THE EFFECTS OF *N*-ACETYLCYSTEINE INFUSION ON PERFORMANCE, GLUTATHIONE AND ION REGULATION DURING VOLUNTARY EXERCISE IN HUMANS.

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This thesis is dedicated to my family and friends

ABSTRACT

The production of reactive oxygen species in skeletal muscle has been linked with muscle fatigue. Infusion of the antioxidant compound *N*-acetylcysteine (NAC) reduced fatigability in electrically-evoked human muscle contractions, but numerous adverse reactions were reported. No studies have investigated NAC infusion effects during voluntary exercise in humans. This thesis investigated whether an NAC infusion protocol that was modified to minimise these adverse reactions, would augment time to fatigue, enhance glutathione availability and enhance potassium (K^+) regulation during voluntary fatiguing exercise.

Three separate studies were conducted in healthy human volunteers, ranging from untrained to endurance trained. In all studies, subjects participated in a double-blind, randomised, crossover design, receiving either a NAC or saline (CON) infusion into a superficial forearm vein. NAