOS is the predominant isoform in skeletal muscle, more specifically the splice variant nNOSμ has been found to be the main isoform activated during muscle contraction with muscle lacking the nNOSμ isoform showing no downstream activation of cGMP [76,106]. However, a recent study by Hong et al. [78] found using nNOSμ knockout mice that nNOSμ was not required for the normal increase in glucose uptake with contraction [121]. Interestingly, glucose uptake during contraction remained to be attenuated with the addition of L-NMMA in nNOSμ knockout mice indicating NO was still being generated from another NOS isoform, or splice variant of nNOS. To determine if this may have been due to a non-specific effect of the NOS inhibitor, L-Arginine was co-administered to overcome the competitive inhibition, which did restore glucose uptake during contraction to normal. This suggests that it was indeed the reduction in NO synthesis from NOS that impaired glucose uptake, but not via nNOSμ.

Despite the recent doubt on where NO is sourced from during skeletal muscle contraction, the evidence continues to indicate that NO is a key signalling molecule in the up regulation of glucose uptake independent of blood flow during exercise and muscle contraction.

1.3.2 Nitric oxide and efficiency during exercise

1.3.2.1 What is Efficiency, and how is it measured?

The primary function of muscle is the transduction of chemical energy, in the form of ATP to mechanical form i.e. force and work. The fraction of this energy used for work is termed efficiency [74] i.e. work performed/energy expended.

A number of methods have been developed to determine exercise/contraction efficiency ranging from *in vivo* techniques such as the analysis of respiratory gases (indirect calorimetry) to *ex vivo* methods where isolated animal muscles are contracted in a physiological buffer and heat or metabolites are analysed. The methods that will be briefly discussed in the following section include; Direct and indirect calorimetry (*in vivo),* magnetic resonance spectroscopy (*in vivo),* analysis of metabolic substrates (*ex vivo*) and thermodynamics analysis (*ex vivo).*

1.3.2.1.1 Direct and Indirect calorimetry – in vivo

The method of direct calorimetry involves measuring the heat generated by the body within an insulated environment. This method has several limitations such as the requirement of bulky equipment and the need to keep the participant in a physically confined environment for long periods [160]. Consequently, direct calorimetry is mainly used for thermoregulation studies rather than studies assessing exercise efficiency [154].

Indirect calorimetry has become the popular method to examine metabolic efficiency during exercise as the method has the added benefit of being able to calculate the ratio of substrates oxidised, in regards to carbohydrates and fat. The method is referred to as "indirect" because the method measures O_2 consumption (VO_2) and carbon dioxide (CO_2) production (VCO_2) instead of directly measuring energy transfer. However, as all energy is ultimately produced by the process of oxidation, both methods yield very similar results as first demonstrated by Atwater et al. in 1899 [4].

The common indirect method utilised is the use of a non-rebreathing mask where atmospheric air is breathed through a one-way valve and expired out of another one-way valve where it either collected in a Douglas bag, or measured breath-by-breath and analysed for O_2 and CO_2 concentration. If work is quantified along with calculating energy cost measurements then net exercise efficiency can be calculated indirectly. For example on a cycling ergometer, power produced can be measured thus work calculated whilst performing VO_2 and VCO_2 analysis. However, being an *in vivo* method only overall exercise efficiency can be quantified, and skeletal muscle efficiency cannot be distinguished from other "over head" energy costs, such as cardiovascular energy demands. Therefore, in assessing skeletal muscle efficiency per se, this method may be lacking.

1.3.2.1.2 Magnetic resonance spectroscopy – in vivo

The use of ^{31}phosphorous magnetic resonance spectroscopy (^{31}P-MRS) for metabolites in muscle was pioneered in the 1970s by Hoult et al. [79] and Dawson et al. [48,49] and has since become a popular method to determine the metabolic cost of muscle contraction under varying conditions [174]. This is likely due to the method being non-invasive with the ability to analyse muscle as a whole and measure some metabolites in real time and *in vivo* [174].

Measuring metabolites such as PCr and ATP during exercise enables the rate of ATP turnover to be calculated [101], combined with measuring work via an ergometer exercise/contraction efficiency can be calculated. In addition, mitochondrial function can also be assessed by analysing the rate of PCr resynthesis [93] after an acute bout of exercise or contraction thus making such a method a valuable tool in understanding muscle physiology. One downfall of ^{31}P-MRS is poor sensitivity, resulting in the need to average repeated signals to increase sensitivity, which results in a reduction in time resolution [12,174] especially in comparison to measuring heat generated which will be discussed later. Other disadvantages of using ^{31}P-MRS are restrictions in the type of exercise performed with having to contain the exercising limb within the chamber, which needs to be relatively fixed. In addition no metal material can be close to the MRI. Consequently limiting exercise to exercises such as leg extension, or ankle flexion. One last inhibitory factor with the use of the ^{31}P-MRS is the very high cost of the MRI as well as the expertise required to operate the equipment.

1.3.2.1.3 Analysis of metabolic substrates in muscle – ex vivo

The key metabolic pathways that are involved in the generation of energy for muscle contraction were all discovered with the analysis of extracts from muscle [37]. This method involves a brief stimulation of muscle *ex vivo* before being frozen rapidly at varying time points post contraction as described by Carlson [37]. Only a very brief contraction can be performed, typically a single contraction to limit rephosphorylation of phosphocreatine (PCr) via aerobic and anaerobic metabolic pathways [36]. In comparing the concentration of PCr after contraction to rest, energy cost can be calculated. In order for this method to achieve accurate results, the muscle needs to be frozen very rapidly as ATP hydrolysis will continue to occur until freezing is complete [16,35]. Therefore, methods such as the use of Freon pre cooled in liquid nitrogen [35] are used which allows for a more rapid freezing than liquid nitrogen, which has now been deemed inadequate for the analysis of muscle energetics [35]. This requirement for rapid freezing post contraction limits this method to *ex vivo.* Afterwards, analysis of adenosine triphosphate (ATP), creatine (Cr) and phosphocreatine (PCr) is performed. By comparing the amount of PCr and ATP present in resting muscle to muscle that has contracted, often the left and right muscle of the same animal, the amount of PCr and ATP broken down can be calculated. Since the amount of energy that is released upon the hydrolysis of PCr and ATP is known (ΔH =-34 kJ/mol [47]), the amount of energy used for contraction can be calculated, and contraction efficiency can be determined by

simultaneously measuring work. However, the major draw back in such a method is that each muscle only provides the metabolite concentration at one specific point in time, thus requiring multiple muscles in order to determine factors such as rate of PCr breakdown and resynthesis. In addition, due to the need to combine the values from multiples muscle error is introduced from inter variability between muscles, which is an issue when differences in metabolites can be very small [12,47]. These factors make it a time consuming, and logistically challenging process in order to get useful data. On the contrary, measuring heat, which will be discussed next, allows repeated measures to be performed on the same muscle.

1.3.2.1.4 Thermodynamic analysis – ex vivo

One of the main methods for investigating muscle energetics *ex vivo* is the use of a thermopile to measure heat production during and post contraction [174]. The heat production of a bundle of muscle fibres, or a single intact muscle fibre can be measured with the preparation being mounted in contact with the plastic film of the thermopile containing a series of metal film thermopiles [174]. Using such a method net mechanical (ε_{Net}) efficiency can be established based on the Equation 1, ΔH_{Net} is net enthalpy, the sum of the heat produced and W performed during a series of contractions.

$$\varepsilon\mathrm{Net} = \frac{W}{\Delta \mathrm{H_{Net}}}$$

Equation 1.1: Formula to calculate net efficiency (ε_{Net}). W: total work performed in series of contractions. ΔH_{Net}: total heat and work generated during series of contractions.

Like ^{31}P-MRS method, mitochondrial function can also be determined by analysing the rate of heat production post contraction, which is analogous to oxidative re-phosphorylation [12], yet with far greater sensitivity and time resolution. This is a major advantage of measuring heat [12]. In addition, it is a non-destructive method, thereby enabling multiple measurements to be performed on the same muscle [173] making it possible to analyse multiple acute treatments, assuming treatments can be washed out, on the same muscle. These advantages at least in part, contribute to it being the main method to investigate muscle energetics *ex vivo* [12]. However, the downfall of the method is the fact is that it is *ex vivo,* thus factors such as blood flow, motor unit recruitment patterns, and endocrinological factors are eliminated.

In summary, all methods have their advantages and downfalls, yet all have added valuable knowledge to the area of muscle energetics therefore highlighting the importance of each method in understanding muscle energetics [174].

1.3.2.2 The role of Nitric oxide in efficiency during exercise

The inhibition of NOS has been shown to increase whole body oxygen consumption at rest in anaesthetised dogs [155]. The proposed mechanism for this effect is that NO is able to competitively and reversibly bind to cytochrome oxidase (COX) resulting in the inhibition of mitochondria function [43], which was first demonstrated using isolated mitochondria [29]. On the contrary, energy cost for force development is reduced with L-NMMA using a perfused-hind limb model in rats via the measurement of metabolites and VO_2 [8,98]. However, as only an isometric contraction was performed it cannot be said that contractile efficiency is enhanced with NOS inhibition as previously defined. In regards to exercise efficiency, Mortensen et al. [129] found that co-infusing L-NMMA and a prostaglandin inhibitor into the femoral artery whilst performing knee extensor exercise (19 ± 1 Watts) reduced oxygen cost. However, as L-NMMA was not infused alone it is not clear if the increase in exercise efficiency was due to the reduction in NO synthesis or due to the prostaglandin inhibitor. Indeed, when L-NMMA alone has been used in other studies examining the effect of NOS inhibition on contractile efficiency, no significant effect on efficiency was found [24,95,144]. Although blood flow was marginally reduced in the study by Radegran & Saltin [144], oxygen extraction was increased resulting in no net change in oxygen consumption across the working muscle i.e. the quadriceps muscle.

Overall these results suggest that an acute reduction in NO does not effect exercise efficiency. However, I am not aware of any studies that have examined the effect of increasing NO availability during muscle contraction or exercise on efficiency.

1.4 NITRATES AND EXERCISE

1.4.1 Nitrates and efficiency during exercise

Larsen et al [104] in 2007 were the first to investigate the effect of NO_3^- ingestion on exercise efficiency. They demonstrated that NO_3^- supplementation for 3 days increased muscle efficiency by showing a reduction in oxygen (O_2) consumed at a given sub-maximal workload. Subsequent research has shown that the ingestion of beetroot juice, rich in NO_3^- also increased exercise efficiency [6,7,165] as illustrated in Fig. 7. This effect has also been demonstrated after one acute dose of NO_3^- ingested 2.5 hours prior to exercise [165,175] indicating the effects of NO_3^- are rapid. This finding is intriguing since oxygen consumption has been previously been known to be tightly coupled to workload irrespective of training [130] and age [3].

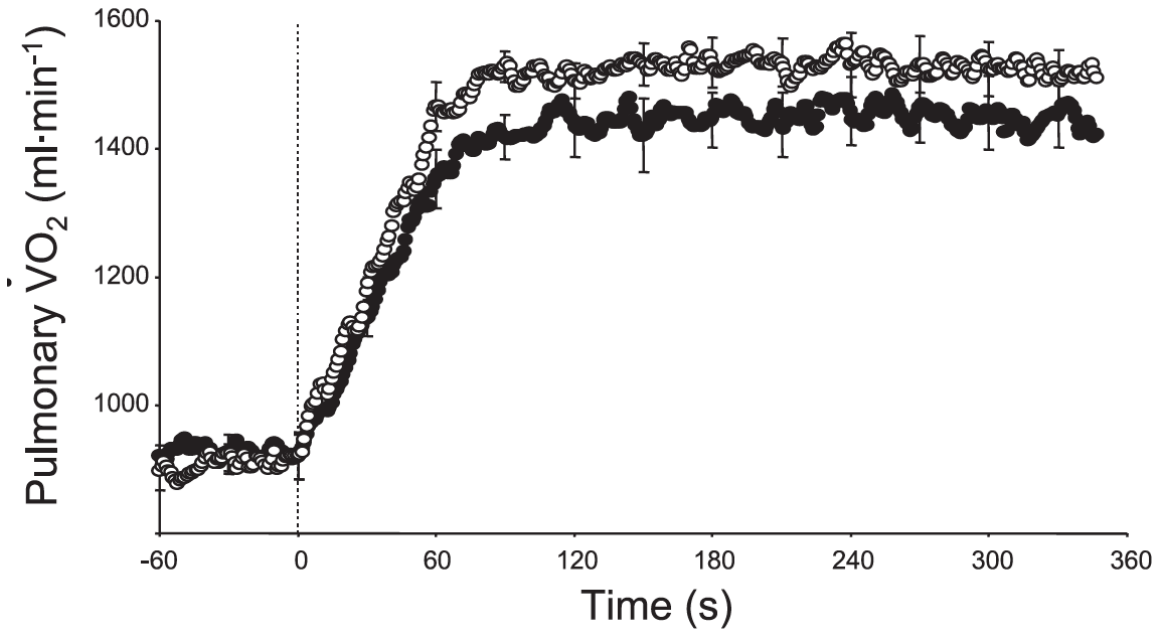

Figure 1.5: VO_2 response to a submaximal bout of cycling. Oxygen cost is significantly reduced following beetroot juice supplementation (closed dots) compared to placebo (Open circles). From Bailey et al. [7]

However, not all studies show an increase in exercise efficiency at a given submaximal workload [18,20,27,92,156,175]. Bescos et al. [20] found in trained cyclists (65.1 ± 6.1 ml.kg.min^{-1}of VO_2 peak) no significant difference in oxygen consumption at a given sub-maximal workload 3 hours after ingestion of sodium nitrate at a dose of 10 mg/kg (~8mM NO_3). Similar findings have been supported by Christenson et al [41] in elite cyclists (>70 ml.kg.min^{-1}of VO_2 peak).

This finding has been suggested to occur due to the trained status of the individuals [19]. However, a study by Cermak et al. [39] found that 6 days of supplementation in the form of Beetroot juice (NO_3^- dose of ~8mM per day) in amateur cyclists (VO_2 peak 58 ± 2 $ml.kg.min^{-1}$) did reduce sub-maximal oxygen consumption compared to control. A multiday supplementation may be needed to enhance efficiency in individuals of a trained status. Although in regards to recreationally active individuals, other recent studies have also shown no effect after 3-6 days of NO_3^- supplementation on exercise efficiency [27,92,156,175]. Consequently, other authors have investigated whether this lack of effect on sub-maximal exercise efficiency may be related with the amount of NO_3^- supplemented. Regarding this, Wylie et al. [175] only found there to be a significant reduction in VO_2 2.5 hours post beetroot juice ingestion at a dose equivalent to 16 mM of NO_3, and not at 4 or 8 mM, suggesting that a higher dose may be required to induce an increase in exercise efficiency in recreationally active individuals. All these studies discussed are summarised in Table 1.1.

Study	Form	Dose of NO_3^-	Training Status	Duration of Supplementation	Effect on submaximal exercise efficiency at normoxia
Larsen et al. 2007	$NaNO_3$	0.1 $mM.kg^{-1}$	Recreation	3 days	↑
Bailey et al. 2009	Beet Juice	~11.2 $mM.day^{-1}$	Recreation	6 days	↑
Bailey et al. 2010	Beet Juice	~5.1 $mM.day^{-1}$	Recreation	6 days	↑
Vanhatalo et al. 2010	Beet Juice	~5.2 $mM.day^{-1}$	Recreation	6 days	↑
Cermak et al. 2012	Beet juice	~8 $mM.day^{-1}$	Trained	6 days	↑
Breese et al. (2013)	Beet juice	~8 $mM.day^{-1}$	Recreation	4-6 days	↔
Kelly et al (2014)	Beet juice	~8 $mM.day^{-1}$	Recreation	3 days	↔
Bescos et al. 2011	$NaNO_3$	0.1$mM.kg^{-1}$	Trained	3 hours	↔
Wylie et al 2013	Beet juice	~4 & 8 $mM.day^{-1}$	Recreation	2.5 hours	↔
Wylie et al 2013	Beet juice	16 $mM.day^{-1}$	Recreation	2.5 hours	↑
Vanhatalo et al. 2010	Beet Juice	~5.2 mM	Recreation	2.5 hours	↑

Table 1.1 – Summary of studies examining the effects of single and multiday ingestion of NO_3^- on exercise efficiency in recreational and trained individuals. ↑: Increase in exercise efficiency ↓: Decrease in exercise efficiency ↔: No change in exercise efficiency. Note this table is not exhaustive of all studies examining the effect of NO_3^- on exercise efficiency.

The first clinical study to investigate the effect of NO_3^- ingestion on exercise efficiency in chronic obstructive pulmonary disease (COPD) patients found no reduction in oxygen cost with moderate intensity cycling [131]. In addition there was no reduction in blood pressure as has been previously documented [91,102,142]. The lack of effect on exercise efficiency of NO_3^- supplementation in COPD patients [131], was suggested to be related to the elevation in oxidative stress that occurs in COPD patients [42], which may have led to damage at the mitochondrial level and consequently reducing the effect of NO_3^- supplementation on mitochondrial function. Another clinical study examined the acute effects of NO_3^- ingestion (ingested 3 hours prior) in the form of beetroot juice on peripheral arterial disease (PAD) patients in response to exercise. They found an increase in walking time with NO_3^- supplementation before the onset of claudication pain.

In addition, via the use of near infrared spectroscopy (NIRs), oxygen extraction was found to be significantly lower indicating an increase in muscle blood flow. This study highlights the potential for NO_3^- to be used in a therapeutic setting.

1.4.2 Nitrate and metabolism during exercise

The mechanism(s) by which NO_3^- may increase skeletal muscle efficiency during exercise is poorly understood. There are currently three main hypotheses. The first relates to the efficiency of mitochondria to generate ATP, the second relating to substrate utilisation, and the last relates to the efficiency of the skeletal muscle contraction. In regards to the effects of NO_3^- on mitochondria, Larsen et al. [103] demonstrated a significant decrease in mitochondrial proton leak isolated from skeletal muscle and a significant increase in exercise efficiency in humans supplemented with NO_3^- (0.1 mmol $kg^{-1}day^{-1}$) for 3 days. Furthermore, a reduction in the abundance of the protein ADP/ATP translocase (ANT) was also found, a major protein proposed to contribute to the proton leakage back across the inner mitochondrial membrane [21,137]. Based on their findings Larsen et al. suggest NO_3^- ingestion may enable the mitochondria to generate ATP more efficiently i.e. tighter coupling between O_2 consumed and ATP generated. However, the down regulation in ANT expression does not seem likely to explain the increase in exercise efficiency demonstrated after one dose of NO_3^- ingested 2-3 hours prior to exercise [165,175] which is likely insufficient time for a change in ANT protein expression to occur. A possible mechanism for such an acute effect could be attributed to NO ability to reversibly inhibit cytochrome c oxidase (Complex IV) in the electron transport chain [157]. A number of studies suggest that NO inhibition of Complex IV can occur to a degree without affecting ATP production [26,61]. Therefore, this may explain the potential acute effects of NO_3^- on exercise efficiency in relation to the mitochondria.

In regards to the second possible mechanism involving substrate utilisation, a significant ($p<0.05$) increase in the respiratory exchange ratio (RER) from 0.88 ± 0.01 (Placebo) to 0.91 ± 0.01 with the ingestion of nitrates has been shown during exercise [103]. This indicates a slight shift towards greater CHO oxidation. This slight increase in CHO oxidation would correspond to a reduction in metabolic cost of 1%, due to carbohydrates being a slightly more efficient metabolic fuel in regards to ATP generated per O_2 consumed [28].

Important to note, no other studies to date have shown this increase in RER with NO_3^- ingestion, suggesting such a mechanism may not be critical with respect to the effects of NO_3^- on exercise efficiency. Furthermore, even if this shift in substrate utilisation did occur with NO_3^-, it can only partially account for the 3.4% reduction exhibited with NO_3^- ingestion in this study [103].

The final hypothesis proposed is that NO_3^- ingestion reduces ATP cost i.e. the energy cost of force production, [6,7]. Supporting this theory, Bailey et al. [6] demonstrated using ^{31}P-MRS, that estimated ATP turnover during exercise was significantly reduced after 6 days of NO_3^- (5.1 mM/day) supplementation. Bailey et al. [7] speculated this reduction in ATP cost of force production may be the result of a decrease in excess calcium being secreted from the sarcoplasmic reticulum (SR) during contraction. It has been shown that up to 40% of total ATP turnover during contraction is attributed to the reacquisition of cytosolic calcium via the SR calcium pumps [12]. They [7] propose that the reactive oxygen species generated during contraction increase the open probability of the SR Ca^{2+} release channels [128]. A small elevation in NO may protect the calcium release channel from oxidation without affecting function [147], therefore preventing excessive Ca^{2+} being released. NO has also been shown to reduce calcium cycling [67,167] and slow cross bridge cycling kinetics [60,71]. Therefore, the NO generated from the reduction of NO_2^- may reduce excess calcium release from the SR, thus reducing the ATP required by the SR-Calcium pumps to reacquire the excess calcium released thereby reducing the oxygen cost. However, at this stage this proposal remains speculative. Therefore, the mechanism(s) by which NO_3^- may decrease ATP cost of skeletal muscle contractions remains unclear at this stage.

1.5 RATIONALE FOR STUDIES OF THIS THESIS

Thus far, important metabolic signalling pathways have not been explored. This includes the pathway involving the energy-sensing enzyme AMP-activated protein kinase (AMPK) [136]. If indeed less ATP is consumed for a given sub-maximal workload, there would likely be less activation (phosphorylation) of AMPK, as AMPK phosphorylation is an indicator of metabolic disturbance, increasing with exercise intensities [170]. Therefore, there is a need to investigate the effect of NO_3^- ingestion on AMPK activation, which will help further elucidate the effects of NO_3^- on exercise metabolism.

Currently it is assumed that the mechanism by which NO_3^- ingestion increases exercise efficiency is via nitric oxide, the more reactive product of NO_3^- metabolism [7,104]. Supporting this assumption, previous studies have shown that preventing the oral conversion of NO_3^- to NO_2^- using antiseptic mouthwash abrogates the beneficial effects of NO_3^- on blood pressure and gastro-intestinal health [142] where NO is attributed to such effects [115,127]. Nevertheless, the effect of preventing the oral conversion of NO_3^- to NO_2^- has not been determined in regards to skeletal muscle metabolism or efficiency during contraction. In addition as mentioned earlier, NOS inhibition has not been shown to alter exercise efficiency *in vivo* [24,95,144]. Preventing the bacterial conversion of NO_3^- to NO_2^-, as used in the studies on blood pressure would be beneficial in distinguishing the effects of NO_3^- from NO_2^- and NO, thereby helping in determining the mechanism(s) by which NO_3^- may affect skeletal muscle metabolism and/or efficiency. Given that NO_3^- has been shown to increase force production and glucose permeability of skeletal muscle *ex vivo* [30,77,88], albeit at supra physiological levels, the effects on skeletal muscle efficiency during contraction may be a direct result of NO_3^- rather than NO itself. Furthermore, there is evidence to indicate that NO_2^- independent of NO can act as a signalling molecule [32]. Bryan et al. [32] demonstrated that heme S-nitrosation and nitrosylation proceeds in the presence of the NO scavengers carboxy-PTIO and oxyhemoglobin indicating NO_2^- as the active substrate. Therefore, further research into the direct effects of NO_3^- and NO_2^- on skeletal muscle metabolism is warranted.

In summary, it is evident that further studies are required to investigate the metabolic effects of NO_3^- ingestion on skeletal muscle metabolism during exercise/contraction. Using methods discussed earlier such as measuring heat generated *ex vivo* during contraction to determine contraction efficiency, analysing metabolites and protein signalling will aid in elucidating the mechanism(s) by which NO_3^- has effects on skeletal muscle metabolism during contraction/exercise.

1.6 AIMS

The general aim of this thesis was to determine the effects of inorganic NO_3^- on exercise metabolism and efficiency in contracting/exercising skeletal muscle. The specific aims were:

1. To determine whether there are direct acute effects of NO_3^- and NO_2^- on skeletal muscle glucose uptake during *ex vivo* mouse muscle contraction with, and without NOS inhibition (Study 1)
2. To investigate the direct acute effects of NO_3^-, NO_2^- and NO on net thermodynamic efficiency and force dynamics during *ex vivo* mouse muscle contraction (Study 2).
3. To examine the effects of 6 days of NO_3^- feeding on net thermodynamic efficiency and force dynamics during *ex vivo* mouse muscle contraction (Study 2).
4. To assess exercise efficiency, glucose kinetics, muscle metabolism and AMPK signalling in recreationally active males performing submaximal endurance exercise after an acute dose of NO_3^- in the form of beetroot juice (Study 3).

1.7 HYPOTHESES

The hypotheses tested were that:

1. NO_3^- and NO_2^- would stimulate *ex vivo* muscle skeletal glucose uptake in mice during contraction. Additionally, we hypothesised that the NOS inhibitor would attenuate glucose uptake, which would not be normalised with the addition of NO_3^- and NO_2^- (Study 1). This would demonstrate that the effects of NO_3^- and NO_2^- are independent of NO
2. Acute application of NO_3^- would not alter contractile efficiency of skeletal muscle *ex vivo*. NO_2^- would increase contractile efficiency, and L-NMMA would have no effect. In addition we hypothesise force production will not be altered with any of the acute treatments. (Study 2). This would demonstrate that NO_3^- effects on exercise efficiency are independent of NO.
3. Six days of NO_3^- supplementation in mice would increase the thermodynamic efficiency of skeletal muscle along with a con-current increase in force production (Study 2).
4. Acute ingestion of NO_3^- in the form of beetroot juice would reduce oxygen consumption during submaximal exercise and increase in glucose uptake in healthy humans and this effect will be abolished with the use of mouthwash (Study 3).

CHAPTER TWO:

THE ACUTE EFFECT OF NITRATE AND NITRITE ON GLUCOSE UPTAKE DURING ISOLATED MOUSE MUSCLE CONTRACTION

2.1 INTRODUCTION

There is much interest in the emerging beneficial effects of inorganic nitrate (NO_3^-) ingestion which include increasing exercise efficiency [6,7,104,165], reducing blood pressure, [102,142,165] and improving glucose regulation in diabetic mouse models [38,135]. NO_3^- is an ion which is readily available in the diet, particularly abundant in green leafy vegetables [113]. NO_3^- is well absorbed in the large intestine, about ~20% of which is secreted back into the oral cavity where bacteria that reside on the dorsal area of the tongue convert NO_3^- to NO_2^- [161]. NO_2^- is then swallowed and subsequently absorbed into the blood stream resulting in an elevation of plasma NO_2^- concentration [104] alongside the con-current rise in NO_3^- concentration.

Whether nitrate supplementation affects glucose metabolism during exercise/contraction has not been well examined. NO_3^- ingestion has been shown by Larsen et al. [103] to increase the RER, indicative of an increase in carbohydrate oxidation. Another study has looked at the effect of NO_3^- ingestion on plasma glucose concentration during sub-maximal, and high intensity intermittent exercise. Mean glucose concentration was found to be significantly less during both exercise intensities after ingesting concentrated beetroot juice the day before, and immediately prior to experimentation [176]. However, measuring plasma glucose concentration gives no real indication of skeletal muscle glucose uptake, as the glucose concentration is the balance of glucose appearance and disappearance from the blood. NO_3^- has been shown to increase muscle force in both frog [77,88] and mouse muscle [31] as well as increasing glucose uptake, even when force is normalised to control values during *ex vivo* contractions [77]. However, supraphysiological concentrations of NO_3^- [77] were used, approximately three orders of magnitude higher than found in the blood after a high NO_3^- meal. As far as we are aware the independent effects of NO_3^- on glucose uptake during contraction, and force at physiological concentrations, have not been explored. However NO_3^- feeding at a physiological level has been shown to increase isometric force production at sub-maximal stimulation, possibly as a result of a greater influx of calcium in single muscle fibres from mice [70].

This is also consistent with the finding in a human study where peak force response was enhanced with low-frequency electrical stimulation after NO_3^- feeding [64]. In addition, NO_2^- has been shown to act as a signalling molecule [32] therefore warranting investigation of whether NO_2^- per se affects contraction stimulated glucose uptake. NO_2^- is able to be further reduced to nitric oxide (NO), which can occur spontaneously under acidic conditions, such as in the gut [115] and is greatly facilitated by hypoxia and a number of proteins and enzymes that include deoxyhaemoglobin [45], deoxymyoglobin [158], and xanthine oxidase [126], as reviewed by Lundberg et al [113].

NO has been shown to be essential for the normal increases in skeletal muscle glucose uptake during contraction in rodents [123,124] and during exercise in humans [24,95]. Inhibition of NO synthase (NOS), the enzyme class that synthesises NO, has consistently been shown to attenuate the increase in skeletal muscle glucose uptake, independent of blood flow *in vivo* [24,95], *in situ* [151] and *ex vivo* [123,124]. Studies have tended to assume that the effects of NO_3^- are due to increases in NO. To examine whether there are direct effects of NO_3^- and NO_2^- on glucose uptake during contraction we used an *ex vivo* contraction model in an attempt to isolate any effect of NO_3^- and NO_2^- on glucose uptake, or force during contraction independent of the effects of NO. In the *ex vivo* preparation, the buffer is bubbled with carbogen (95% oxygen) resulting in an environment that is hyperoxic, and slightly alkaline (pH 7.4). Therefore, the likelihood of reduction of NO_2^- to NO is unlikely. In addition, NO_3^- cannot be reduced to NO_2^- due to the absence of NO_3^-reductase bacteria normally present in the oral cavity.

To confirm that NO_3^- and NO_2^- were not reduced to NO, we investigated whether NO_3^- or NO_2^- could overcome the inhibitory effects of the NOS inhibitor L-^{N}G-monomethylarginine (L-NMMA) on glucose uptake during contraction. We have shown that L-NMMA attenuates the increase in glucose uptake during contraction *ex vivo* so if these inhibitory effects were overcome by either NO_3^- or NO_2^- this would indicate that NO had been formed in our preparation. We hypothesised there would not be any effect of NO_3^-, or NO_2^- on glucose uptake during contraction therefore concluding that the effects *in vivo* are via NO.

2.2 METHODS

2.2.1Animals

All procedures performed in this experiment were approved by the Victoria University Animal Experimental Ethics Committee (AEETH 07/12) and conformed to the Australian code of practice for the use and care of animals for scientific purposes as described by the National Health and Medical Research Council (NHMRC) of Australia. This study used Male C57BL/6 mice at 12-14 week of age, which were sourced from Animal Resource Centre (Perth, Western Australia, Australia). Mice were acclimatised in the animal house for at least one week prior to experimentation. The animal house was maintained at 21°C with a 12:12-h light dark cycle with standard rodent chow and water available ad libitum.

2.2.2 Materials

Radioactive 2-Deoxy-D-[1,2-^{3}H]-glucose and D-[^{14}C] mannitol were purchased from PerkinElmer (Boston, MA, USA). All other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

2.2.3 Muscle Dissection

Mice were anesthetised with sodium pentobarbital (60 mg/kg intraperitoneal injection) prior to both EDL muscles being excised. The EDL, primarily a glycolytic (Type II) muscle was chosen as it has been shown that NO_3^- feeding preferentially effects type II muscle fibres [54,70].

Both tendons were ligated with 5-0 silk suture to a hook. The muscle was suspended horizontally in a bath in incubation solution [(mM: 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCL, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2,5 $CaCl_2$, pH 7.4] with the addition of 0.01% BSA, 8 mM mannitol and 2 mM sodium pyruvate. The solution was maintained at 30°C and continuously oxygenated with 95% O_2 and 5% CO_2 (Carbogen; BOC Gases, Australia) throughout the experiment. One end of the muscle was attached to a fixed hook and the other end being attached to a force transducer (Zulli Technology, VIC, Australia). Muscles were incubated for 30 min in the absence or presence of 500 μM sodium NO_3^- or 500 nM sodium NO_2^-. These concentrations correspond to the peak plasma concentrations reported following acute ingestion of ~6-8mM of NO_3^- in humans [94,165,176].

Other treatment conditions were L-NMMA (100 μM), L-NMMA (100 μM) + NO_3^- (500 μM) and L-NMMA + NO_2^- (500 nM). After the 30-minute incubation period, optimal muscle length was established by changing the muscle length until maximum twitch force was obtained. After completion of the incubation period, muscles were either rested or stimulated to contract. Muscles were stimulated (Zulli Technology, Victoria, Australia) for 10 minutes by square wave electrical pulses (350 ms train; 60Hz; 0.2 ms; 12 V; 12 contractions/min) delivered via two platinum electrodes that ran alongside the muscle without touching it. Contraction forces were recorded, and displayed with software developed by Zulli Technology. Only the first 5 minutes of force was recorded and presented, as this is the period where rate of fatigue is most evident (<20% of initial force at this stage) [78,123,124]. At the end of the 10 minutes of rest or contraction, muscles were washed in ice-cold buffer, blotted on filter paper, the suture and hooks were cut off and muscles were snap frozen in liquid nitrogen. Muscles were stored at -80°C for future analysis.

2.2.4 Glucose uptake measurements

Glucose uptake was measured in the final 5 minutes of contraction or rest. The incubation buffer was quickly exchanged with a radioactive buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.128 μCi/ml) and 8 mM D-[^{14}C] mannitol (0.083 μCi/ml), [123]. To calculate muscle 2-deoxy- glucose uptake muscles were weighed before being digested for 10 min at 95°C in 135 ul of 1 M NaOH. Muscle homogenates were then neutralised with 135 ul of 1 M HCl before being spun at 13,000 g for 5 min. The supernatant (200 ul) was recovered and added to 4 ml of inorganic liquid scintillation counter (PerkinElmer, Boston, MA, USA). Radioactivity was measured using a β-scintillation counter (Tri-Carb 2910TR, PerkinElmer, Boston, MA) with muscle glucose uptake calculated based on [^{14}C] mannitol and [^{3}H] 2-DG counts before being normalised to muscle weight [123].

2.2.5 Statistical analysis

All data are expressed as means ± SEM and results analysed using SPSS statistical package. Glucose uptake and initial force were analysed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis if a treatment effect was established. Normalised force was analysed using one-factor repeated-measures ANOVA (treatment x time). Sample size was based on data from previous research investigating glucose uptake using the same model [68,123] in order to have an adequate statistical power (0.8).

2.3 RESULTS

2.3.1 Force

Initial force was significantly (P<0.05) attenuated by approximately 30% in the NO_3^-, and NO_2^- groups compared with the other contraction groups, as shown in Fig. 2.1A. When contraction force was normalised to initial peak force, there was no significant difference in the rate of fatigue over the first 5 minutes of contraction between the contraction groups as shown in Fig. 2.1B. The initial force and rate of fatigue in all other groups were not different (P>0.05) to contraction alone.

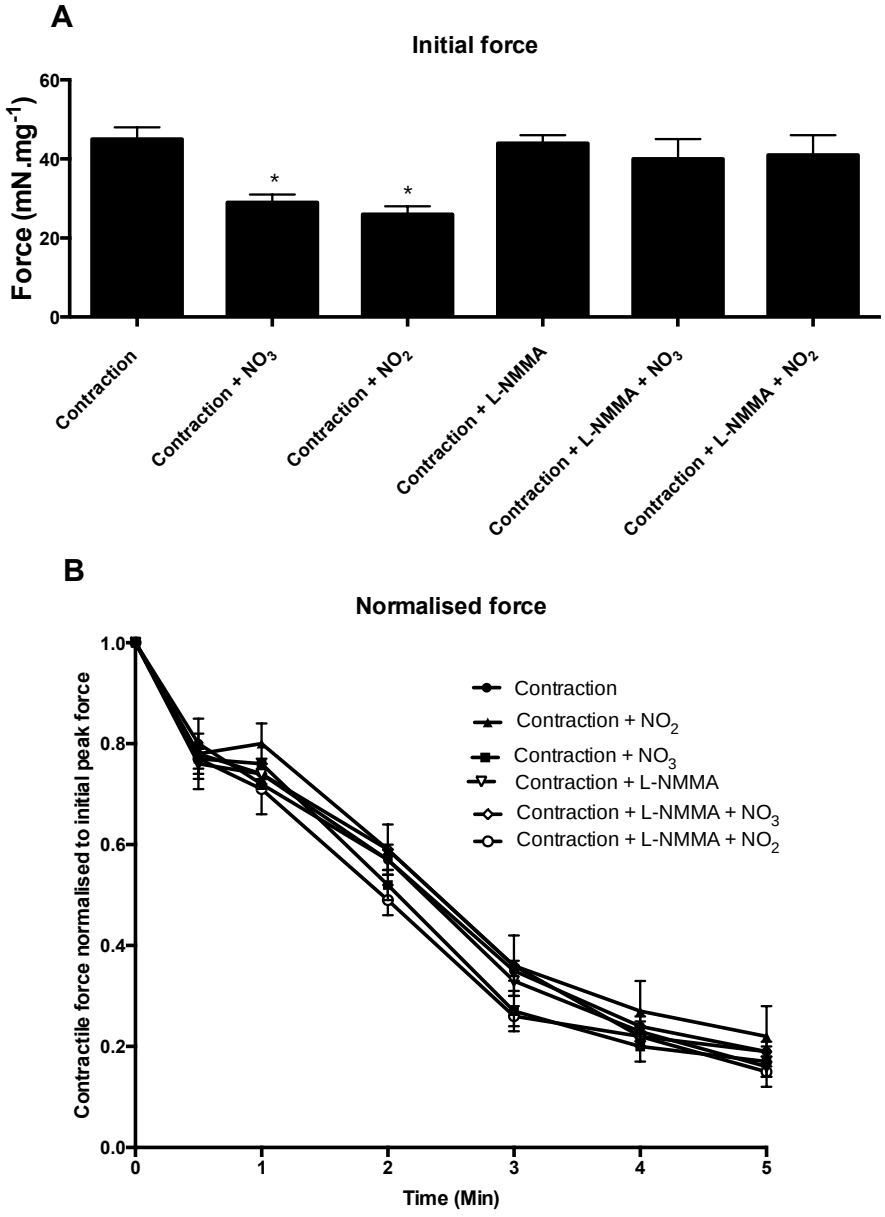

Figure 2.1: (A) Initial force (± SEM) of muscle contraction with the varying conditions and treatments (n=6-18). (B) Force normalised to peak initial force (± SEM) during the first 5 minutes of contraction. (n=6-18) * P<0.05 vs. other groups.

2.3.2 Glucose uptake

As shown in Fig. 2.2, contraction increased glucose uptake approximately 2-fold compared with basal conditions. Contraction + NO_3^- or Contraction + NO_2^- increased glucose uptake similarly to contraction alone. L-NMMA attenuated (P<0.05) the increase in glucose uptake by ap20%, and the co-incubation of NO_3^- or NO_2^- with L-NMMA had no effect, remaining significantly lower than contraction alone.

Glucose uptake for Contraction + NO_3^- or Contraction + NO_2^- was greater (P<0.05*)* when normalised to the area under the curve of the force graph for the first five minutes (Fig. 2.3).

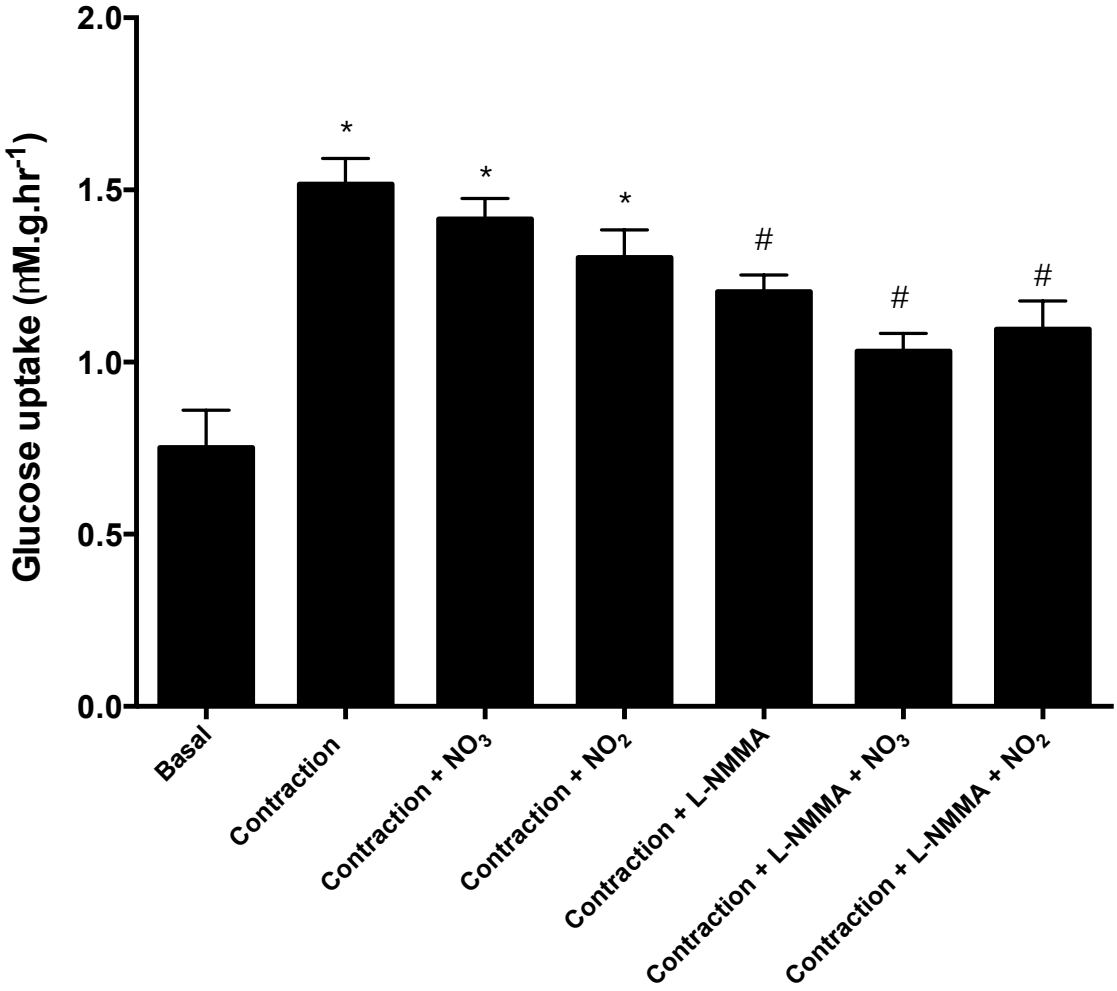

Figure 2.2: Glucose uptake (±SEM) with the varying conditions and treatments (n=5-18). *P<0.05 vs. basal. # P<0.05 vs. contraction alone.

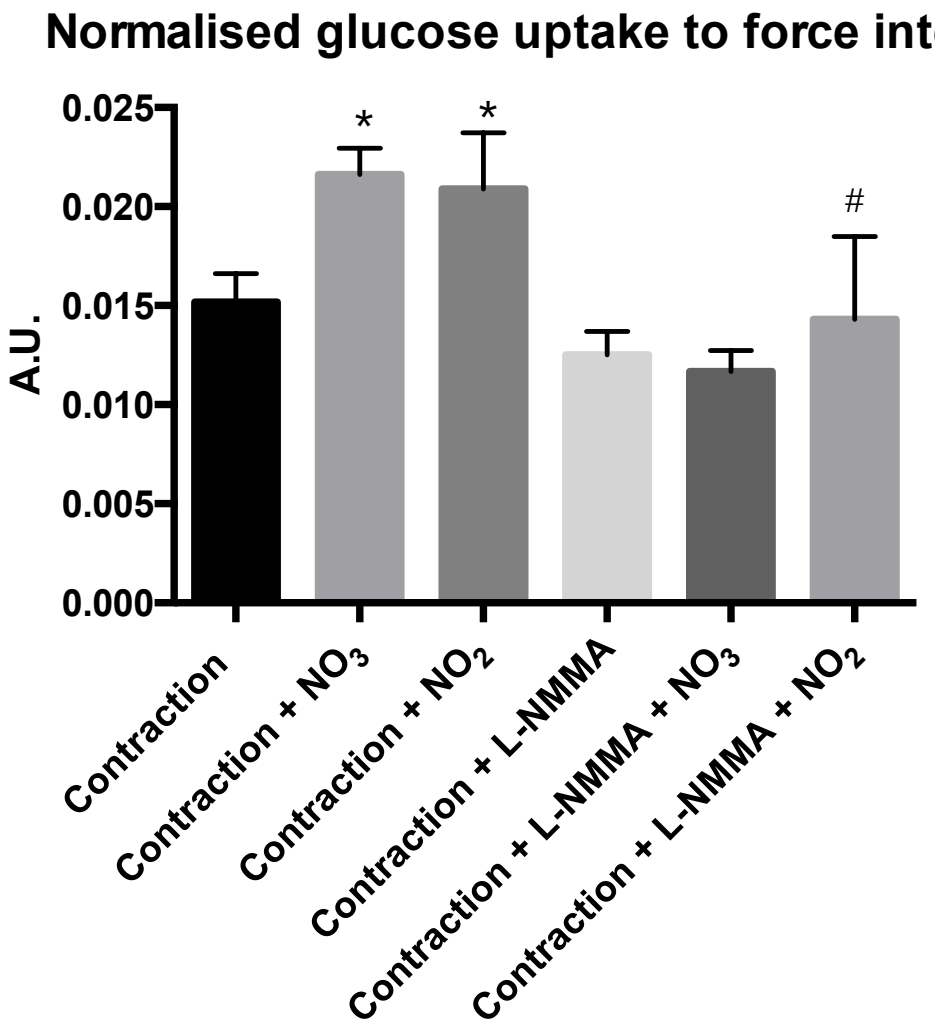

Figure 2.3: Glucose uptake (±SEM) normalised to the force generated during the first 5 minutes of contraction (Area under the curve) with the varying treatments (n=6-18). *p<0.05 vs. Contraction, Contraction + L-NMMA and Contraction +L-NMMA + NO_3. # P<0.05 vs. Contraction + NO_3.

2.4 DISCUSSION

The major findings of this study were that NO_3^- and NO_2^- did not alter glucose uptake during contraction, although glucose uptake was greater than contraction alone when normalised to force. In addition neither NO_3^-, nor NO_2^- altered the attenuation in glucose uptake with L-NMMA.

NO_3^- and NO_2^- at first glance, at physiological concentrations did not appear to affect skeletal muscle glucose uptake during contraction. However NO_3^- and NO_2^- as shown in Fig. 1, both reduced initial peak force without affecting the rate of fatigue over the first 5 minutes. A study by Ihlemann et al [84] demonstrated that glucose uptake into skeletal muscle *ex vivo* is directly proportional to the contraction force generated.

They found that glucose uptake increased from 27 ± 2 nmol.g.5 min^{-1} basally to 68 ± 3 and 94 ± 3 nmol.g.5 min^{-1} during contraction at 50, and 100% of peak initial force, respectively. We found that despite a approximately 30% reduction in initial peak force during contraction in the NO_3^- and NO_2 treatments there was no corresponding reduction in glucose uptake during contraction$^{-\cdot}$ This suggests that both treatments actually had a relative stimulatory effect on glucose uptake during contraction. Indeed, when glucose uptake was normalised to the area under the curve of the force graph for the first five minutes, the contraction + NO_3^- and contraction + NO_2^- groups did have greater glucose uptake compared with contraction alone, as illustrated in Fig. 2.3.

The attenuation in force generation with NO_3^- and NO_2^- is interesting. We are not aware of previous research that has examined the direct acute effects of physiological concentrations of NO_3^- or NO_2^- on force generation. Although, as mentioned previously, NO_3^- feeding has been shown to increase low-frequency electrically evoked twitch force, contrary to our findings. A reduction in endogenous NO via the inhibition of NOS, and NO scavengers has been shown to increase sub-maximal force [97,146], suggesting NO can be inhibitory to force generation. This suggests NO was generated in these treatment groups. However, further reduction of NO_2^- to NO is unlikely using isolated muscle considering a hyperoxic environment was present, the opposite environment shown to enhance this reduction [112]. Additionally, in the absence of nitrate reductase bacteria, normally present in the mouth *in vivo*, the reduction of NO_3^- to NO_2^- should not be possible. Nonetheless, the addition of the L-NMMA to both NO_3^- and NO_2^- does appear to normalise the force generating capacity of the muscle. This suggests that NO_3^- and NO_2^- are somehow reduced to NO and L-NMMA inhibits additional NO from NOS.

However, if NO_3^- and NO_2^- were in fact reduced to NO, one would expect the co-incubation of NO_3^- and NO_2^- with L-NMMA would partially at least, restore glucose to levels obtained in contraction alone, but this was not the case. Therefore, the reason for the decrease in force with NO_3^- and NO_2^- and restoration of force with the addition of L-NMMA is unclear, and requires further investigation. Our results tend to contrast with the results obtained by Holloszy et al. [77] where contraction force and glucose uptake were enhanced with the addition of NO_3^- but as mentioned previously the level of NO_3^- used then was supra-physiological

Currently we are assuming that NO_3^- is able to permeate the skeletal muscle, as no studies could be found investigating if skeletal muscle is able to transport NO_3^- across the plasma membrane.

This assumption is simply based on the fact that NO_3^- has been shown to affect force production and glucose uptake in skeletal muscle [77,88]. However, as described earlier NO_3^- is concentrated and resecreted into the oral cavity before being taken up by the salivary glands, reduced to NO_2^-, and subsequently absorbed in the GI tract. A study by Qin et al [143] investigated how NO_3^- is taken up by the salivary glands, and based on their findings propose the protein Sialin (SLC17A5) is likely to fulfil such a function. However, no studies could be found investigating if this same protein is expressed in skeletal muscle. In regards to the absorption of NO_2^- by tissues, it is known that NO_2^- is rapidly distributed in different tissues after intratracheal, intravenous [32] or intraperitoneal administration [32,138] of NO_2^-. Therefore, based on this, it is assumed NO_3^- and NO_2^- are taken up by the muscle *ex vivo.* However, the thirty-minute exposure time prior to contraction may not be long enough and would require further experimentation to establish if this is so.

In summary, physiological levels of NO_3^- and NO_2^- are associated with normal increases in mouse skeletal muscle glucose uptake during *ex vivo* contraction despite a significant reduction in force production. Additionally, NO_3^- and NO_2^- do not overcome the attenuation of the increase in skeletal muscle glucose uptake during contraction caused by NOS inhibition. This suggests that the effects of NO_3^- and NO_2^- were independent of NO and due to NO_3^- and NO_2^- per se.

CHAPTER THREE:

THE ACUTE AND CHRONIC EFFECTS OF NITRATES ON CONTRACTION EFFICIENCY OF ISOLATED SKELETAL MUSCLE

3.1 INTRODUCTION

Inorganic nitrate (NO_3^-) is an anion found in abundance in green leafy vegetables, and at a high concentration in some root vegetables, such as beetroot [111]. The ingestion of NO_3^- as a salt or from foods containing a high concentration of NO_3^- reduces oxygen consumed (VO_2) for a given sub-maximal workload [7,100,103-105,165], although, not all studies support this [18,20,27,92,156]. This indicates that exercise efficiency (the ratio of power output to rate of energy consumption) may be improved with NO_3^- supplementation. For example, after three days of inorganic NO_3^- supplementation Larsen et al. [104] found that oxygen consumed (VO_2) for a given sub-maximal workload was significantly reduced by ~3-4% in healthy males. This increase in exercise efficiency was attributed to a reduction in mitochondria uncoupling since an increase the ratio of adenosine triphosphate (ATP) generated to O_2 consumed (P:O ratio) was observed [103]. An alternate possibility is that the contractile machinery itself is more efficient [6]. Supporting such a proposal, Bailey et al [6] found an attenuation in the reduction in phosphocreatine (PCr) and estimated ATP turnover during steady state knee-extensor exercise.

Currently it is assumed that the effects of NO_3^- on exercise efficiency are via the stepwise reduction of NO_3^- to NO [116]. The basis for this is that NO is known to compete with O_2 at complex IV in the mitochondria, inhibiting mitochondrial respiration [29]. Larsen et al. [103] propose the inhibition of mitochondria respiration results in the mitochondria sensing a state of hypoxia. A molecular response is in turn initiated to decrease leak respiration, indicated with a decrease in the protein ANT, a protein attributed to proton leak [103]. In addition they demonstrated an increase in mitochondrial efficiency via measuring respiration in isolated mitochondria.

However, similar decreases (approximately 3-4%) in VO_2 for a submaximal workload are also shown after just one dose of NO_3^- given 2.5 hours prior to exercise [165,175]. With such a rapid effect there is unlikely to be sufficient time for a significant change in ANT protein expression to occur suggesting that an alternate mitochondrial effect is occurring, or that the contractile machinery is becoming more efficient as has been indicated by a reduction in calculated ATP turnover [7] for given power output after NO_3^- ingestion.

NO_3^- ingestion also affects muscle force dynamics. Using isolated mouse muscle fibres, Hernandez et al. [70] observed an increase in sub-maximal (stimulation frequency ≤50Hz) force generation along with an approximate 35% increase in rate of force development in fast twitch muscle (EDL). This was only observed at a stimulation frequency of 100Hz (near maximal stimulation for this muscle) after 7 days of nitrate supplementation [108]. They also found an increase in myoplasmic free calcium during tetanic stimulation, accompanied by an increase in the calcium handling proteins calsequestrin 1 and the dihydropyridine receptor. They propose the effects on force dynamics are a result of these changes to calcium handling. This indicates the potential for NO_3^- ingestion to alter the contractile mechanism, which may have some relevance regarding its effects on exercise and/or contractile efficiency. Furthermore, NO_3^- increases twitch force by approximately 300% in isolated amphibian and mammalian muscle, albeit at supra physiological concentrations [75,88,145]. In these experiments the effect of NO_3^- was rapid and reversible with the effect being lost after transitioning back to regular buffer. Such a rapid response in circumstances where little conversion of NO_3^- to NO_2^-/NO would be expected, indicates that NO_3^- may have effects on the contraction machinery independent of NO. Furthermore the metabolic intermediate NO_2^- has also been shown to have physiological effects independent of NO [32]. Therefore it is necessary to perform further investigation into the direct effects of NO_3^- and NO_2^- on force dynamics and contractile efficiency at physiological concentrations.

The proposed mechanisms are currently lacking. It is either insufficient to explain both the acute and chronic effects of NO_3^- on exercise efficiency, or has not been explored further i.e. the proposed increase in efficiency of the contractile machinery in skeletal muscle. Therefore, we investigated the effects of the acute application of NO_3^- and its metabolites on the contractile efficiency of isolated mouse muscle.

Contractile efficiency was defined as the ratio of the amount of work performed to the total enthalpy output i.e. heat + work during series of contraction cycles [12]. With this approach, it is possible to measure contractile efficiency precisely, whilst also being able to assess mitochondrial function [15]. In addition the effects of 6 days of NO_3^- supplementation was examined using the same isolated mouse model. On the basis of the work performed with NO_3^- in exercising humans [7,100,103-105,165] that did find an increase in exercise efficiency with NO_3^- ingestion, we hypothesised that acute application NO_3^- would not alter contractile efficiency but NO_2^- would as it is the more reactive metabolite. In addition we hypothesised that 6 days of supplementation of NO_3^- in mice would result in an increase in contractile efficiency as has been shown in some human studies.

3.2 METHODS

3.2.1 Muscle preparation

Adult male mice (Swiss strain) were rendered unconscious by the inhalation of a mixture of 20% O_2 and 80% CO_2 and then killed by cervical dislocation. The fast-twitch extensor digitorum longus (EDL) muscles were dissected from the hind limbs and placed in Krebs-Henseleit solution with the following composition (in mM): NaCl, 118; KCL, 4.75; KH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; $CaCl_2$, 2.5; glucose, 10. The EDL muscle was chosen as effects of NO_3^- feeding in mice have been shown to be exclusive to fast twitch muscle [70]. The solution was aerated (95% O_2- 5% CO_2) before and throughout experiments. All procedures were approved by Griffith University Animal Ethics Committee (PES/02/13/AEC). Experiments were performed using muscle fibre bundles (mean mass ± SEM, 3.58 ± 0.32mg; length, 9.03 ± 0.31mm). The size of the fibre bundles were quantified prior to contraction to ensure diffusive O_2 supply to the muscle was adequate [11]. Aluminium foil clips were crimped onto each tendon to provide a low-compliance link between the preparation and the apparatus. The preparations were mounted between a fixed clamp and the lever of a servo-controlled ergometer (300C, Aurora Instruments, Ontario, Canada) and lay along the active thermocouples of a thermopile used to record changes in muscle temperature [13] as shown in Fig. 4.1. The recording region of the thermopile was 6mm in length, contained 24 antimony-bismuth thermocouples (Barclay's Laboratory, Queensland, Australia) and produced 2.05 mV $°C^{-1}$. There were eight thermocouples at either end of the thermopile that were not connected to the recording circuit to ensure uniform heat

loss along the length of the preparation. This ensures that sections of the muscle that move on to the recording section of the thermopile during shortening are at the same temperature as the parts of the muscle on the recording section prior to shortening [74]. Muscle preparations were stimulated via fine platinum wires that were lightly touching either side of the muscle with rectangular pulses of 2 ms duration delivered at a frequency of 150Hz [108]. All experiments were performed at 35°C, which is near the physiological temperature of this muscle [2].

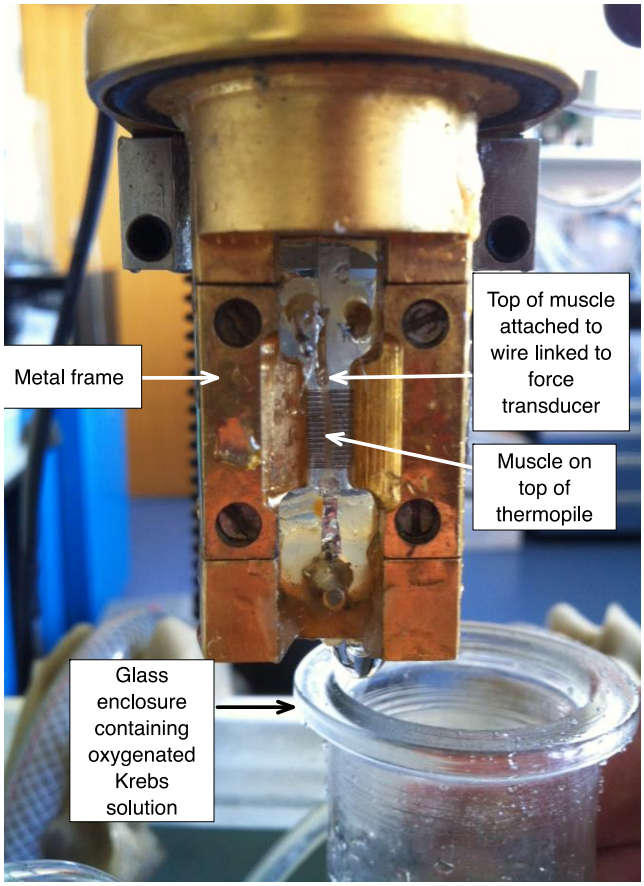

Figure 3.1: Thermopile setup to measure thermodynamics and work.

3.2.2 Treatment protocol

3.2.2.1 Acute study

Eight muscle preparations were obtained from five adult male mice (Swiss strain). The force and heat produced by each muscle was measured in standard Krebs solution and in each of three solutions designed to manipulate the nitrate or nitrite status of the muscle.

The three treatments were: (1) 500 μM $NaNO_3$ (NO_3^-) (2) 500 nM $NaNO_2$ (NO_2^-) and (3) 100 μM of the nitric oxide synthase inhibitor L-N^G-monomethylarginine (L-NMMA). Treatment stock solutions (x1000 working concentration) were prepared with MilliQ water and added to the standard Krebs-Henseleit solution. The concentration of NO_3^- and NO_2^- were based on reported peak plasma concentrations of NO_3^- and NO_2^- measured after ingestion of NO_3^- in humans [94,165]. The concentration of L-NMMA chosen has been previously shown to decrease endogenous NO production via nitric oxide synthesis enzyme (NOS) activity by approximately 90% [73]. Nitrite is unlikely to be reduced further to NO as it can do *in vivo* due to the hyperoxic environment present in this *ex vivo* model. The intent of the L-NMMA treatment group was to determine the role of endogenous NO production on contractile efficiency by inhibiting enzymatic production of NO. Muscles were exposed to each treatment solution for 30 min before measurements were made. Between each treatment, muscles were bathed in fresh Krebs-Henseleit solution for 10 min. The control measurements were always carried out first to ensure measurements were not affected by treatments. This was also done to enable the use of the control group for the chronic study. The order of exposure to the three treatments was counter-balanced to accommodate any crossover in treatments effects.

3.2.2.2 Chronic study

Six muscle preparations were obtained from six separate adult mice, which were given 1 mM $NaNO_3$, dissolved in distilled water for 6 days. The total decrease in water volume was measured after the 6 days, and average dose for each mouse calculated assuming no water loss. The dose of NO_3^- consumed was 0.29±0.03 μM/g body mass (n = 6; mean ± SE). This is similar to that previously used in human NO_3^- and exercise studies, which corresponds to a daily ingestion of about 200-300 g of spinach in a 70 kg person [70]. The results were compared to the data from the control group in the acute study.

3.2.3 Experimental protocols

At the beginning of each experiment, stimulus strength was set to 10% above that required to elicit maximum twitch force. After stimulus strength had been set, a brief series of maximal tetani was used to set muscle length to that at which force output was maximal (L_0).

A digital photograph was taken of the muscle at L_0 and was used to measure muscle length. Muscle length was then set to 110% of L_0 so that muscle contracts across L_0 during the series of isokinetic contractions.

The contraction protocol consisted of a series of 10 contractions at a frequency of 0.2 Hz. Each contraction consisted of 12 stimulus pulses delivered at 150 Hz (total duration of stimulation, 73 ms). Each muscle performed a series of isometric contractions, and a series of isokinetic, using the work loop method [87], under each treatment. Force dynamics i.e. maximum force in each cycle, rise rate (rate of force development) and relaxation rate were measured during the isometric contraction. In the isokinetic protocol, each contraction commenced with 20 ms of isometric contraction, to allow force to develop, followed by isovelocity shortening with an amplitude of 15% L_0 as shown in Fig. 4.2. The shortening velocity was 2.8 L_0 s^{-1} (isokinetic) which is the velocity where contractile efficiency of these muscles is maximal at 35°C [14,108]. Once shortening was complete, the muscle was slowly lengthened back to the starting length. This duration of stimulation ensured the muscle contracted throughout the shortening phase while allowing sufficient time for relaxation to be complete before lengthening phase began as shown in Fig. 3.2. This maximised the net work output in each length change cycle.

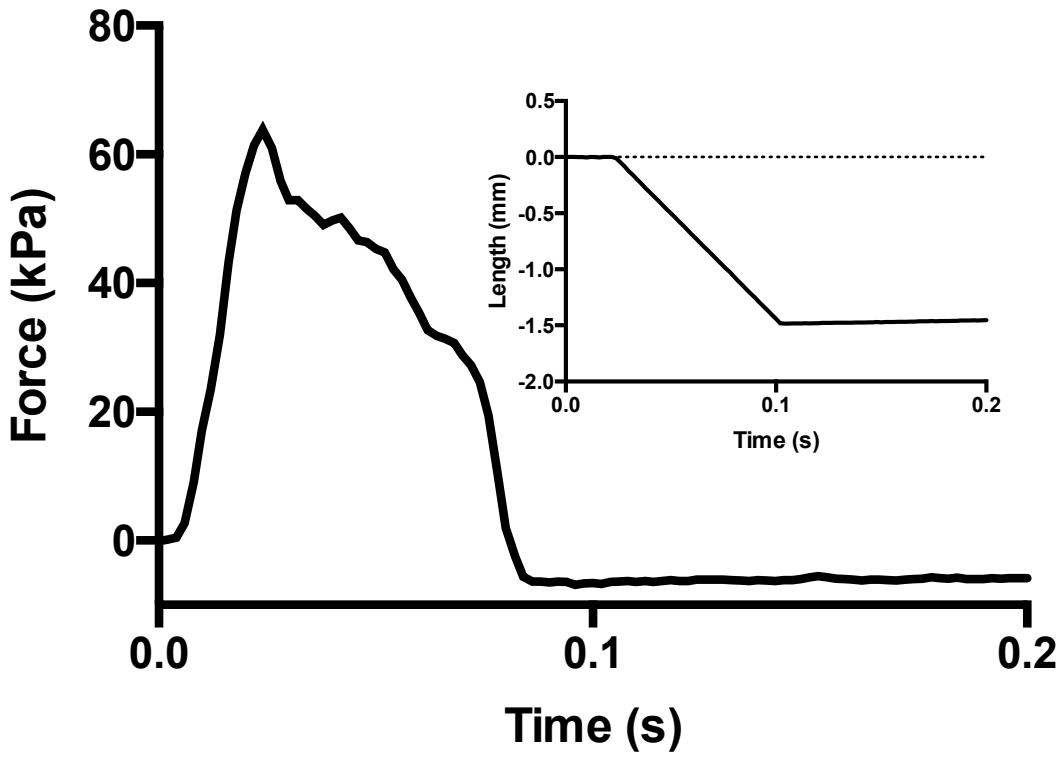

Figure 3.2: Force output during isokinetic contraction from EDL muscle preparation (mass, 5.08 mg; length 9.01 mm) from the 1st of 10 contraction cycles (12 pulses stimulus pulses delivered at 150 Hz). Insert shows change in length with the initial 20ms of contraction being isometric contraction to allow for force to develop before commencing to shorten at 25.2 mm. s^{-1}(2.8 L_0 s^1) at an amplitude of 1.35 mm (15% L_0).

Muscle enthalpy production was used as the index of energy cost for the contraction series [12]. Enthalpy output is the sum of the heat and work produced and is proportional to the ATP turnover and O_2 consumed [173]. Work output was determined from records of force and change in muscle length. Heat production was determined from the change in muscle temperature, which was measured throughout the contraction series and for a further 180 s by which time heat production associated with the series of contraction had ceased. Before each contraction series, muscle heat capacity and the rate of heat loss from the preparation were measured [17]. This was performed utilising the Peltier effect by heating and cooling the muscle and measuring the initial rate of cooling or heating to determine the time constant. From the heating and cooling time constant the heat capacity can be quantified [99]. At the end of the experiments the heat produced by the stimulus passing through the preparation (Q_S) was determined. This was done by rendering the muscle inexcitable by exposure to procaine (20 mM in saline) and then measuring the heat produced in response to the same stimulation protocol used in the experiments [85].

Once all recordings were completed, the muscle was removed from the experimental apparatus, the tendons were cut from the preparation, the muscle was blotted and wet mass was measured.

3.2.4 Data recording and analysis

Signals of force output, length change and temperature change were sampled at 500 Hz and digitised (PC16221, National Instruments, Austin, TX, USA). Force measurements were normalised by fibre bundle cross-sectional area, which was calculated as muscle mass/(length x density) and muscle density was taken to be 1.06 g mL^{-1}[108]. Work output was calculated by integrating the force output with respect to the change in muscle length; this gives the net work output, which is the difference between the work performed during shortening and work done on the muscle during lengthening. The total work performed during the series of contractions (W_T) was the cumulative work output measured at the end of the final contraction cycle (Fig. 3.3) [14].

To calculate the heat produced by the muscle, the change in muscle temperature was corrected for heat lost from the preparation during recording, multiplied by the heat capacity of the preparation and any adhering Krebs-Henseleit solution and the stimulus heat was subtracted. The total, supra-basal heat produced by the muscle in response to the contraction series (Q_M) was taken as the cumulative heat produced between the start of the contraction series and 150s after the end of the contractions (Fig 3.3). The enthalpy output (ΔH_T), which is the energetic equivalent of the oxygen consumed [108], was equal to $Q_M + W_T$ (Fig. 3.3). Both W_T and ΔH_T were normalised by the blotted wet weight of the preparation. The mechanical efficiency (ε) was defined as the ratio of W_T to ΔH_T.

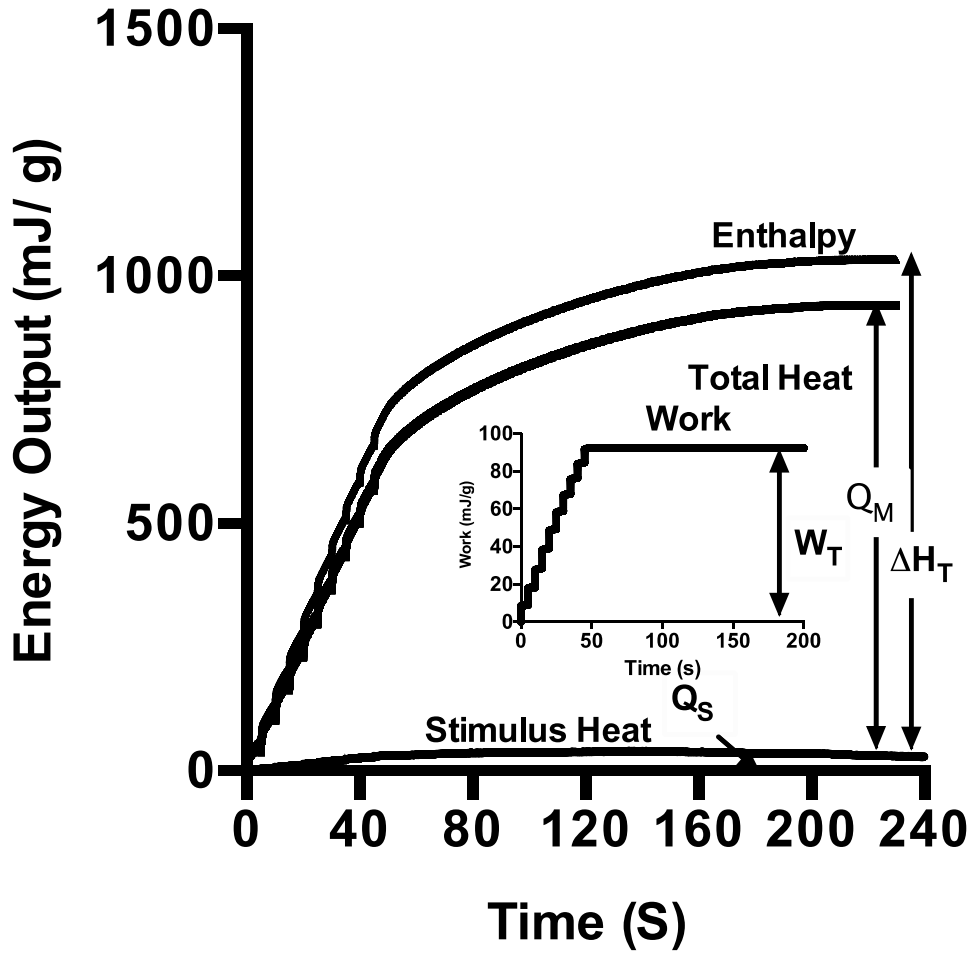

Figure 3.3. Time course of energy output. Energy output from an EDL muscle preparation (mass, 5.08 mg; length 9.01 mm) during and after performing 10 contractions, each consisting of 12 pulses stimulus pulses delivered at 150 Hz at intervals of 5 s. Heat is generated from biochemical reactions in the muscle and from the electrical stimulus passing through the muscle. The lower plot shows heat from the stimulus itself, measured at the end of the experiment once the muscle had been rendered inexcitable with procaine. The middle plot shows total heat generated from the muscle and from the stimulus. Heat continued to be generated by the muscle, approximately 150 s after the contraction series had ceased; this due to the ongoing oxidative reversal of the biochemical changes involved in the series of contractions. The upper record is the sum of the heat and work (shown in inset). The total muscle heat produced (Q_M) is the difference between the total heat recorded and the stimulus heat (Q_S). The total enthalpy output (ΔH_T) was Q_M + the total work done (W_T; inset).

Since the EDL was contracted under aerobic conditions and overall activity was moderate, it can be assumed that recovery metabolism is almost exclusively a result of oxidative metabolism [46,107]. Therefore, analysing the heat rate immediately after the last contraction cycle (i.e. recovery phase) provided a means to assess mitochondrial function. Maximum recovery heat rate was determined by the rate of heat produced 0.5s after the final contraction cycle i.e. the beginning of the recovery phase. The time constant for the time course for the recovery phase was determined by fitting an exponential through the first 30s after the end of the contraction series as shown in red in Fig. 3.4.

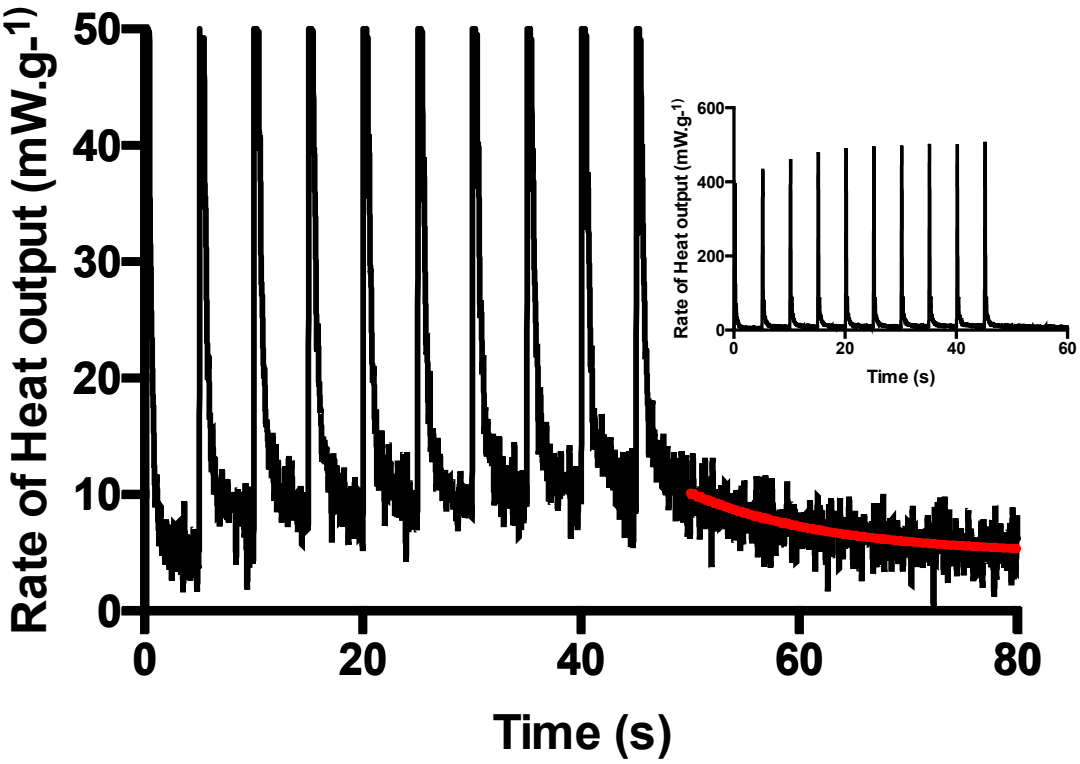

Figure 3.4: Rate of Heat output (mass, 3.21 mg; length 11.21 mm) during and after performing 10 contractions, each consisting of 12 pulses stimulus pulses delivered at 150 Hz at intervals of 5 s (Heat rate truncated at 50 $mW.g^{-1}$). As shown in the figure insert, heat rate peaks with each contraction, indicative of the rapid breakdown in ATP. This is subsequently followed by an interval of low heat rate during the recovery phase when metabolites to fuel ATP generation is resynthesised. An exponential line is fitted through the first 30 seconds of data post last contraction, as shown in red, to determine the time constant for the decline in heat rate post contraction. Maximum heat rate was determined at 0.5 seconds (start of the fitted red line in fig.) post last contraction cycle. Insert showing peak heat rate for each contraction cycle.

3.2.5. Statistical analysis

A one-way repeated measures ANOVA was used for the acute study to analyse net contractile efficiency and recovery heat (maximum and time constant). Force dynamics were analysed with a two-factor repeated-measures ANOVA with the factors being treatment and contraction cycle. The contractile efficiency and recovery heat (maximum and time constant) data for the chronic study were analysed using a one-way ANOVA and force dynamics were analysed using a two-factor ANOVA with the factors being treatment and contraction cycle. Statistical significance was set at $p<0.05$. When a significant interaction was observed post-hoc analyses were performed using Tukey's test. Data are reported as mean ± standard error mean (SEM). Power calculations were performed using the software G*Power. Using a statistical power of 0.8 as commonly performed our results were able to distinguish a difference in efficiency of ±4%.

3.3 RESULTS

3.3.1 Acute study

The mechanical efficiency of the muscle was initially measured in standard Krebs solution (control) followed by treatment solutions containing one of the following NO_3^-, NO_2^- or L-NMMA in a randomised counterbalanced design. The mechanical efficiency of the EDL muscle in control conditions was 12.9% ± 2.0 %. This value is comparable to values previously reported with this muscle at this specific temperature [108]. There was found to be a significant overall effect on mechanical efficiency with the treatments (Fig. 3.5). Post-hoc analysis indicated the only treatment to be significantly different from control was NO_3^- where mechanical efficiency was reduced by approximately 30% compared to control (Fig. 3.5).

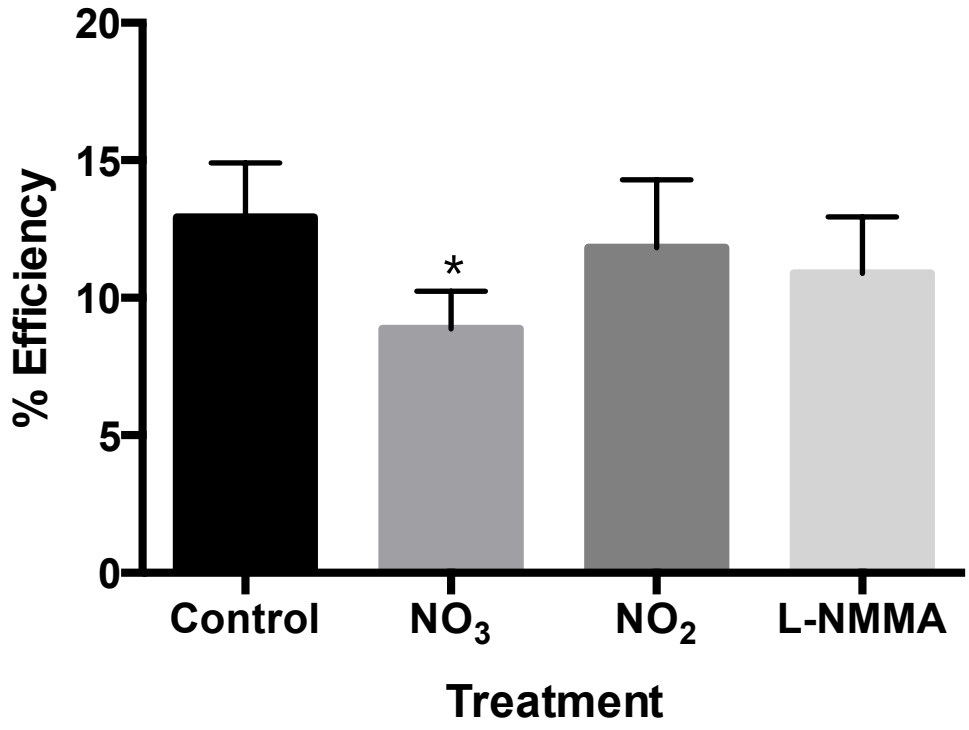

Figure 3.5: Net mechanical efficiency ± SEM with each acute treatment (n=6). Significant ($p<0.05$) treatment effect was found. *Significantly ($p<0.05$) different from all other treatment groups.

In addition, there was found to be a significant effect on W_T between groups, with subsequent post-hoc analysis showing that NO_3^-, NO_2^- and L-NMMA treatment groups to be significantly less than control (Fig 3.6A), although ΔHT was not significantly different between groups (Fig. 3.6B).

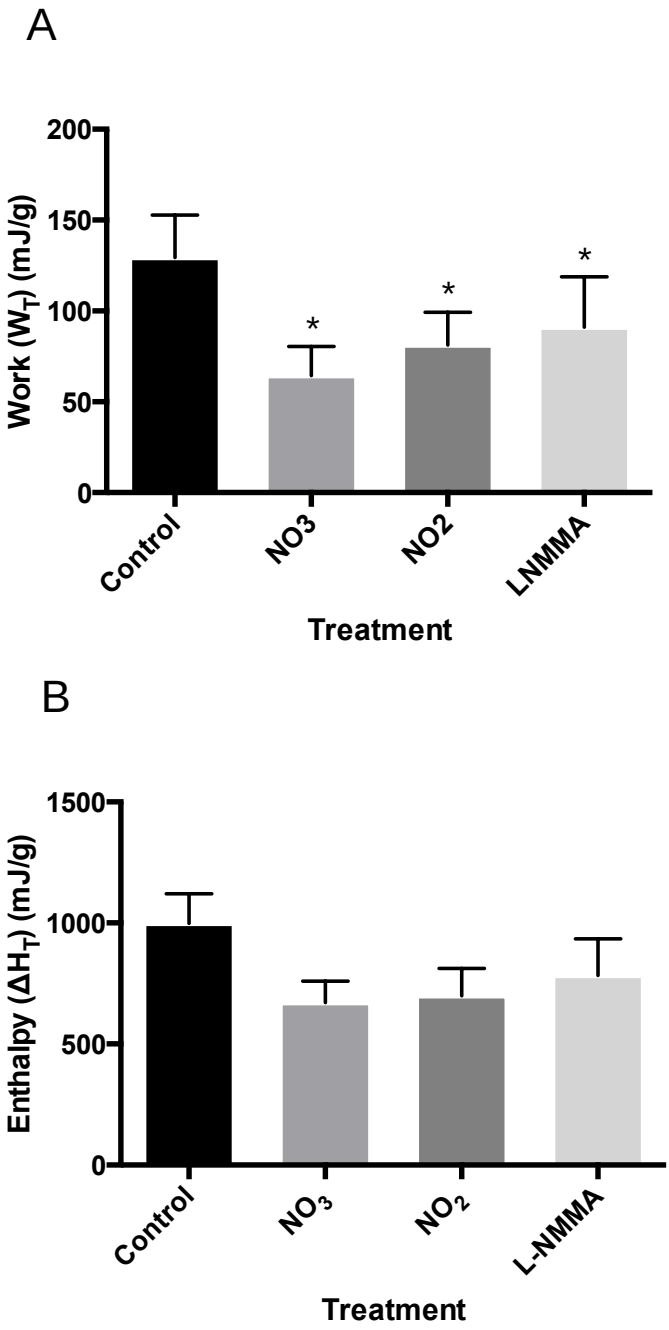

Figure 3.6: (A) Net work (W_T) ± SEM with each acute treatment (n=6.) Significant ($p<0.05$) treatment effect was found. *Significantly ($p<0.05$) different from placebo. (B) Net enthalpy (ΔH_T) ± SEM with each acute treatment (n=6). No significant ($p>0.05$) treatment effect was found.

There was found to be no significant effect with any of the treatments on either the maximum rate of recovery heat output, or the rate constant in the decline in recovery heat (Fig. 3.7). This indicates that mitochondrial function was not affected by the treatments.

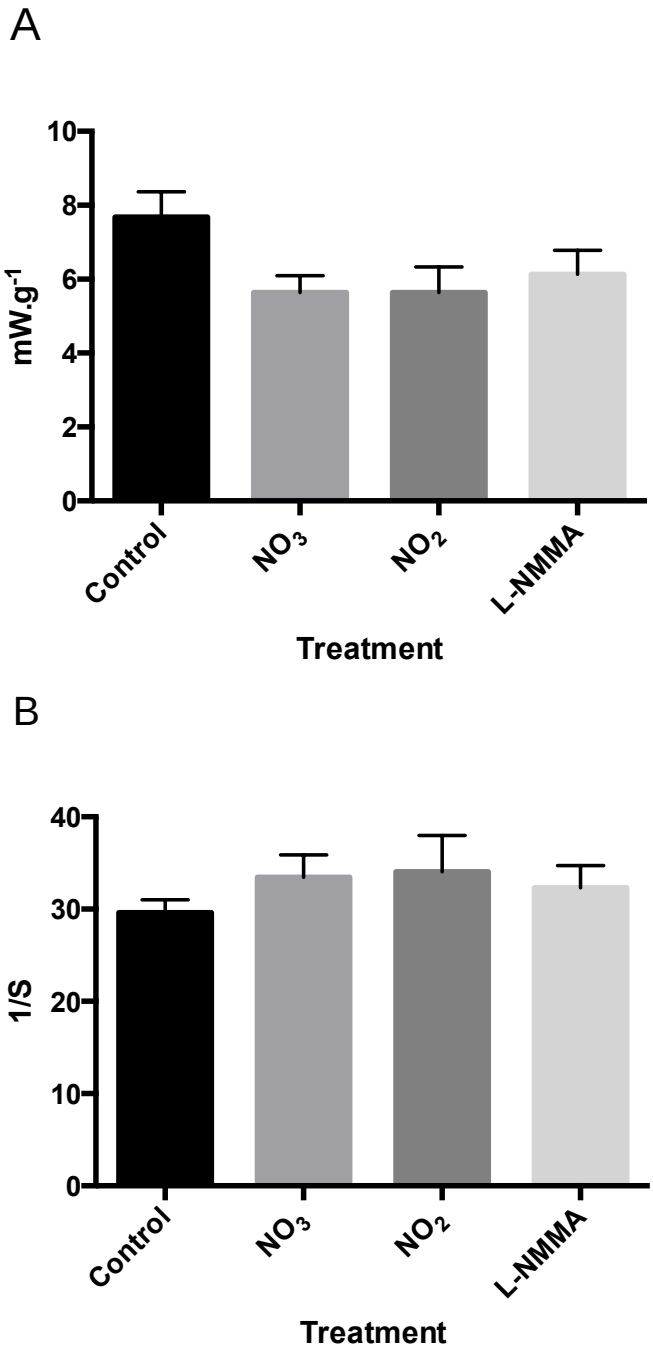

Figure 3.7: (A) Maximum recovery heat rate ± SEM with each acute treatment (n=7). No significant treatment effect ($p>0.05$) was found. (B) Time constant for recovery heat rate ± SEM with each acute treatment (n=7). No significant treatment effect ($p>0.05$) was found.

The incubation of the muscle in treatment$^-$ solutions did not significantly alter maximum force (Fig 3.8A) or alter the time course of change in rate of force development (Fig 3.8B). There was no significant interaction between treatment and contraction cycle. Relaxation rate was not significantly effected (Fig 3.8C).

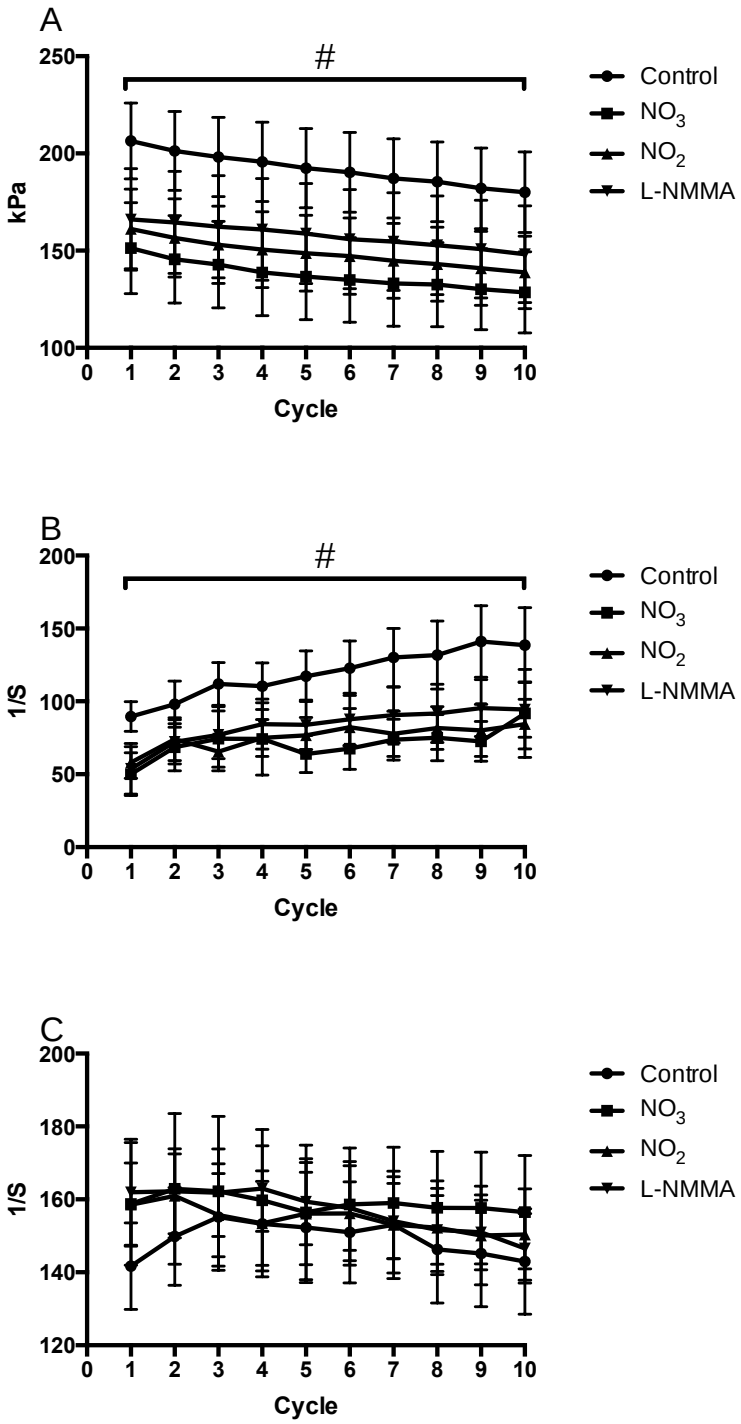

Figure 3.8: (A) Maximum force ± SEM with each acute treatment (n=7). # Significant treatment effect ($p<0.05$) was found. No significant interaction between treatment and contraction cycle. (B) Time constant for rise rate ± SEM with each acute treatment (n=7). # Significant treatment effect ($p<0.05$) was found. No significant interaction between treatment and contraction cycle. (C) Time constant for relaxation rate ± SEM with each acute treatment (n=7). No significant treatment effect ($p>0.05$) was found.

3.3.2 Chronic study

To more closely simulate the route NO_3^- follows in human studies i.e. the stepwise reduction to NO_2^- and potential further reduction to NO under hypoxic conditions [112], the second part of the experiment was performed where mice ingested NO_3^- via their drinking water.

The mechanical efficiency of the EDL muscle was measured after 6 days of NO_3^- feeding and compared to the mean mechanical efficiency achieved in the control conditions used in the acute part of the study, which was 12.9 ± 2.00 %. There was no effect of NO_3^- feeding on mechanical efficiency with a mean mechanical efficiency of 12.9 ± 2.2% as shown in Fig. 3.9.

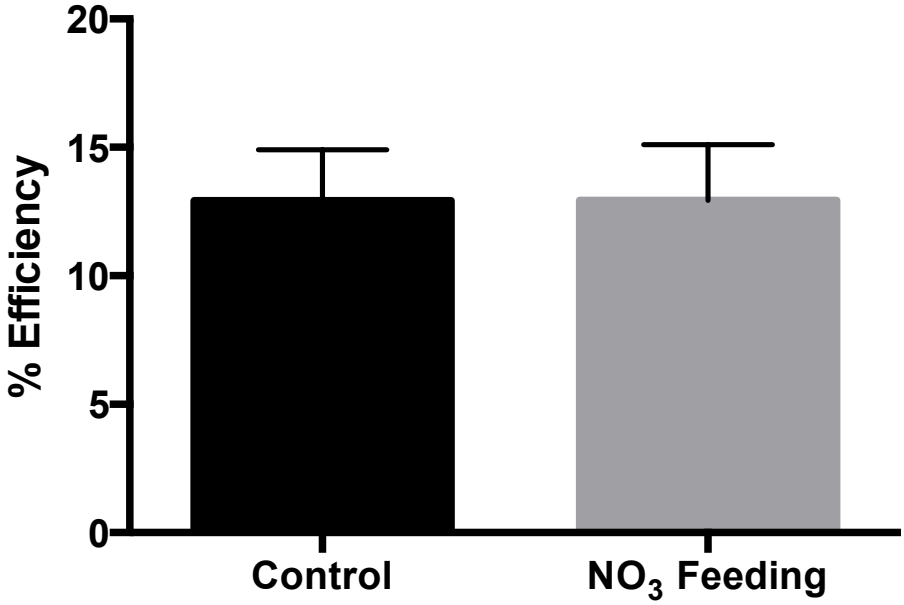

Figure 3.9: Mean net mechanical efficiency ± SEM for control (n=7) and Six days of NO_3^- feeding (n=6) group. No significant ($p>0.05$) differences between NO_3^- feeding group and control.

Again mitochondrial function was assessed from the magnitude and kinetics of recovery heat production. There was no significant effect of NO_3^- feeding on either the maximum rate of recovery heat output, nor the rate constant for the decline in recovery (Fig. 3.11).

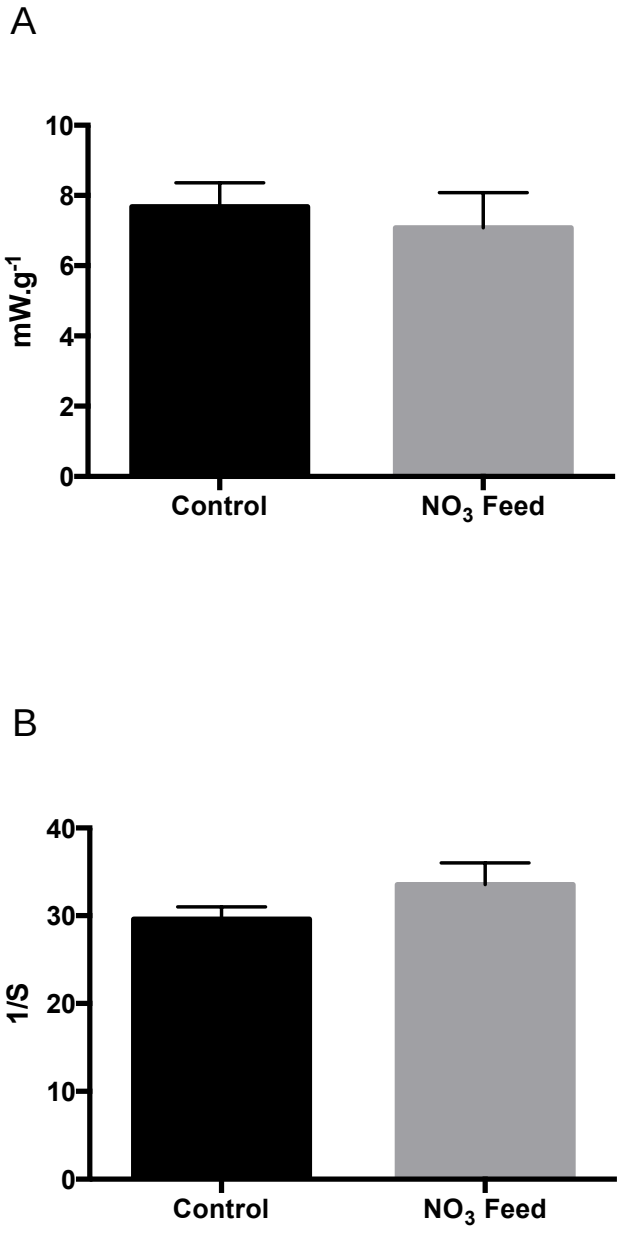

Figure 3.11: (A) Maximum recovery heat rate ± SEM for control (n=7) and NO_3^- feed (n=6). No significant ($p>0.05$) differences between groups were found. (B) Time constant for recovery heat rate ± SEM for control (n=7) and NO_3^- feed (n=6). No significant ($p>0.05$) differences between groups were found.

NO_3^- feeding also did not significantly alter maximum force (Fig 3.12). There was a significant effect of NO_3^- feeding on the time course of changes in rise rate (rate of force development) with post hoc analysis indicating that the rate of force development was significantly less (at cycles 7-10) as shown in Fig 3.12B. There was no effect with NO_3 supplementation on rate of relaxation (p=0.29) (Fig 3.12C).

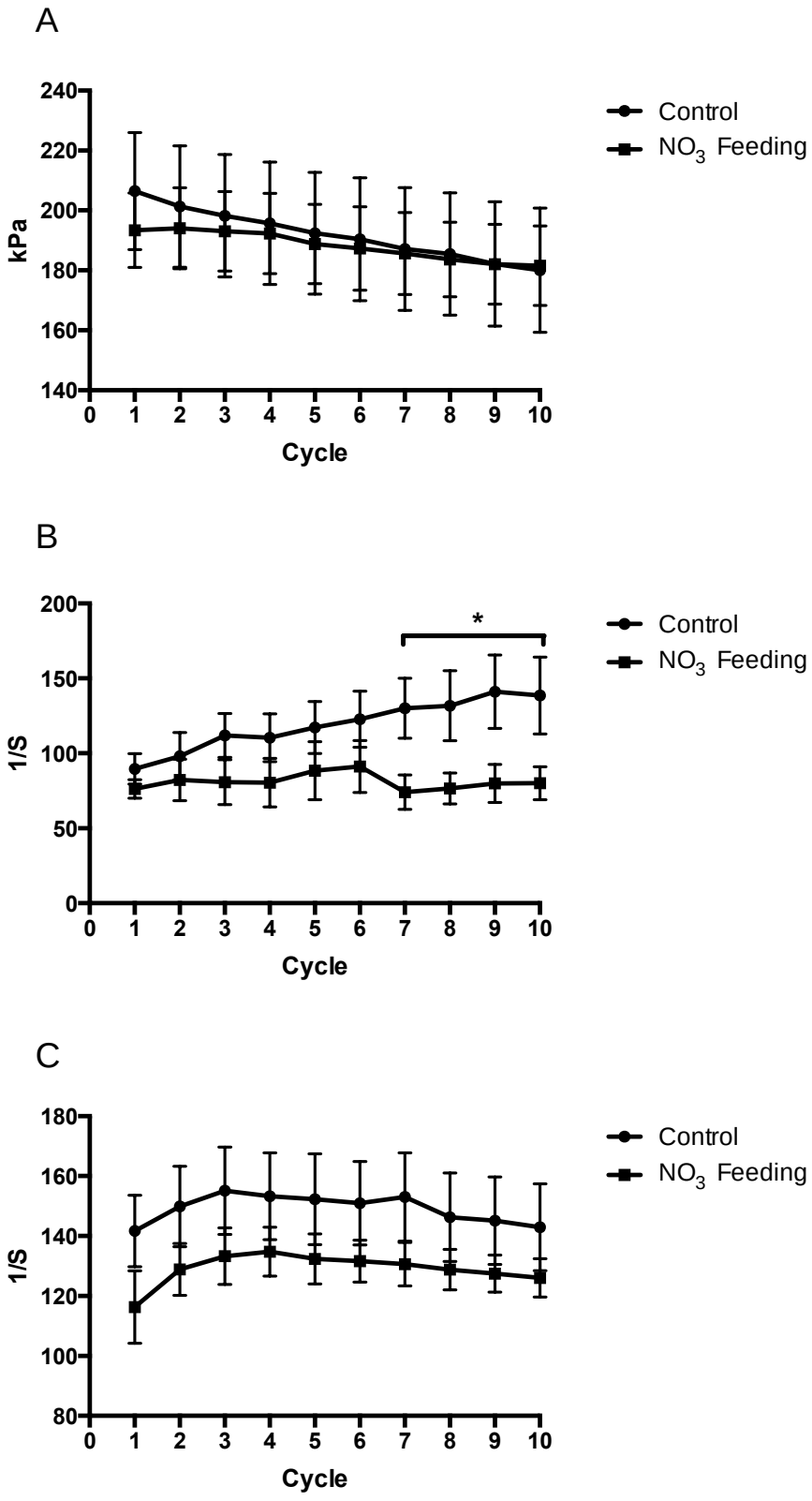

Figure 3.12: Mean maximum force (A), rise rate (rate of force development) (B) and relaxation rate (C) ± SEM for control (n=7) and the NO_3^- feeding group (N=6). *Significant difference ($p<0.05$) between control and NO_3^- feeding group for rise rate. No significant ($p>0.05$) difference between NO_3^- feed group and control for maximum force or relaxation rate.

3.4 DISCUSSION

On the basis of previous human studies, we hypothesised that acutely applying NO_2^- to an isolated muscle would increase contractile efficiency with NO_3^- having no effect. However, no increase in contractile efficiency was found with the application of NO_2^- or NO_3^-. In order to more closely simulate the route of NO_3^- ingestion in human studies a second experiment was undertaken in which mice ingested NO_3^- via their drinking for 6 days before muscles were isolated and mechanical efficiency measured. Again, no increase in mechanical efficiency was observed. Therefore, it is concluded that the contractile efficiency of isolated fast-twitch mouse muscle is not increased by either acute application of NO_3^- or NO_2^-, or by the ingestion of NO_3^-. In addition, the acute application of the NOS inhibitor L-NMMA had no effect on the contractile efficiency of isolated fast-twitch mouse muscle.

Despite the interest regarding the positive effects of NO_3^- on exercise efficiency, there are a number of studies where no enhancement in exercise efficiency was observed after NO_3^- ingestion [8, 12-16, 82]. For example, some studies have shown that NO_3^- is ineffective in well-trained individuals (VO_2 peak >60ml/kg) [20,41]. In addition, recent studies have also demonstrated no effect on exercise efficiency in recreationally active individuals under normoxic conditions with 3-6 days of NO_3^- supplementation (6-8mM per day) [27,92,156,175]. In some cases a dose higher than ~8mM NO_3^- may be required to elicit an effect on efficiency. Supporting this Wylie et al. [175] demonstrated a dose of 4 and 8 mM of NO_3^- was ineffective, and a dose of 16 mM of NO_3^- was required to significantly enhance exercise efficiency. The current study was designed on the maximum rise in [NO_3^-] and [NO_2^-] elicited by 4-8 mM of nitrates being ingested in humans [94,165,176]. Although the dose given in the chronic part of the study has been previously shown to have an effect on other parameters of muscle contraction in mice [70], this dose may be insufficient to elicit an effect on contractile efficiency in EDL muscles from mice.

The lack of effect on contractile efficiency may also be due to a species difference. In a long term NO_3^- supplementation study in healthy mice no alteration in mitochondrial efficiency or function was found [72], contrary to the findings by Larsen et al. in people [103]. As the effect on mitochondrial function is proposed to be one of the mechanisms that accounts for NO_3^- induced improvements in exercise/contractile efficiency, this finding raises the possibility that mice may not respond as people do in regards to the effects of NO_3^- on exercise efficiency.

Being the first study to our knowledge to examine the effects of NO_3^- on contractile efficiency in mice, further studies would be needed to clarify this point. For example measuring whole body VO_2 consumption during controlled exercise i.e. treadmill running (*in vivo*) or measuring the arterial-venous O_2 difference across the working muscle (*in situ*) in mice.

If NO_3^- or NO_2^- do enhance exercise efficiency *in vivo*, then the lack of effect in our study may be related to the use of isolated muscles. Two potentially important aspects of isolated preparations that differ from *in vivo* are that they do not have an functional circulation, and that they are studied in a relatively high O_2 environment. Therefore, if an increase in muscle blood flow, which may occur with NO_3^- ingestion [54], is an important factor in enhancing exercise efficiency *in vivo*, then any consequences of altered blood flow will not be seen in isolated muscle. A second possible reason for the lack of effect *ex vivo* is related to the hyperoxic environment created with the solution being continually bubbled with 95% oxygen. This is needed to ensure that the diffusive supply of O_2 from the muscle surface is sufficient to prevent the core of the muscle becoming hypoxic [11] since blood flow is absent. The PO_2 at the muscle surface is over 500 mmHg [11], far greater than that found *in vivo* (~5 mmHg) during exercise [34]. The proposed active substrate in NO_3^- metabolism is NO [114], and the stepwise reduction of NO_2^- to NO is enhanced under hypoxic conditions [114]. Therefore, it is possible that the reduction of NO_2^- will be attenuated in such an environment, if not abolished. However, the presumed reduction in endogenous NO via L-NMMA [73] did not alter contractile efficiency in our study, thus not supporting NO being a critical factor in contractile efficiency. Furthermore, human studies have found no effect of NOS inhibition on exercise efficiency [24,95,144].

In contrast to our hypothesis that NO_3^- would enhance contractile efficiency, we found a decrease in contractile efficiency (~30%) with the acute application of NO_3^- to isolated skeletal muscle compared to control. In other words, work performed (W_T) decreased relative to the energy cost i.e. the heat + work generated during contraction ($W_T + Q_M$), (Equation 3.1). Q_M is the sum of the heat generated by the cross bridges (Q_{CB}) and activation heat (Q_A) which is mainly due to ATP use for calcium pumping [16] (Equation 3.2) and is proportional to the number of cross-bridge cycles that occur during a contraction series [16].

$$\varepsilon = W_T/(W_T+Q_M)$$

Equation 3.1: Formula to calculate contractile efficiency (ε). W_T: Total work performed in series of contractions. Q_M: Heat generated by the muscle during contraction series.

$$Q_M = Q_{CB} + Q_A$$

Equation 3.2: Q_M is the sum of the heat generated by cross-bridge formation (Q_{CB}) and heat generated from the activation processes of muscle contraction (Q_A).

Therefore, one possibility for the attenuation in contractile efficiency with NO_3^- is that Q_M increased i.e. the energy consumed by cross-bridge cycling (Q_{CB}) and/or energy for activation of contraction (Q_A) increased. In order for contractile efficiency to be reduced by 30% Q_A would need to increase by a factor of 2.5, based on equation 4.1 and 4.2, and assuming Q_{CB} and W_T did not change. Alternatively, if a change in Q_{CB} were responsible for the reduction in contractile efficiency, it would need to increase by 1.8 fold. It is difficult to envisage mechanisms that could account for changes of these magnitudes. A more probable reason for the reduction in contractile efficiency is a reduction in W_T with no alteration in Q_M. Indeed, the current data support such a possibility with the observation that there was no significant effect of NO_3^-on enthalpy, but a significant reduction in work output (Fig. 3.6). Furthermore, no significant effects on maximum recovery heat rate, and recovery heat rate kinetics (Fig. 3.7), indicative of no change in mitochondrial oxidative phosphorylation, hence, no change in ATP cost.

The energy required for filament movement is generated from ATP hydrolysis, and the principle factor in determining the conversion of energy from ATP into work is the amount of filament movement produced for each ATP consumed [10]. Therefore, the data suggest that NO_3^- reduced the work completed in each cross bridge cycle with no significant alteration in the number of cross-bridge cycles. A possible basis for such a change can be suggested using the conceptual models of Huxley [80] and Huxley & Simmons [81]. In general terms, the work performed by a cross-bridge is the product of the force it generates and the amount of filament sliding it generates in one attachment cycle.

It seems unlikely that cross-bridge force output changed with the direct application of NO_3^- because there was no change in the maximum isometric force (Fig. 3.8A). Therefore, the most likely basis for the decline in work output and contractile efficiency was that in the presence of NO_3^- each cross-bridge generated less filament sliding during each attachment cycle. The rate of force development with a twitch has been shown to be longer with the application of NO_3^- [75,145], and force development in a twitch is mainly determined by the time course with which different cross-bridges attach and start to generate force [81]. Therefore, NO_3^- may decrease the rate of cross-bridge attachment, which in turn may reduce the capacity of cross-bridge to generate filament movement.

Aside from contractile efficiency, an interesting finding is the significant difference in time course of rate rise, otherwise known as rate of force development in the NO_3^- chronic group compared to control illustrated in Fig. 3.12. Typically as illustrated in the control group, rise rate increases as the series of contraction progresses. This has been shown previously by Barclay et al. [9]. A number of possible explanations are put forward by Barclay et al. [9], however the explanation that seems most consistent with our findings is the proposed calcium (Ca^{2+}) buffering by parvalbumin. The proposed mechanism is based on the fact that parvalbumin is present in high concentrations in mouse EDL muscle, and that parvalbumin affects the rate of transfer of Ca^{2+} from the sarcoplasmic reticulum (SR) to the myofibrils. With subsequent contractions, the degree of saturation of parvalbumin binding sites would increase until a steady state is reached, thus allowing a more rapid transfer of Ca^{2+} to the myofibrils resulting in an increasing rate of force development. In addition to the enhancement in sub-maximal force shown by Hernandez et al. [70] mentioned earlier, NO_3^- feeding co-currently increased calcium release with a tetanic stimulation, as well as basal [Ca^{2+}] tending to be higher compared with control [70]. Therefore, the parvalbumin may already be partially saturated allowing for a greater transfer of Ca^{2+} to the myofibrils from the initial contraction.

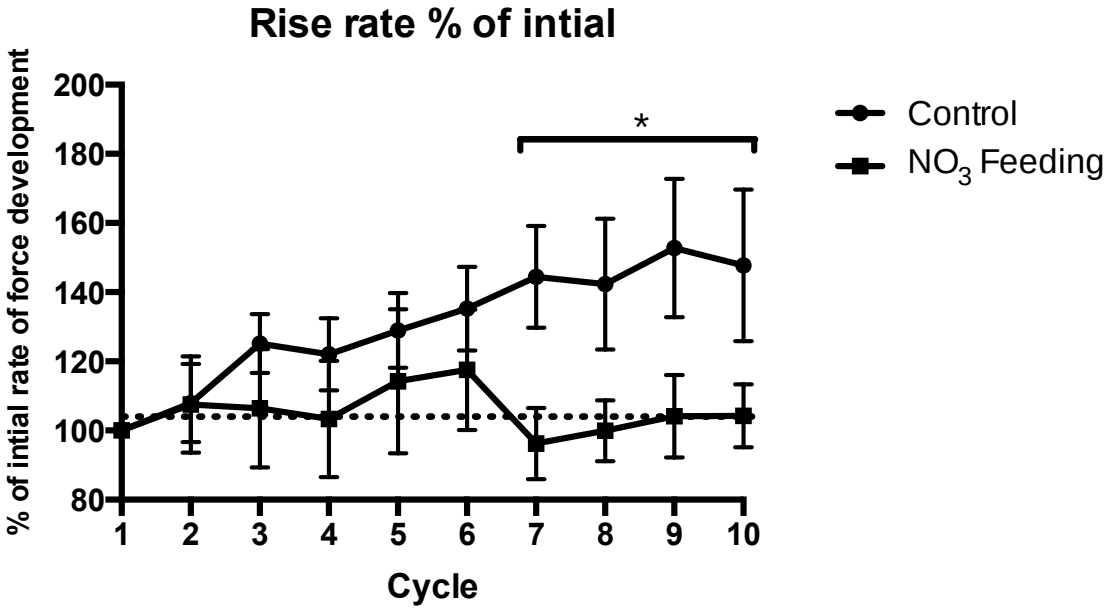

Figure 3.13 Mean percentage (%) of initial rate of force development for control (n=7) and NO_3^- feeding (n=6) ± SEM for each cycle. *Significant difference ($p<0.05$) between control and NO_3^- feeding group for rise rate.

However, the rate of force development after chronic NO_3^- supplementation remained significantly lower than control, contrary to the findings of Hernandez et al. [70] where rate of force development was increased by approximately 35% at 100 Hz. Furthermore, if in fact a greater calcium was present, and parvalbumin was partially saturated prior to contraction, there would be expected to be a greater rate of force development at the beginning of the contraction series, which was not evident (Fig. 3.13). In fact rate of force development started at approximately the same rate if not lower, and remained lower throughout the series of contractions, becoming significantly less than control from cycle 7-10 (Fig 3.12B & Fig. 3.13). Therefore, an increase in calcium seems unlikely to explain this alteration in the time course of rate of force development with NO_3^- feeding leaving the reason for this effect uncertain.

In summary, in isolated muscle the acute application of NO_3^- decreased contractile efficiency, whereas 6 days of NO_3^- feeding resulted in no change. Neither total enthalpy, nor mitochondrial function were altered with the acute application of NO_3^-, which indicates work performed in each cross bridge cycle was reduced.

Based on the data present the most likely mechanism for this reduction in contractile efficiency is less filament movement per cross-bridge cycle.

The lack of effect on contractile efficiency with 6 days of NO_3^- feeding may have been due to a factor that is missing in isolated muscle, possibly blood flow or a result of the hyperoxic environment present and would require further investigation. Six days of NO_3^- feeding did significantly affect the time course of rate of force development with no effect on maximum force; however, the reason is unclear. Therefore, overall our results do not support that NO_3^-, NO_2^- or NO improve contractile efficiency.

CHAPTER FOUR:

THE ACUTE EFFECTS OF NITRATES AND NITRITES ON SUB-MAXIMAL EXERCISE EFFICIENCY AND SKELETAL MUSCLE METABOLISM IN RECREATIONALLY ACTIVE MALES

4.1 INTRODUCTION

There has been much interest over recent years in the potential of inorganic NO_3^- to increase exercise efficiency and exercise performance [114]. Based on this, beetroot juice (Beet) has been used as an ergogenic aid and health supplement because it contains large amounts of inorganic NO_3^-. It is now known that dietary inorganic NO_3^- can be reduced to NO_2^- and NO as well as other bioactive nitrogen species *in vivo* [104]. The bio-activation of NO_3^- requires the formation of NO_2^- as an intermediate, a reaction that is facilitated by anaerobic oral bacteria [111]. The critical role of oral bacteria has been supported by studies demonstrating that antiseptic mouthwash abolishes the increase in plasma levels of NO_2^- after the consumption of NO_3^- [63]. In addition, the reduction in blood pressure and gastro protective effects that have been shown with NO_3^- ingestion are abolished when an antiseptic mouthwash is used prior to NO_3^- ingestion in healthy participants [89,142].

In regards to physical exercise, some [6,7,103,104,165,175], but not all studies [20,27,92,175], have found that Beet/ NO_3^- ingestion decreases VO_2 (~3%) during sub-maximal and maximal exercise in healthy subjects. This increase in efficiency during exercise following Beet/NO_3^- ingestion has been hypothesised to be due to one or more of three possible mechanisms. The first proposes that Beet/NO_3^- ingestion induces a reduction in the ATP cost of force production [6]. In support of this, Bailey et al. [6] found using phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) that there was an attenuation of the decrease in PCr and estimated ATP turnover during low and high intensity knee-extensor exercise after several days of Beet ingestion.

The second possible mechanism is related to the efficiency in which the mitochondria produce ATP [103]. In humans, the ratio of ATP generated to oxygen consumed (P/O ratio) during exercise increased after 3 days of NO_3^- supplementation in healthy subjects [103].

There was also a significant decrease in basal leak respiration and an increase in expression of the mitochondrial protein ADP/ATP translocase (ANT), one of the main proteins attributed to leak respiration [103]. These findings suggest that NO_3^- supplementation enables the mitochondrial to better maintain the proton gradient across the membrane. However, an increase in mitochondrial efficiency via this mechanism is unlikely to explain comparable increases in efficiency (~3-4%) after one dose of NO_3^- 2.5 hours prior to exercise [165,175] and following multi-day supplementation [6,7,103,104]. This suggests that the acute effects of NO_3^- ingestion on energy efficiency and/or muscle metabolism may involve another mitochondrial effect and/or an effect that does not involve the mitochondria.

The third potential mechanism proposed to explain a decrease of oxygen consumption during exercise following NO_3^- ingestion is related to substrate utilisation. One study found the respiratory exchange ration (RER) during submaximal exercise was increased from 0.88 ± 0.01 to 0.91 ± 0.01 after 3 days of NO_3^- ingestion [103]. Although small, this indicates a slight increase in carbohydrate oxidation, which is a slightly more efficient fuel [28] in regards to oxygen consumption per unit of ATP. It should be noted however, that to the best of our knowledge no other human exercise study has found an effect of Beet/NO_3^- ingestion on RER during exercise [6,7,100,105,165,175]. There is, however, evidence in animals that nitrate can alter substrate oxidation during isolated muscle contraction [77]. Holloszy and Narahara [77] found that NO_3^-, albeit at a supra-physiological dose, can increase force production and glucose uptake acutely in frog skeletal muscle *ex vivo* [77]. This is interesting since there is also evidence showing that NO plays a key role in glucose uptake during contraction in skeletal muscle [24,121,122,124]. However, to the best of our knowledge, no previous study has investigated the effect of Beet/NO_3^- ingestion on glucose kinetics during exercise in humans. Additionally, no studies to date that we are aware of have examined the effects of Beet/NO_3^- ingestion on the AMP kinase (AMPK) signalling during exercise, which is surprising given that AMPK is an energy sensor in skeletal muscle and activated by exercise [65,170]. Therefore, we examined the effect of a single dose of Beet with, and without mouthwash on glucose kinetics, muscle metabolism, AMPK signalling (ACCβ phosphorylation) and oxygen consumption in healthy humans during submaximal exercise.

We hypothesised that a single dose of Beet would decrease oxygen consumption, increase glucose uptake and attenuate the reduction in PCr during exercise in comparison with placebo. We also hypothesised that antiseptic mouth wash with Beet would prevent these effects by greatly attenuating the conversion of NO_3^- to NO_2^-, thus implying that the effects of NO_3^- are not direct but via NO_2^- or NO. In addition, we hypothesised that the better maintenance of skeletal muscle energy balance during exercise with Beet would result in a reduced activation of the AMPK signalling (ACCβ phosphorylation) during exercise.

4.2 METHODS

4.2.1 Participants

Eight healthy recreationally active males (mean ± SE, age 27±1 years, height 178±2 cm, body mass 77±6 kg; VO_{2peak} 46±3 ml·kg^{-1}·min^{-1}) volunteered to participate in this study. The procedures carried out in this study were approved by Victoria University Human Ethics Committee (HRETH 11/292). Before commencing the study participants were informed about the associated risks and potential benefits of participation, and they gave their written informed consent.

4.2.2 Procedures

Participants were required to report to the laboratory on five occasions. During the first visit participants performed a ramp incremental exercise test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) in order to determine their peak pulmonary oxygen consumption during cycling (VO_{2peak}). Total expired gas volumes were measured using a turbine flow meter (KL Engineering, Sunnyvale, California). Expired oxygen (O_2) and carbon dioxide (CO_2) fractions were continuously analysed by O_2 and CO_2 analysers (Amtek S-3A/II and Ametek CD-3A, respectively; Process Instruments, Pittsburgh, Pennsylvania, USA), which were calibrated using gases of known composition. Oxygen consumption (VO_2), CO_2 production (VCO_2) and RER were calculated every 15 sec using Turbofit computer software (Vacumetrics Inc., Ventura, California, USA). Heart rate was measured using a Polar hear rate monitor RS800cx model. The test commenced with participants cycling in a stepwise manner at 50, 100 and 150 Watts (W) for 3 minutes each.

The power output was then increased by 30 $W \cdot min^{-1}$ until exhaustion. Participants cycled at a self-selected cadence between 80-90 rpm. The VO_2 peak was determined as the average of the VO_2 over the final 30 s of exercise.

A few days after completion of the VO_2 peak test participants completed a 30-minute familiarisation trial in order for the participant to be accustomed to the bike setup and intensity of the experimental trial, as well as to confirm the workload estimated to elicit 65% VO_2 peak for the exercise trials was correct.

Participants were then randomly assigned in a single blind crossover design to attend the laboratory for three experimental trials during which they received one of three different treatments with a washout period of at least 1 week between each trial: (1) 140 ml of concentrated organic beetroot juice rich in NO_3^- (Beet; ~8 mMol) (Beet It, James White Drinks, Ipswich, UK); (2) 140 ml of concentrated organic beetroot juice depleted of NO_3^- (Placebo; ~0.01 mmol) (Beet It, James White Drinks, Ipswich, UK); and (3) 140 ml of concentrated organic beetroot juice rich in NO_3^- (~8 mmol) followed by rinsing mouth with mouthwash [Chlorohexidine Gluconate 2mg/ml; 20ml of Colgate™ Savacol] for 1 minute (Beet + MW). This dose has been shown to abolish the reduction in blood pressure with NO_3^- ingestion [142]. Participants were provided with a list of foods rich in nitrates to refrain from in the 24 hours prior to each trial and told not to use mouthwash the morning of the trials. Participants were asked to complete a 24 h food diary to record what they consumed prior to the first trial in order to replicate their diets as close as possible in subsequent trials (food diary was photocopied and returned to them to replicate).

The participants attended the laboratory in an overnight fasted state. A cannula (Optiva IV catheter 20GX1-1/4") was then inserted into an antecubital vein of both arms, one for the infusion of the [6,6-^{2}H]-glucose isotope tracer (Cambridge Isotope Laboratories, Andover, MA) for glucose kinetic determination, and the other for blood sampling. After the cannulas had been inserted the first blood sample was taken and treatment ingested (-150 min). The [6,6-^{2}H] glucose isotope was infused using a syringe pump (Terumo™ Syringe pump TE-331) and commencing 2 hours prior to exercise (-120 min). A primer dose of 54$\mu mol.kg^{-1}$ [120] was infused over first 5 min followed by a continuous infusion rate of 0.62μmol.kg.min [120] which continued over the remaining of the experiment (120 min of rest and 60 min of exercise).

Blood samples were taken at the following time points -150, -120, -60, -30, -20 and 0 min (just prior to exercise). Exercise involved cycling at the power output determined in the preliminary testing to elicit 65% of VO_2 peak for 1 hour.

During exercise, blood samples were obtained at 15, 30, 45 and 60 min, spun down and stored for later analysis of plasma glucose, percent enrichment of [6,6-^{2}H]-glucose and plasma lactate. Blood samples at -150, 0, 30 and 60 min were additionally spun down and stored for later analysis of non-esterified fatty acids (NEFA), nitrate, nitrite and insulin. Blood for glucose and lactate determination was placed in fluoride heparin tubes; blood for NEFA, NO_3^-, and NO_2^- analysis was placed in EDTA tubes [20]; and blood for insulin was placed in lithium heparin tubes. The blood samples for nitrate and nitrite were spun down and plasma extracted within 10 min of collection due to its rapid degradation [7]. The remaining blood samples were spun down at the end of the trial. Respiratory gas analysis was performed at the time points 10-15 and 45-50 min during exercise.

Skeletal muscle biopsy samples (~150 mg) were obtained from vastus lateralis just prior to exercise, and immediately (<30 sec) after exercise as previously described [125]. The leg which the sample obtained taken was alternated for each trial. Muscle samples were obtained after a skin incision had been made under local anaesthesia (Xylocaine™ 1%). Pre and Post exercise muscle biopsy incisions were prepared at the same time. Muscle samples were immediately frozen in the needle in liquid nitrogen and were later transferred to cryotubes for storage at -80°C.

4.2.2 Analytical Techniques

4.2.2.1 Blood analysis

Plasma glucose and lactate concentration were determined in duplicate using an automated glucose oxidase and L-lactate oxidase method respectively (model YSI 2300 Stat, Yellow Springs Instrument, Yellow Springs, OH). Plasma NEFA content was analysed in duplicate using an enzymatic colorimetric assay (NEFA-C test, Wako, Osaka, Japan). Plasma insulin was determined in duplicate using an ultrasensitive ELISA assay (Mercodia AB, Uppsala, Sweden). Plasma nitrite levels were determined in duplicate by detecting liberated NO in a gas-phase chemiluminescence reaction with ozone using a NO analyser (NOA 280i; Sievers, GE Power & Water, Boulder, CO) as described previously [20].

4.2.2.2 Glucose kinetics

The method to determine percent enrichment of [6,6-^{2}H]-glucose has been previously described [118]. Briefly, 50 µl of plasma was deproteinised with $Ba(OH)_2$ and $ZnSO_4$ and centrifuged. Supernatant was placed in glass vials, dehydrated overnight then derivatised to the pentacetate derivative with the use of pyridine and acetic anhydride.

The derivatised glucose was measured with a gas chromatography mass spectrometer (Shimadzu Model GMS-QP2010 Plus, Kyoto, Japan) using a selected ion-monitoring mode to determine the relative abundance of the selected ions with mass-to-valence ratios of 98 and 100. Glucose kinetics were estimated using a modified one-pool non steady-state model proposed by Steele et al. [163] with the assumption of 0.65 as the rapidly mixing portion of the glucose pool, and estimating the apparent glucose space as 25% of body weight. During cycling at 60% of VO_2 peak, 80-85% of tracer-determined whole-body glucose uptake is attributed to uptake by the legs [85]. Rates of plasma glucose appearance (Ra) and glucose disappearance (Rd) were calculated from the change in percent enrichment of [6,6-^{2}H] glucose and the plasma glucose concentration. The glucose clearance rate (GCR) was calculated by dividing Rd by the plasma glucose concentration.

4.2.2.3 Muscle analysis

A portion of each muscle sample (~20mg) was freeze-dried and subsequently crushed to a powder and any visible connective tissue was removed. The extraction of muscle glycogen commenced by incubating the sample in HCL before being neutralised with NaOH and subsequently analysed in triplicate for glucosyl units using an enzymatic flurometric method [85]. The metabolites (ATP, CrP, Cr, and lactate) were extracted firstly with precooled PCA/EDTA before the addition of precooled $KHCO_3$ to the supernatant. The metabolites were analysed in triplicate using an enzymatic flurometric method used by Harris et al. [66]. PCr, Cr and ATP were normalised to the participant's highest total creatine (Cr + CrP) obtained across the 3 trials.

4.2.2.4 Western blotting

The method used for western blotting is similar to the method previously described [132]. Briefly, a small portion (5μg) of muscle sample was added to 200ul of sample buffer, which was composed of 0.125 M TRIS-HCL (pH 6.8), 4% SDS, 10% Glycerol, 10mM EGTA and 0.1M DTT. This was then left at room temperature for 1hr before being vortexed and stored at -80°C.

Protein concentration was determined using the Red 660 protein assay kit (G-Biosciences, A Geno technology, Inc, USA). Two uL of 1% bromophenol blue was added to the sample. Samples were analysed for total acetyl CoA carboxylase (ACCβ) and phosphorylated ACCβ (Ser^{221}) (Cell Signalling Technology, USA), a protein that is phosphorylated by AMPK [136]. An optimisation gel was carried out for each protein to determine the optimal protein to load.

For the determination of total and phosphorylated ACC, samples were heated for 5 minutes at 95°C. Proteins were separated on 18 well 7.5% Criterion Stainfree gels (BioRad, Hercules, CA). Following electrophoresis, proteins in gels were transferred to nitrocellulose using the Trans-Blot®Turbo™ transfer packs and system. After transfer membranes were imaged following UV activation using a Stainfree Chemidoc (BioRad™) to quantify total protein in each lane. Membranes were subsequently blocked in 5% skim milk in TBST for 1 hour on a rocker at room temperature before being washed in TBST 4 times, 5 minutes each time. Membranes were then cut below the 250kD mark on the ladder with each portion placed in the appropriate antibody to incubate overnight at 4°C on the rocker. The next day membranes were washed 4 times in TBST for 5 minutes before being washed in TBS for 5 minutes. Images were then collected following exposure to SuperSignal West Femto (Pierce) using ChemiDoc (BioRad™) and analysed using Quantity One software (BioRad™).

4.2.3 Data analyses

All data are expressed as mean ± SEM. The data was analysed using the statistical software SPSS Version 21 (IBM™) using a two-factor repeated measures ANOVA. When a significant interaction (Time x Treatment) was found, post-hoc analysis was performed using Tukey post-hoc test. The level of significance was set at $p<0.05$.

4.3 RESULTS

4.3.1 Nitrite

Plasma levels of NO_2^- (Fig. 4.1) increased significantly ($P<0.05$) by approximately 130% above baseline during exercise at time points 30, and 60 minutes in beetroot juice (Beet) with no changes from baseline in placebo and MW+Beet.

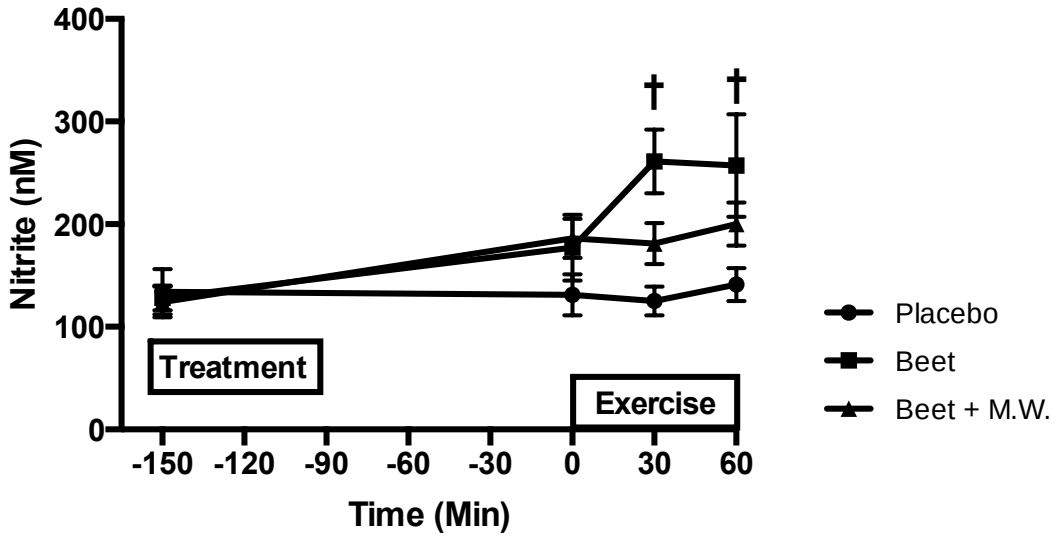

Figure 4.1: Plasma NO_2^- at rest and during 60 min of cycling at approximately 65%VO_2 peak after ingestion of either Beet, Beet + MW or placebo. A significant treatment and treatment by time interaction was found ($p<0.05$). Values are means ± SEM, n=8. † Significant ($p<0.05$) difference between Beet vs. placebo.

4.3.2 Glucose kinetics, plasma glucose, insulin, non-esterified free fatty acids (NEFA) and lactate

Glucose appearance (Ra), glucose disappearance (Rd) and glucose clearance rate (GCR) increased similarly during exercise in each of the three trials (Fig 4.2). In addition, plasma levels of glucose, lactate, insulin and NEFA were similar in each of the three trials (Fig 4.3).

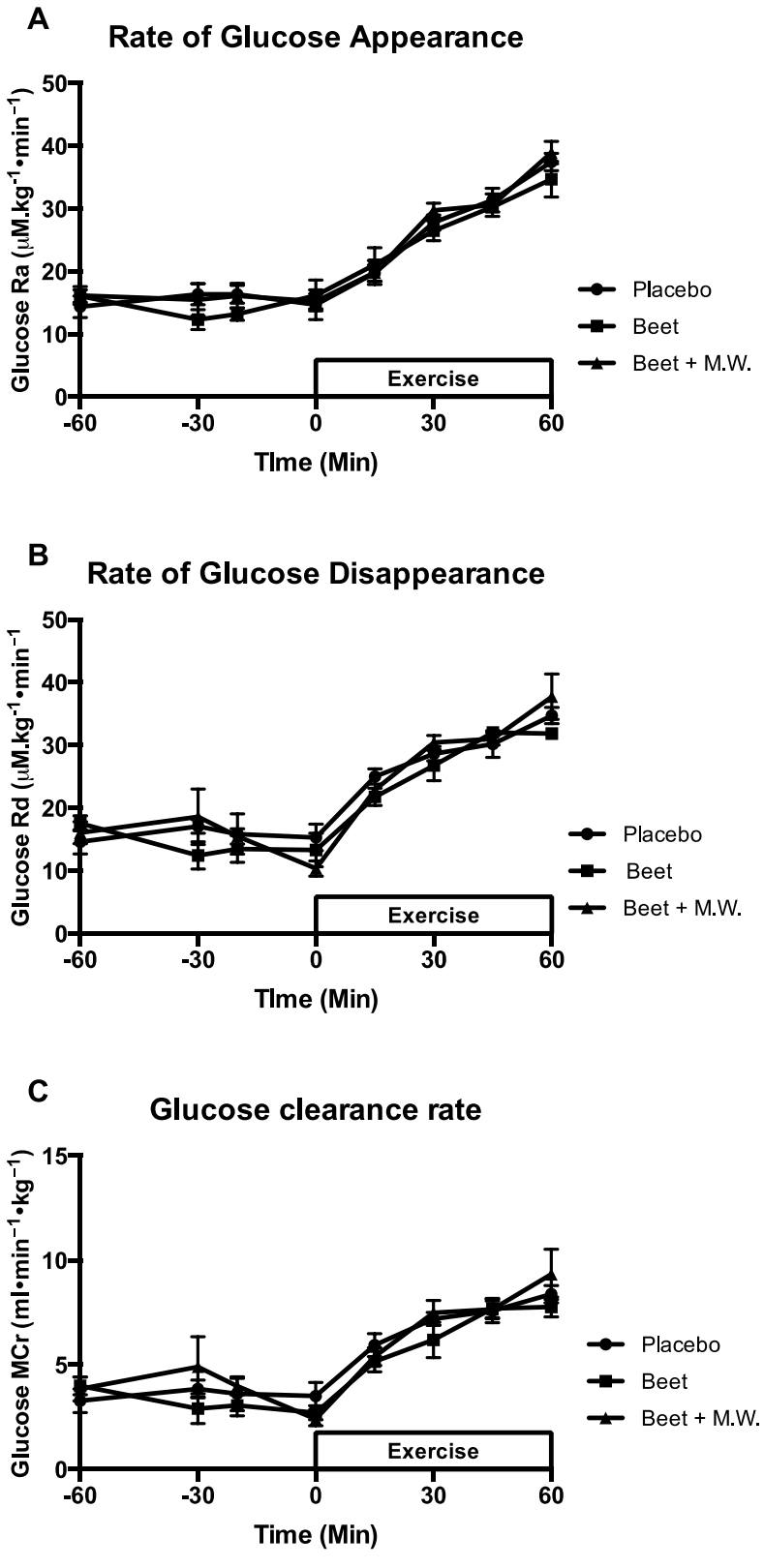

Figure 4.2: (A) Rate of glucose appearance (B) Rate of glucose disappearance and (C) Mean glucose clearance rate (glucose Rd/plasma glucose) at rest and during 60 min of cycling at approximately 65%VO_2 peak after ingestion of either Beet, Beet +MW or placebo. All increased significantly ($p<0.05$) during exercise. Values are means ± SEM. n=8.

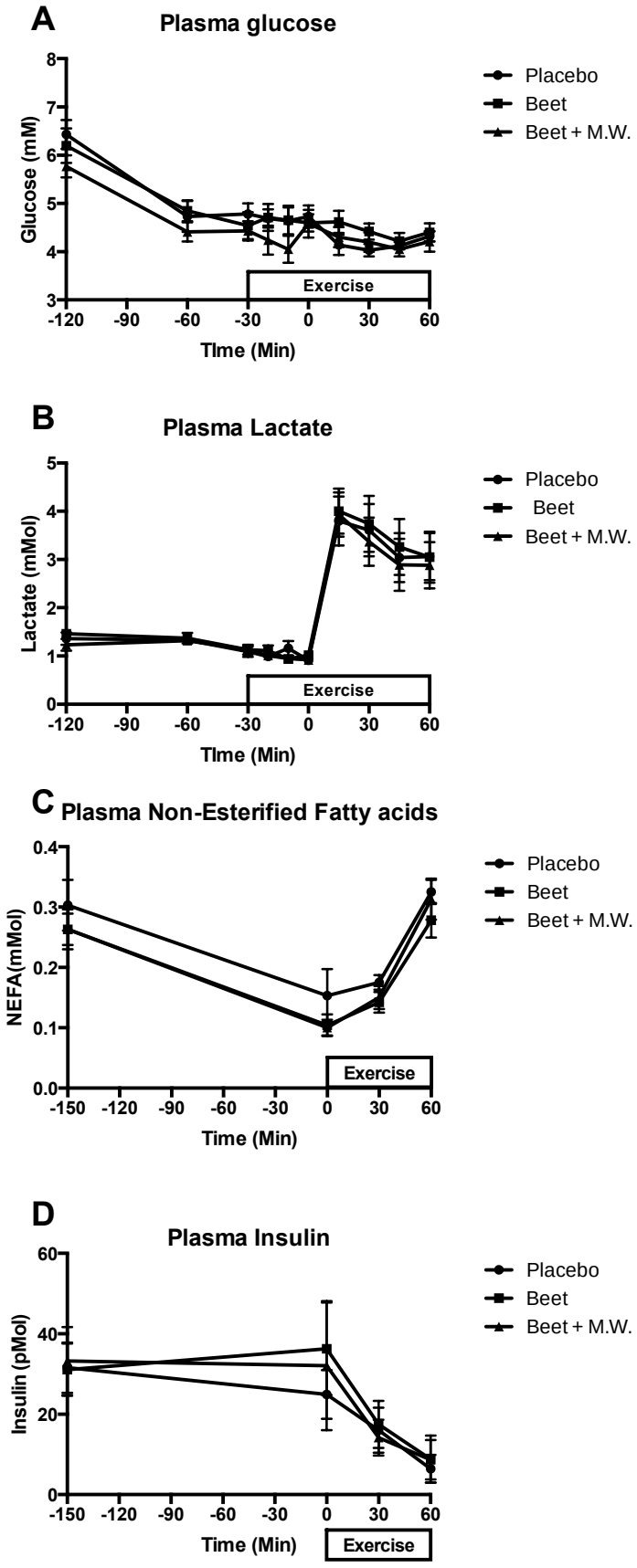

Figure 4.3: (A) Plasma glucose (B) Plasma lactate (C) Plasma non-esterified fatty acids and (D) Plasma lactate at rest and during 60 min of cycling at approximately 65%VO_2 peak after ingestion of either Beet, Beet +MW or placebo. No significant ($p>0.05$) difference at rest or during exercise between groups. Values are means ± SEM. n=8.

4.3.3 Muscle glycogen, lactate and metabolites

Muscle contents of glycogen (Figure 4.4A) and PCr (Figure 4.4C) decreased, and muscle lactate increased with exercise similarly in the three trials (Figure 4.4). Muscle ATP content did not change significantly during exercise in any trials (Figure 4.4C).

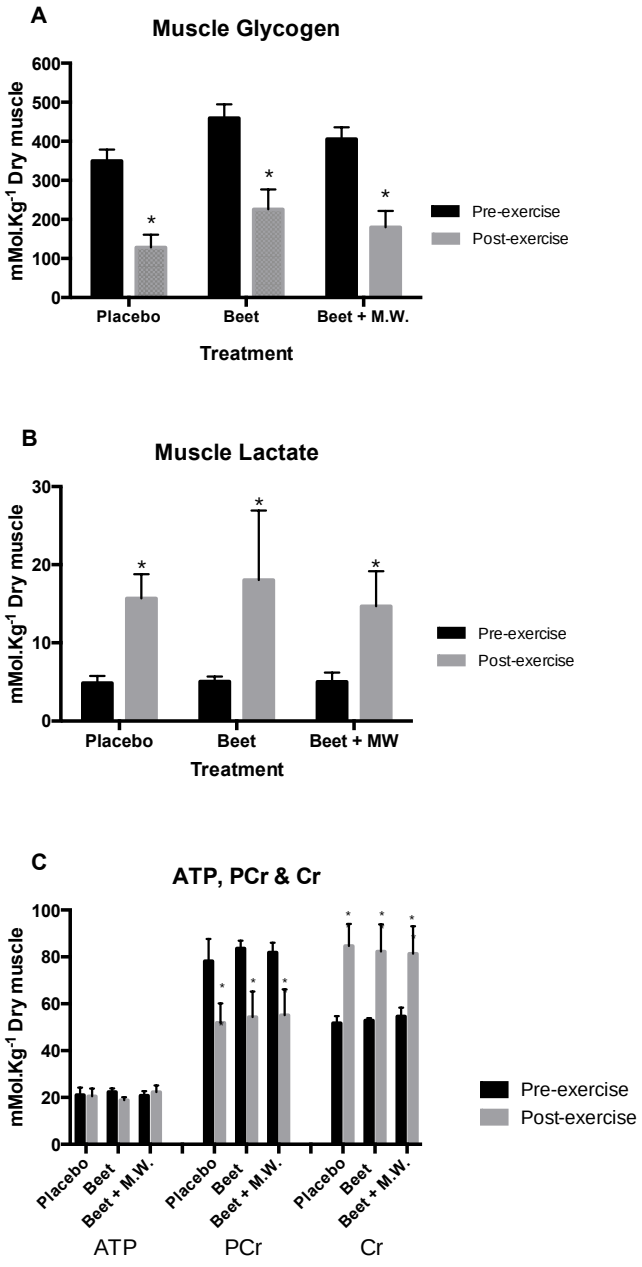

Figure 4.4: Muscle glycogen (A), lactate (B) adenosine triphosphate (ATP), phosphocreatine (PCr) and creatine (Cr) at rest and immediately following 60 min of cycling at approximately 65% VO_2 peak after acute ingestion of either Beet, Beet +MW or placebo. Values are means ± SEM. n=6-7. * Significant ($p<0.05$) difference from pre-exercise.

4.3.4 Cardio-respiratory measures

There was no significant effect of Beet or Beet+MW on exercise VO_2, VCO_2, RER (Table 4.1) or HR (Fig 4.5).

Table 4.1: Respiratory response to exercise and treatments. VO_2, oxygen consumption; VCO_2, carbon dioxide production; RER, respiratory exchange ratio.

Exercise Time	**10-15 min**			**45-50 min**		
Treatment	Placebo	Beet	Beet + M.W.	Placebo	Beet	Beet + M.W.
VO_2 ($L \cdot min^{-1}$)	2.20 ± 0.06	2.21 ± 0.07	2.20 ± 0.07	2.33 ± 0.07	2.35 ± 0.08	2.30 ± 0.07
VCO_2 ($L \cdot min^{-1}$)	2.14 ± 0.06	2.11 ± 0.07	2.12 ± 0.06	2.14 ± 0.06	2.10 ± 0.08	2.01 ± 0.07
RER	0.97 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	0.92 ± 0.01	0.90 ± 0.01	0.92 ± 0.01

Values are means ± SEM. n=8.

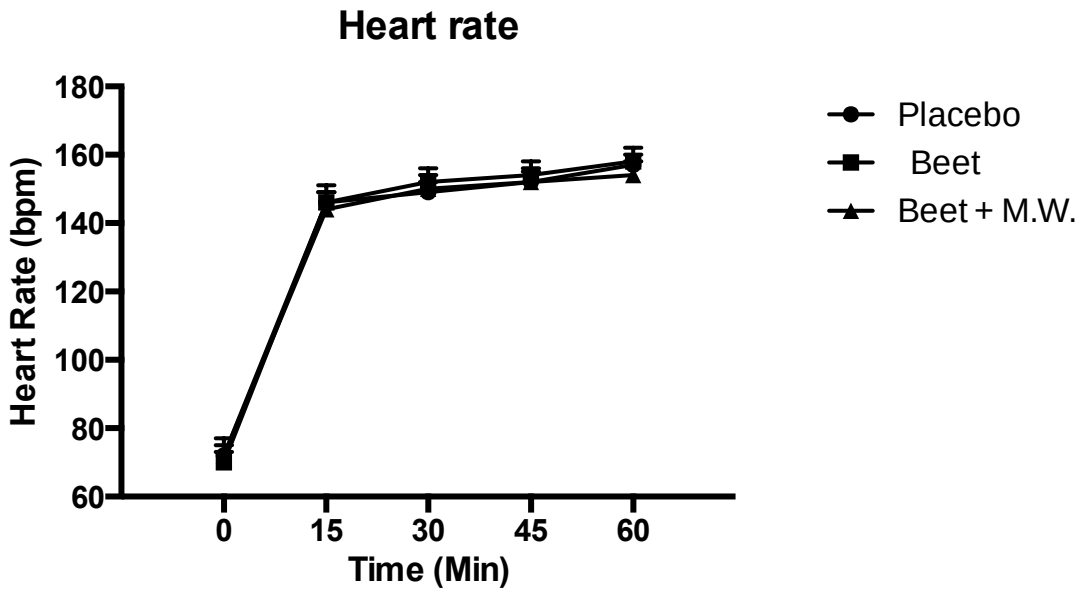

Figure 4.5: Heart rate prior to and during 60 min of cycling at approximately 65% VO_2 peak after ingestion of either Beet, Beet +MW or placebo. No significant ($p>0.05$) difference between groups. Values are means ± SEM. n=8.

4.3.5 Acetyl Co carboxylase (ACC)

Total ACC protein content was unchanged with treatment and exercise (data not shown). Exercise significantly increased phosphorylated ACC relative to total ACC, with no difference between trials (Fig. 4.6).

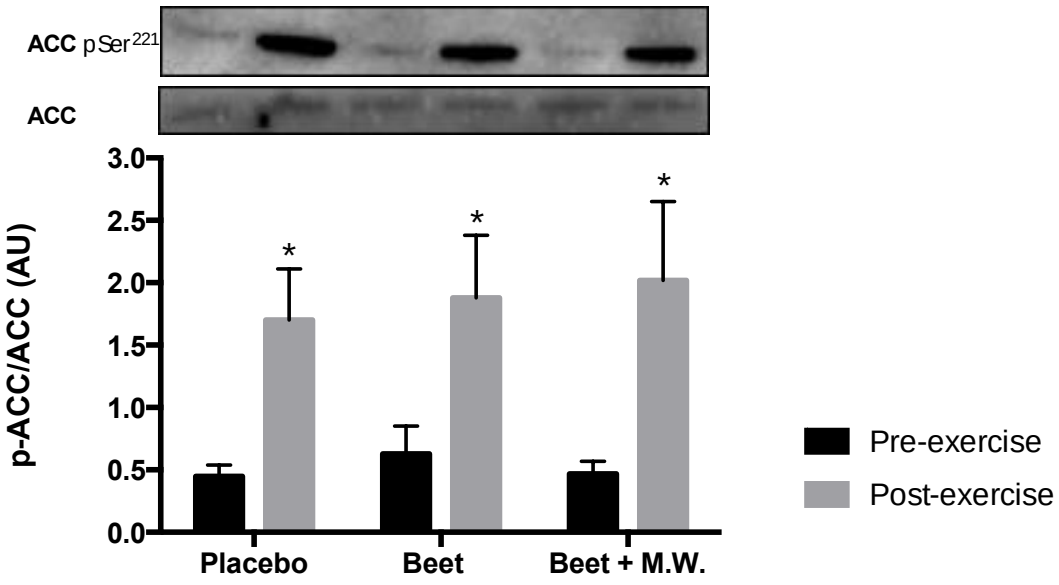

Figure 4.6: Phosphorylated ACC (Ser221) relative to total ACC protein at rest and immediately following 60 min of cycling at approximately 65% VO_2 peak after acute ingestion of either Beet, Beet +MW or placebo. Values are means ± SEM. n=7. *Significantly different from pre-exercise.

4.4 DISCUSSION

The main finding of this study was that contrary to our hypothesis acute ingestion of beetroot juice (Beet) had no significant effect on glucose disposal, muscle metabolism, ACCβ phosphorylation, oxygen consumption or RER during moderate exercise in healthy males. In addition, the combination of Beet and MW also had no effect on any of the measures.

At first we were surprised that we observed no effect of acute ingestion of Beet on oxygen consumption during exercise since two previous studies found lower VO_2 during exercise after an acute dose of beetroot juice [165,175]. However, several studies have also found no effect of either beetroot juice [23,41] or NO_3^- in pharmacological form [19,20,139] on VO_2 during exercise.

The concentration of plasma NO_2^- in our study increased to a similar if not greater extent than previous studies that have observed reductions in oxygen consumption during exercise following Beet/ NO_3^- supplementation [103-105,165,166]. For example, Vanhatalo et al. [165] found that 2.5 hours after beetroot juice ingestion (approximately 5.2 mmol NO_3^-) plasma values of NO_2^- were raised by approximately 160 nM above baseline and this was associated with a significant increase in exercise efficiency (approximately 4%), however, we found no effect on exercise efficiency despite an almost identical increase in plasma NO_2^- after approximately 8 mmol of NO_3^- ingestion in beetroot juice. Certain populations may require a higher dose than others. As demonstrated by Wylie et al. [175] 8 mmol of NO_3^- acutely was insufficient for a reduction in VO_2 during exercise to be evident and required a dose of 16 mmol. Consequently, more research is needed to clarify the effect of NO_3^- and NO_2^- on VO_2 during exercise in healthy humans.

There is evidence from NOS inhibition studies that the generation of NO in skeletal muscle during contraction plays a key role in skeletal muscle glucose disposal during contraction in rodents and during exercise in humans [122,123,148,149,151]. Given this, and the fact that Holloszy and Narahara [77] found that nitrate increased glucose uptake in isolated frog Sartorius muscles during contraction, we predicted that Beet would increase glucose disposal during exercise. However, Beet had no effect on glucose disposal during exercise. This does not mean that NO is not important for glucose uptake during exercise, since increasing levels of NO from NO_3^- above the normal level of NO produced during contraction from NOS may be in excess of requirements. Future studies should examine if NO_3^- can increase or normalise skeletal muscle glucose uptake during contraction or exercise in situations where skeletal muscle NOS levels are reduced, such as in mdx mice [140] or in diabetes [25,90,141]. It should also be noted that the study that found that NO_3^- increased isolated frog muscle glucose uptake during contraction [77] used a dose of NO_3^- that was 3 orders of magnitude higher than that found after NO_3^- supplementation.

We also found no effect of Beet on RER during exercise, which fits with the lack of effect of Beet on glucose disposal and muscle glycogen use during exercise. Other studies have also found no effect of acute Beet/ NO_3^- ingestion of RER during exercise. Larsen et al. [103] found an increase in carbohydrate oxidation (higher RER) during exercise after three days of NO_3^- supplementation, but as far as we are aware this is the only study to find an effect of NO_3^- ingestion on RER during exercise.

Therefore, taken together, the lack of effect of acute Beet/ NO_3^- supplementation on glucose disposal, muscle glycogen use and RER suggests that acute NO_3^- supplementation does not affect carbohydrate metabolism during exercise in healthy recreationally active males. Future studies should examine these measures during exercise at a higher acute dose and after several days of Beet/ NO_3^- supplementation.

Bailey et al [5] found using ^{31}P-MRS, that 6 days of Beet ingestion attenuated the reduction of skeletal muscle PCr content and estimated ATP turnover during both low and high intensity exercise compared with placebo. Rodent studies suggest that NO_3^- feeding effects skeletal muscle blood flow [54] and force production [70] only in fast-twitch skeletal muscles. Therefore, it would appear that the attenuated reduction in PCr with Beet ingestion observed by Bailey et al. [5] during low intensity exercise, which predominantly recruits slow-twitch fibres [62], are likely independent of blood flow and force. To further explore this, we analysed PCr ,Cr and ATP content in skeletal muscle biopsies performed pre and post exercise. In contrast with the *in vivo* data by Bailey et al [5], and in line with the lack of effect of Beet on exercise VO_2, we found that Beet did not induce any effect on these measures. This is consistent with our finding of no effect of Beet on the increase in p-ACC during exercise, a protein phosphorylated by the energy-sensing enzyme AMPK [136]. Although the limitations with measuring metabolites via muscle biopsy due to rapid PCr recovery kinetics [56] and the time (approximately 30 sec) it takes to obtain and freeze the muscle sample is acknowledged, it is in agreement with the recent study by Fulford et al [58]. They found that the ingestion of beetroot juice did not significantly reduce mean PCr cost after a series of maximum voluntary contractions in the Beet trials compared to placebo despite a daily dose (approximately 10.2 mmol) approximately double that used by Bailey et al. [5]. The reason for the varying results is unclear, and cannot be explained by the differing intensities of exercise as Bailey et al [6] investigated both a low and high intensity protocol and found a reduction in PCr attenuation with Beet in both. In addition, the daily dose of NO_3^- used is unlikely to explain the lack of effect by Fulford et al. [58] as a far higher dose was used compared with Bailey et al. [5], although a different knee-extensor protocol was used.

In summary, despite a similar increase in plasma NO_2^- as previous acute Beet/NO_3^- ingestion studies, we found no effect of beetroot juice ingestion on oxygen consumption, glucose disposal, muscle metabolites (glycogen, PCr, ATP, lactate) or AMPK signalling during submaximal exercise. Further research is required to justify the continued use of acute beetroot juice ingestion prior to exercise in humans.

CHAPTER 5:

DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

5.1 OVERVIEW

One of the major findings of this thesis was that NO_3^- did not enhance exercise, or contractile efficiency in healthy males (Table 4.1) or mice (Fig 3.5, 3.7). This was consistent with no effects of beetroot juice ingestion on metabolites (ATP, PCr, Cr and glycogen) or the AMPK signalling pathway (as indicated by ACC phosphorylation) during exercise in men in *Study 3*. During *ex vivo* muscle contractions the direct application of NO_3^- actually resulted in a decrease in mechanical efficiency (Fig. 3.5). In addition, NO_3^- did not affect glucose disposal in healthy recreationally active males during exercise (Fig 4.2), or *ex vivo* in mice (Fig 2.2). Therefore, in summary, the results of thesis do not support a positive effect of NO_3^- on contraction/exercise efficiency or exercise metabolism.

5.2 EFFECT OF NITRATE ON CONTRACTION/EXERCISE EFFICIENCY

The results regarding contractile/exercise efficiency in this thesis differ from a number of studies that have found an enhancement in exercise efficiency after acute [105,165] or multiday [6,7,104,165] supplementation with NO_3^- in human studies. NO_3^- supplementation, in the form of Beet in this thesis resulted in similar increases in plasma NO_2^- as have been observed in studies showing a significant effect of NO_3^- supplementation on exercise efficiency during exercise in humans [7,104,105] suggesting the dose used was sufficient to elicit an effect if one was present. In agreement with current data, there are a number of studies that have not shown any effect of dietary NO_3^- supplementation on exercise efficiency when given acutely [19,156,175] or for multiple days[18,27,92] under normoxia. The reason or reasons for this discrepancy in results is unclear, but may be due to number of differing factors between studies such as fitness status of participants, dose given, and whether NO_3^- was given as a salt, such as sodium nitrate or in a food source rich in NO_3^-. However, from reviewing the literature there appears to be no consistent factors where an effect has been shown or not.

Certain individuals/populations may require a higher dose of NO_3^- in order to elicit an effect on efficiency as shown by Wylie et al. [175] where 16 mM of NO_3^- acutely was required to significantly decrease VO_2 at a given sub-maximal workload. However, in a previous study from the same group a dose of approximately 5.2 mM was effective in eliciting such an effect [165]. Therefore, further research is needed to investigate the role and importance of NO_3^- in respects to exercise efficiency in humans.

In regards to the effects of NO_3^- on *ex vivo* contractile efficiency measured directly in mice (*Study 2*), the acute application of NO_3^- actually resulted in a decrease in contractile efficiency (Fig 3.5), and 6 days of feeding resulted in no effect on contractile efficiency (Fig 3.9). The decrease in *ex vivo* contractile efficiency found after the application of NO_3^-, is most likely due to less filament movement in each cross-bridge cycle i.e. less work performed in each cross-bridge cycle. This is based on the fact there was no alteration in mitochondrial function as assessed by the measurement of recovery heat immediately post contraction or total enthalpy between the treatment groups.

The lack of an enhancement in contractile efficiency in mice fast twitch muscle may be due to a species difference. To the best of our knowledge, no other studies have investigated NO_3^- effects on muscle contractile or exercise efficiency in mice. In support of our findings a study found no effect on mitochondria efficiency, measured *ex vivo* in mice muscle after long term NO_3^- feeding [72]. Previous research performed in humans [103] partially attributed the increase in exercise efficiency they found with the increase in *ex vivo* mitochondrial efficiency also found in their study. However, if indeed there was an effect on contractile efficiency *in vivo* this effect may be lost *ex vivo* due to factors such as a lack of blood flow and the hyperoxic environment present in the *ex vivo* preparation [11]. NO_3^- supplementation in mice has been shown to enhance skeletal muscle blood flow during contraction [54,55], therefore if this effect is critical in enhancing contractile efficiency this would be lost in an *ex vivo* model where blood flow is not present. Another possible limitation of the experiment was the hyperoxic environment present in an *ex vivo* model. The reduction of NO_2^- to NO is enhanced under hypoxia [112]. Therefore, if NO is the active substrate in regards to the effect of NO_3^- on efficiency, as has been proposed [105], this will likely not occur in the hyperoxic *ex vivo* environment. Consequently studies investigating the effects on exercise efficiency *in vivo/in situ* in mice are required to provide clarification as to whether there is a species difference and if the hyperoxic environment is responsible for the lack of effect of NO_3^- on contractile efficiency.

However more importantly, further studies in humans examining different doses and supplementation periods while examining exercise metabolism as was performed in *Study 3* are required to further elucidate the effects of NO_3^- on exercise metabolism.

5.3 EFFECT OF NITRATE ON MUSCLE METABOLITES AND DOWNSTREAM AMPK SIGNALLING DURING EXERCISE

Consistent with the finding of no effect on exercise efficiency in *Study 3* there was also no effect compared to placebo of NO_3^- (Beet) with, or without mouthwash on muscle metabolites (Fig. 4.3) or protein signalling (Fig. 4.4) during exercise. It was hypothesised that the reduction in PCr with exercise would be attenuated with NO_3^- supplementation as previously shown with ^{31}P-MRS [6]. Furthermore, no effect was shown with exercise, after NO_3^- (Beet) ingestion on ACCβ phosphorylation, a protein downstream of AMPK, a major protein affected by disruption in energy homeostasis [136]. We proposed if an enhancement in efficiency were induced by NO_3^- ingestion, skeletal muscle energy balance would be better maintained resulting in less activation of the AMPK signalling cascade, but our experiments did not support this hypothesis.

5.4 EFFECT OF NITRATE ON FORCE DYNAMICS DURING CONTRACTION

In *Study 1* acute incubation of NO_3^- and NO_2^- prior to the *ex vivo* contraction of mouse EDL muscle, reduced force production without altering the rate of fatigue. This reduction in force was not mirrored in *Study 2,* where again the EDL muscle from mice was used and acutely incubated in the same treatments and concentrations as in *Study 1*. One of the main differences between the studies was that bundles of muscle fibres were used in *Study 2*, rather than the whole EDL as used in *Study 1*. Bundles of fibres were used in *Study 2* to reduce the distance that O_2 needs to diffuse in attempt to reduce, if not eliminate the core of the muscle, where metabolic demands are greatest, becoming hypoxic during contraction. Therefore, in *Study 1* hypoxia may have developed at the core of the muscle during contractions, which in turn may have enhanced the reduction of NO_2^- to NO which then caused a reduction in contraction force. Equivocal results have been shown regarding the effects of NO on force with some demonstrating an increase in NO production (NOS activity) reduces contraction force *ex vivo* [97], while others have shown an enhancement in contraction with NO donors [53].

However, even if we assume NO does attenuate force, this does not explain the decrease in force with NO_3^-, since bacteria residing in the oral cavity are required to reduce NO_3^- to NO_2^-, and on the contrary NO_3^- has been associated with an increase in force [70,77]. Therefore, the reason for this similar attenuation in contraction force *ex vivo* in mouse muscle with both NO_3^- and NO_2^- is unclear.

Due to time restrictions further clarification regarding the reasons for this effect could not be pursued. For example performing additional experiments so that protein analysis could be performed and cell signalling examined to determine mechanisms.

Another interesting finding regarding force dynamics was the effect of six days of NO_3^- feeding on the time course of rate of force development in *Study 2.* The usual increase in rate of force development with subsequent contraction cycles was abolished with NO_3^- supplementation in mice (Fig 3.11). This effect may be related to a previous finding by Hernandez et al. [70] in mice where basal, and sub-maximally stimulated calcium concentrations in skeletal muscle were increased after the same NO_3^- supplementation protocol used in this study i.e. same dose and supplementation period, however as discussed in Chapter 3 if this was the case there would be expected to be a greater increase in rate of force development from the very first contraction, which did not occur. In fact the opposite was shown, with the rate of force development being significantly attenuated after NO_3^- feeding compared with control. Therefore, the reason for this alteration in time course of rate of force development is uncertain and further investigation is required. For example, determining the effects of sub-maximal stimulation using the same contraction protocol used by Hernandez et al. [70], on rate of force development. Examining such effects will shed further light on the effects of multiday NO_3^- ingestion on the contractile function of skeletal muscle.

5.5 EFFECT OF NITRATE ON GLUCOSE UPTAKE DURING CONTRACTION/EXERCISE

The acute application *ex vivo* of NO_3^- and NO_2^- to mice EDL muscle (*Study 1)* resulted in no alteration in glucose uptake during contraction (Fig. 2.2) compared to contraction alone. This finding was consistent with there being no effect on glucose disposal (Fig 4.2B), RER (Table 4.1) or glycogen utilisation (Fig. 4.4A) during a submaximal bout of exercise after the acute ingestion of NO_3^- in the form of beetroot juice in humans (*Study 3*). Furthermore, the attenuation in glucose uptake during *ex vivo* contraction in mice (*Study 1*) with the NOS inhibitor L-NMMA was not restored with the addition of either NO_3^- or NO_2^- suggesting that, as designed, neither was

further reduced to NO. However, as mentioned earlier, even though glucose uptake was normal, force was attenuated with NO_3^- and NO_2^-. As previously shown, glucose uptake is related to contractile force [84]. Therefore in light of this, glucose uptake relative to contractile force was enhanced with the acute incubation of NO_3^- and NO_2^- (Fig. 2.3). However, the mechanism for such an effect is unclear and requires additional investigation where force is normalised across groups allowing glucose uptake to be compared directly without the need to correct for the different forces. This has been performed previously by altering the muscle length during contraction as done previously [84].

5.6 LIMITATIONS

The studies in chapter 2 and 3 were both performed using an *ex-vivo* model. The buffer was bubbled with 95% oxygen due to the lack of blood flow and as a result created a hyperoxic environment at the surface of the muscle. Despite this, the core may remain to be hypoxic. The combined result may be a "non-physiological" environment, possibly abolishing any effect that may be present in the exercising human model. Furthermore, in such a model there is no blood flow, therefore any effect of NO_3^- and/or NO_2^- via blood flow will be lost. However, such a model is required to investigate mechanism/s of action. As discussed in the next section "Future directions", further research utilising different models is required to help elucidate the effects of NO_3^- ingestion on exercise efficiency and metabolism.

The exposure time to NO_3^- and NO_2^- prior to contraction in Chapter 2 and 3 may not be sufficient time for NO_3^- and NO_2^- to penetrate the muscle and would require further experimentation with greater exposure time to establish if this is so.

The use of only EDL muscle (a fast twitch muscle) in Chapter 2 and 3 is a potential limitation in examining the effects of NO_3^- and NO_2^- on skeletal muscle.

To further explore the effects on force in Chapter 2, additional experimentation is required, such as muscle-signalling analysis via Western blot to identify possible mechanisms.

The dose and time frame used in Chapter 4 is a limitation of the study. Ideally a higher dose along with a multiday supplementation period would have also been investigated.

5.7 FUTURE DIRECTIONS

As outlined above further investigation is required to further clarify the effects of NO_3^-on muscle metabolism during exercise. In regards to the effects of NO_3^- on exercise efficiency in humans, a study undertaking a similar design as *Study 3* (Chapter 4) i.e. using a stable glucose tracer to examine glucose kinetics and analysis of muscle metabolites, cell signalling and blood markers, but with the addition of a number of different treatment groups would be insightful. For example adding a group where 16 mM of NO_3^- is ingested, as used by Wylie et al. [175] prior to exercise as well as a group where NO_3^- was ingested for a number of days allowing time for changes in protein expression to occur. A greater dose or multiday supplementation period may also lead to an effect in regards to glucose metabolism. Using a power out relative to the individual's critical power would be another consideration for future research. This would better ensure all individuals are working at the same relative intensity and therefore ensuring a uniform metabolic response.

To further clarify if there is a species difference regarding the effect of NO_3^- on contraction/exercise efficiency in mice (*Study 2*- Chapter 3*),* and help explain the findings in terms of efficiency, further experiments are needed. For instance, it would be useful to use indirect calorimetry in mice after a single and/or multiday ingestion of NO_3^- whilst some form of controlled exercise is performed such as submaximal treadmill running. Depending on the results of such studies i.e. if an enhancement in exercise efficiency is established in mice, further mechanistic studies could be undertaken such as the use of *in situ* models, where NO_3^- and NO_2^- can be infused across the contracting muscle to further examine the effect of NO_3^- and subsequent metabolites on muscle metabolism. Furthermore the method utilising contrast-enhanced ultrasound and the infusion of micro bubbles to look at micro capillary blood flow [151] would be a valued addition to such a model and will help determine if any effects of NO_3^- or NO_2^- are via a change in blood flow.

Future studies examining the effects of NO_3^- and NO_2^- as performed in Chapters 2 and 3 should consider using a longer exposure time prior to contraction to allow more time for NO_3^- and NO_2^- to penetrate the muscle. In addition, repeating the studies as performed in Chapter 2 and 3 with a slow twitch muscle such as the soleus would be interesting to determine whether the effects differ between fast and slow twitch muscles. It would also be of interest to determine the effect of varying doses to establish a dose response.

Following on from the effects of NO_3^- and NO_2^- on force, and glucose uptake relative to force, future studies need to examine whether normalising the force between groups enhances glucose uptake during contraction with the acute application of NO_3^- and NO_2^- compared with the control. If such an effect is evident, it will confirm the findings from *Study 1* (Chapter 2) where glucose uptake relative to force is enhanced with NO_3^- and NO_2^- exposure.

5.8 CONCLUSIONS

In summary, the major conclusions of this thesis are:

1) Exposure of isolated mouse muscle to physiological levels of NO_3^- and NO_2^- do not enhance absolute skeletal muscle glucose uptake during contraction *ex vivo* nor does it restore glucose uptake when attenuated with the NOS inhibitor L-NMMA.
2) Exposure of isolated mouse muscle to physiological levels of NO_3^- and NO_2^- attenuated force during *ex vivo* conditions without diminishing glucose uptake during contraction therefore showing glucose uptake relative to force is enhanced.
3) Acute ingestion of Beet did not induce changes to VO_2, VCO_2, RER and exercise efficiency in healthy recreationally active males. This finding was consistent with there being no effect of Beet on skeletal muscle metabolites, downstream AMPK signalling, or glucose kinetics during exercise.
4) Exposure of mouse muscle bundles to physiological levels of NO_2^- does not improve contractile efficiency, and exposure to physiological levels of NO_3^- decreases efficiency
5) Six days of NO_3^- supplementation in mice does not effect *ex vivo* contractile efficiency, although the time course of the rate of force development was attenuated over the later contractions of a series of contractions.

Taken together, the results of this thesis suggest that at the doses used, there are few beneficial effects of nitrate ingestion or exposure to nitrate on muscle contraction efficiency or metabolism.

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APPENDIX A:

Notification of animal approval for Study 1 performed at Victoria University, Melbourne, Australia.

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

MEMO

TO Prof. Glenn McConell
Victoria University

DATE 10/8/2012

FROM Dr Kerry Dickson
Chair, Victoria University AEEC

SUBJECT Ethics Application AEETH 07/12

Dear Glenn,

AEETH 07/12 The acute effects of nitrates, nitrites and nitric oxide on glucose uptake during ex-vivo muscle contraction.

The AEEC Committee reviewed your application at the meeting held on August 8th 2012 and approved your request.

Advisory
• in section 2.1 "dislocation" is spelt incorrectly • given that the animal is already anaesthetised there is no need for the 2nd dose of pentobarbitone prior to cervical dislocation

The application was assessed and is **approved** between 10th August 2012 and 9th August 2015.

Continued approval of the project is conditional upon the following:

- Any variation proposed to the project, and the reasons for that change, must be submitted to the AEEC for approval and must not be implemented until approval is granted.
- Annual and Final Reports should be supplied promptly to the Secretary of the AEEC.
- The project should only be conducted in approved premises nominated on the Licence SPPL 77. Use of other premises would constitute a variation and relevant details are to be notified to the AEEC for approval as "field work".
- The AEEC must be notified in writing of:
  - Any changes to the following approved personnel listed on the application
  - Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.
- Should the numbers of animals treated exceed that estimated for the first year of the ethics application, the primary investigator should submit a request for a minor amendment to update the numbers accordingly.

Please submit a hard copy and e-copy of the revised application to the Ethics Officer in the following manner:

- Memo outlining the changes made according to the Committee's requests (one copy).
- Tracked changes copy of amended application (one copy)

- One clean copy of the amended application (one original copy signed by the Chief Investigator)

If you have any further queries, please do not hesitate to contact me on 9919 2574

On behalf of the Committee, I wish you all the best for the conduct of the project.

Kind Regards,

Dr Kerry Dickson
Chair
AEEC
Victoria University

Mean data ± SEM for glucose uptake, glucose uptake normalised to force, and force from Study 1 in table format.

Glucose Uptake (µM.g.hr $^{-1}$)

Treatment/Condition	N	Glucose uptake	SEM
Basal	9	0.752	0.108
Contraction	15	1.517	0.075
Contraction + NO_3	6	1.416	0.059
Contraction + NO_2	6	1.304	0.080
Contraction + L-NMMA	18	1.205	0.048
Contraction + L-NMMA + NO_3	5	1.032	0.051
Contraction + L-NMMA + NO_2	6	1.096	0.081

Glucose uptake normalised to force (AU)

Treatment/Condition	N	Glucose uptake	SEM
Contraction	15	0.015	0.001
Contraction + NO_3	6	0.022	0.001
Contraction + NO_2	6	0.021	0.003
Contraction + L-NMMA	18	0.013	0.001
Contraction + L-NMMA + NO_3	5	0.012	0.001
Contraction + L-NMMA + NO_2	6	0.014	0.004

Force **(mn.mg^{-1})**

Time	Contraction			Contraction + NO_3			Contraction + NO_2			Contraction + L-NMMA			Contraction + L-NMMA + NO_3			Contraction + L-NMMA + NO_2		
(Min)	Force (g)	SEM	N	Force (g)	SEM	N	Force (g)	SEM	N	Force (g)	SEM	N	Force (g)	SEM	N	Force (g)	SEM	N
0	45	3	15	29	2	6	26	2	6	44	2	18	40	5	6	41	5	6
0.5	36	3	15	23	1	6	21	3	6	34	2	18	32	4	6	32	3	6
1	32	2	15	22	1	6	22	3	6	33	2	18	30	3	6	29	3	6
2	26	3	15	15	1	6	16	2	6	25	2	18	23	2	6	21	3	6
3	16	2	15	8	1	6	9	1	6	15	1	18	14	2	6	11	2	6
4	11	1	15	6	1	6	7	1	6	10	1	18	9	1	6	9	1	6
5	9	1	14	5	1	6	6	1	6	7	1	16	7	1	6	7	1	6

APPENDIX B:

Notification of animal ethics approval for Study 2 performed at Griffith University, Gold Coast, Australia.

ANIMAL ETHICS COMMITTEE

29 November 2013

Dear Associate Professor Christopher Barclay,

I write further to your application for Animal Ethic Approval for your project "The cellular basis of nitrate-induced increases in muscle efficiency" (GU Ref No: PES/02/13/AEC). This project has been considered for full review by the Griffith University Animal Ethics Committee.

The Animal Ethics Committee thanks you for your detailed and comprehensible application. After thoughtful consideration and discussion the Committee resolved to provisionally approve this application subject to your response to the following matters.

- Has the use of excess breeding stock from the Animal House been considered? Please discuss this possibility with the Animal Facilities Manager.

Your response to these matters will be considered by the Chair.

A Committee member would like to pass on a page from a book titled 'The First 20 Minutes' by Gretchen Reynolds as it may be of interest. This page is attached.

We wish you success in undertaking this important work and look forward to your response to the above matters. You may not commence the proposed project until you provide a response to these matters and are issued with authorisation to do so.

Please forward your response to Dr Amanda Fernie, Research Policy Officer – Animal Ethics, Office for Research, as per the details below.

Regards

[redacted]

Dr Amanda Fernie
Animal Ethics Committee Secretary
Office for Research
Bray Centre, Nathan Campus
Griffith University
ph: 3735 6618
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email: A.Fernie@griffith.edu.au

Mean data ± SEM for max force, rate of force development, rate of force relaxation, enthalpy, work, efficiency, maximum and time constant for recovery heat rate for the acute and feeding study in Study 2 in table format.

Max force – Acute (kPa)

N=7

Contraction Cycle	Control		NO_3		NO_2		L-NMMA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	206.42	19.57	151.25	23.42	161.15	20.53	166.12	26.20
2	201.29	20.23	145.53	22.49	156.51	20.12	164.60	26.24
3	198.23	20.42	142.86	22.33	153.06	19.89	162.35	26.26
4	195.71	20.45	138.81	22.30	150.51	19.56	160.97	26.15
5	192.40	20.36	136.65	22.11	148.69	19.51	158.84	25.84
6	190.36	20.51	134.98	21.74	147.24	19.62	155.95	25.55
7	187.13	20.49	133.19	21.99	144.86	19.29	154.66	25.13
8	185.44	20.45	132.60	21.69	143.12	19.09	152.78	25.35
9	182.09	20.75	130.13	20.97	140.99	19.01	150.83	25.12
10	180.06	20.74	128.62	20.97	138.79	18.64	148.21	24.91

Rise rate/ Rate of force development – Acute (1/S)

N=7

Contraction Cycle	Control		NO_3		NO_2		L-NMMA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	89.50	10.31	50.05	14.75	58.03	10.96	53.77	17.42
2	98.10	15.93	68.56	16.13	74.10	14.73	72.32	15.17
3	111.92	14.64	74.45	22.07	65.53	10.69	77.12	16.45
4	110.44	15.98	74.17	24.90	74.85	12.81	84.44	17.24
5	117.22	17.29	63.91	12.61	76.70	12.16	83.84	17.03
6	122.78	18.62	67.56	14.17	82.32	12.92	87.68	17.98
7	130.14	20.12	73.67	14.03	77.74	15.57	90.66	19.32
8	131.79	23.39	75.10	15.83	81.74	14.71	91.68	20.04
9	141.10	24.56	72.56	13.72	80.03	17.96	95.49	19.55
10	138.55	25.76	91.81	30.22	84.48	17.05	94.42	18.98

Rate of relaxation – Acute (1/S)

N=7

Contraction Cycle	Control		NO_3		NO_2		L-NMMA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	141.73	11.91	158.77	16.83	158.57	11.43	161.95	14.55
2	149.85	13.41	162.89	20.70	160.96	11.57	162.23	11.66
3	155.15	14.64	162.25	20.54	155.71	11.43	161.85	11.99
4	153.31	14.54	159.78	19.43	153.44	11.57	162.97	11.76
5	152.28	15.15	156.44	18.50	156.09	14.04	159.38	11.82
6	150.96	13.90	158.65	15.46	156.14	14.21	157.66	11.64
7	153.06	14.76	159.04	15.26	153.02	13.21	154.05	10.35
8	146.30	14.72	157.68	15.5	152.24	12.89	151.61	11.43
9	145.15	14.60	157.6	15.37	150.10	13.55	150.97	10.30
10	142.97	14.50	156.48	15.55	150.40	12.59	146.56	9.51

Enthalpy, work, maximum recovery heat rate and time constant for recovery - Acute

N=6 – Enthalpy, work and efficiency.
N=7 – Max recovery heat rate and recovery time constant

	Control		**NO_3**		**NO_2**		**L-NMMA**	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Enthalpy ($mW.g^{-1}$)	998.30	122.00	671.04	88.37	699.59	11.98	782.63	151.42
Work ($mJ.g^{-1}$)	129.38	23.30	64.38	15.95	81.30	17.90	91.13	27.54
Efficiency (%)	12.92	1.99	8.88	1.36	11.82	2.48	10.90	2.05
Max recovery heat rate ($mW.g^{-1}$)	7.68	0.68	5.64	0.46	5.64	0.69	6.13	0.65
Recovery time constant (1/S)	29.63	1.37	33.44	2.43	34.05	3.93	32.32	2.40

Max force, rise rate and relaxation rate – NO_3 Feeding study

N=7

Contraction Cycle	Max Force (kPa)		Rise rate (1/S)		Relaxation rate (1/S)	
	Mean	SEM	Mean	SEM	Mean	SEM
1	193.39	12.41	76.18	6.19	116.28	12.08
2	194.04	13.49	82.21	13.91	128.89	8.68
3	193.01	13.24	80.68	15.12	133.26	9.41
4	192.26	13.46	80.35	16.27	134.82	8.22
5	188.80	13.30	88.46	19.34	132.39	8.32
6	187.27	13.93	91.33	17.34	131.65	7.03
7	185.66	13.70	74.08	11.46	130.61	7.23
8	183.64	12.42	76.63	10.36	128.79	6.73
9	182.01	13.39	79.88	12.69	127.45	6.17
10	181.56	13.23	80.10	10.97	126.08	6.49

Enthalpy, work, efficiency, maximum recovery rate and time constant for recovery – NO_3 feeding study

N=6

	Mean	SEM
Enthalpy ($mW.g^{-1}$)	838.48	229.11
Work ($mJ.g^{-1}$)	88.17	13.21
Efficiency (%)	12.94	2.17
Max recovery heat rate ($mW.g^{-1}$)	7.08	1.00
Recovery time constant (1/S)	33.55	2.45

APPENDIX C:

Notification of human ethics approval for Study 3 performed at Victoria University, Melbourne, Australia.

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

MEMO

TO Professor Glenn McConell
Institute of Sport and Active Living
Victoria University
Footscray Park Campus

DATE 19/1/2012

FROM Dr Anthony Watt
Acting Chair
Victoria University Human Research Ethics Committee

SUBJECT Ethics Application – HRETH 11/292

Dear Professor McConell,

Thank you for resubmitting this application for ethical approval of the project entitled:

***HRETH 11/292** The acute effects of nitrates and nitrites on the efficiency and metabolism of skeletal muscle during sub-maximal exercise (HREC 11/184)

The proposed research project has been accepted and deemed to meet the requirements of the National Health and Medical Research Council (NHMRC) 'National Statement on Ethical Conduct in Human Research (2007)' by the Victoria University Human Research Ethics Committee. Approval has been granted from 19th January 2012 to 12th December 2013.

Continued approval of this research project by the Victoria University Human Research Ethics Committee (VUHREC) is conditional upon the provision of a report within 12 months of the above approval date (by **19h January 2013**) or upon the completion of the project (if earlier). A report proforma may be downloaded from the VUHREC web site at: http://research.vu.edu.au/hrec.php.

Please note that the Human Research Ethics Committee must be informed of the following: any changes to the approved research protocol, project timelines, any serious events or adverse and/or unforeseen events that may affect continued ethical acceptability of the project. In these unlikely events, researchers must immediately cease all data collection until the Committee has approved the changes. Researchers are also reminded of the need to notify the approving HREC of changes to personnel in research projects via a request for a minor amendment.

On behalf of the Committee, I wish you all the best for the conduct of the project.

Kind regards,

Dr Anthony Watt
Acting Chair
Victoria University Human Research Ethics Committee

Mean data ± SEM for participant's characteristics, VO_2, VCO_2, RER, HR, plasma glucose, lactate, FFA, insulin, NO_3^-, NO_2^- and rate of glucose appearance, disappearance and glucose clearance rate from Study 3 in table format. Also include is muscle metabolites, and expression of ACC phosphorylated (Ser^{221}) and total ACC pre and post exercise.

Participants Characteristics

N=8	Mean	SEM
Age (Years)	27	1
Height (cm)	178	2
Weight (Kg)	76.7	5.5
VO_2 peak (L/min)	3.43	0.09
Relative VO_2 peak (ml/kg/min)	46.3	2.9

Heart rate (bpm)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
0	72	5	70	3	72	3
15	146	3	146	5	144	5
30	149	3	152	4	150	4
45	152	3	154	4	152	4
60	157	3	158	4	154	4

Plasma Glucose (mM)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	4.47	0.10	4.36	0.11	4.48	0.11
0	4.24	0.15	4.12	0.23	4.04	0.17
30	3.80	0.12	3.92	0.09	3.79	0.16
60	3.77	0.12	3.92	0.09	3.76	0.13

Plasma Lactate (mM)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	0.528	0.041	0.716	0.080	0.614	0.052
0	0.594	0.052	0.706	0.042	0.603	0.026
30	2.259	0.360	2.394	0.379	1.934	0.238
60	1.926	0.329	1.776	0.390	1.572	0.191

Plasma FFA (mM)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	0.303	0.042	0.263	0.033	0.263	0.026
0	0.153	0.044	0.104	0.018	0.100	0.013
30	0.175	0.012	0.142	0.017	0.150	0.019
60	0.325	0.020	0.278	0.029	0.313	0.034

Plasma Insulin (pM)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	31.569	6.253	31.069	6.527	33.223	8.446
0	24.933	6.047	36.278	11.489	32.074	16.048
30	16.005	5.613	17.472	5.859	14.160	4.442
60	6.425	3.465	8.845	5.873	8.664	4.917

Plasma NO_3^- (µM)

N=8

	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	180	19	133	16	138	22
0	126	10	351	41	387	44
30	121	14	340	32	390	31
60	154	20	391	43	394	32

Plasma NO_2^- (nM)

N=8

	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-150	134	22	128	12	124	15
0	131	20	177	32	186	19
30	125	14	261	31	181	20
60	141	16	257	50	200	21

Rate of glucose appearance ($\mu M.kg.min^{-1}$)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-60	14.37	1.71	16.06	1.02	16.15	1.41
-30	16.36	1.68	12.33	1.60	15.48	2.60
-20	16.37	1.43	13.20	1.01	16.10	1.99
0	14.69	2.36	16.14	2.45	15.23	1.21
15	19.72	1.30	21.08	2.73	19.80	1.93
30	27.76	1.23	26.47	1.58	29.71	1.15
45	31.35	1.86	30.26	0.90	30.57	1.82
60	37.42	1.39	34.70	2.86	38.95	1.79

Rate of glucose disappearance ($\mu M.kg.min^{-1}$)

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-60	14.61	1.94	17.58	1.24	16.05	1.76
-30	17.07	1.09	12.42	2.17	18.62	4.43
-20	15.86	3.23	13.48	2.14	15.49	1.24
0	15.3	2.14	13.32	2.67	10.36	1.2
15	25	1.22	21.79	1.41	22.91	0.74
30	28.64	1.19	26.75	2.45	30.45	1.14
45	30.2	2.14	32.04	0.72	31.04	1.03
60	34.71	1.31	31.86	0.78	37.73	3.64

Glucose clearance rate ml.kg^{-1}.min^{-1})

N=8

Time	Placebo	SEM	Nitrate	SEM	Nitrate + MW	SEM
-60	3.28	0.57	3.99	0.43	3.84	0.58
-30	3.85	0.43	2.91	0.72	4.91	1.44
-20	3.62	0.81	3.06	0.5	3.98	0.38
0	3.50	0.65	2.71	0.34	2.41	0.33
15	5.94	0.55	5.15	0.49	5.39	0.43
30	7.19	0.32	6.19	0.85	7.49	0.59
45	7.58	0.57	7.69	0.49	7.65	0.40
60	8.38	0.41	7.77	0.48	9.33	1.20

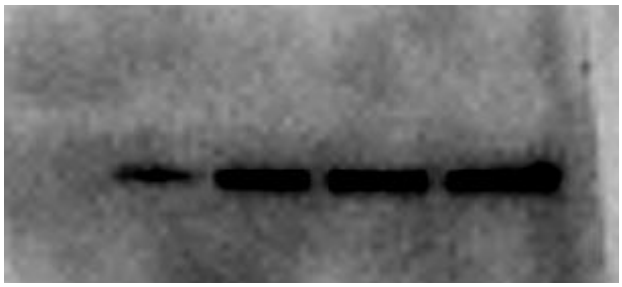
Muscle metabolites (mMol.kg^{-1} Dry Muscle)

	Pre-exercise							Post-exercise					
	N	**Placebo**	**SEM**	**Nitrate**	**SEM**	**Nitrate + MW**	**SEM**	**Placebo**	**SEM**	**Nitrate**	**SEM**	**Nitrate + MW**	**SEM**
Glycogen	7	349.9	29.0	459.1	35.7	405.8	30.2	128.3	32.5	225.7	50.9	179.9	42.0
Lactate	7	4.9	0.9	5.1	0.6	5.0	1.2	15.7	3.1	18.0	8.9	14.7	4.5
ATP (Normalised)	6	20.1	2.8	20.2	1.3	20.1	1.4	18.0	3.0	17.4	0.8	19.0	2.2
PCr (Normalised)	6	77.8	7.7	81.9	3.2	75.1	6.8	39.7	7.5	56.1	9.0	52.7	8.8
Cr (Normalised)	6	52.4	2.5	52.6	0.8	54.3	2.7	81.5	8.3	80.8	9.6	76.8	8.7

Western blotting

Chemiluminescent signal linearity over increasing total protein loadings for the proteins ACC phosphorylated (Ser^{221}) and total ACC.

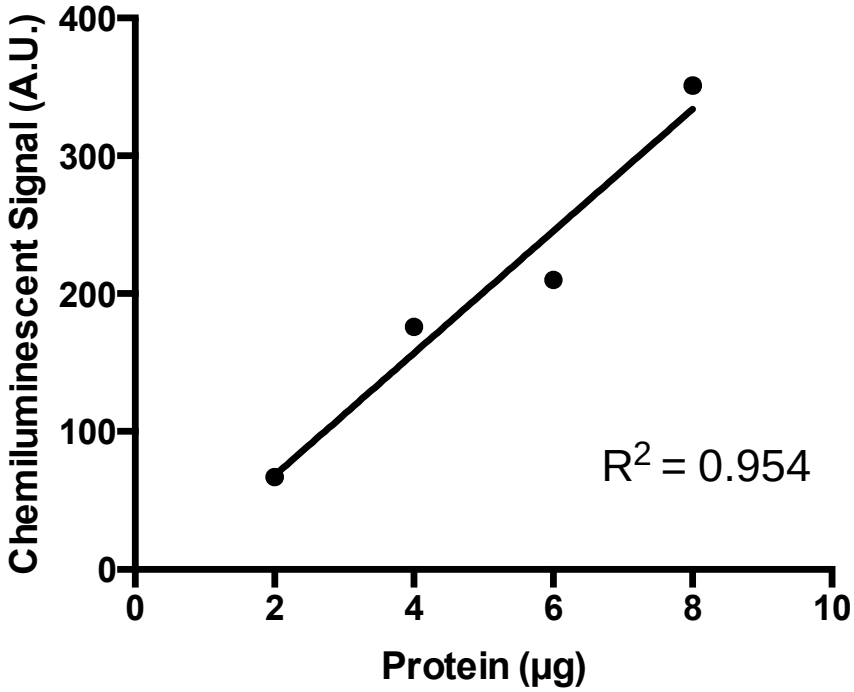
ACC Ser^{221} phosphorylation



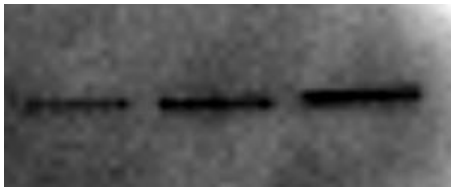
280 kDa

2 4 6 8

Protein loaded (µg)



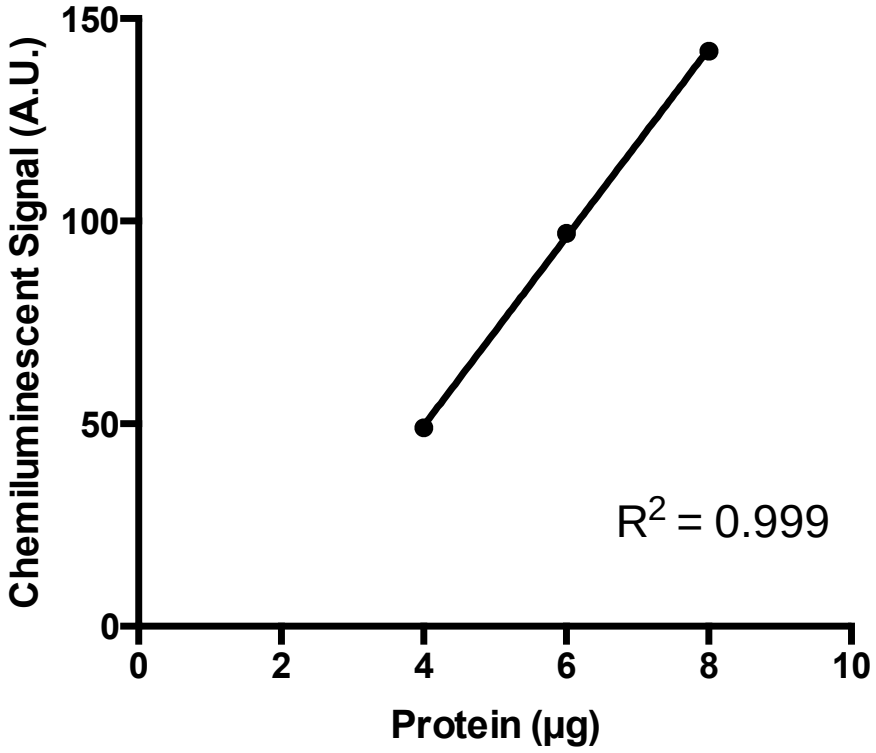
ACC - Total



280 kDa

4 6 8

Protein loaded (µg)



ACC phosphorylated (Ser^{221}) normalised to total ACC (AU)

N=7

	Placebo	**SEM**	**Nitrate**	**SEM**	**Nitrate + MW**	**SEM**
Pre-exercise	0.45	0.09	0.63	0.22	0.47	0.10
Post-exercise	1.70	0.41	1.88	0.50	2.02	0.62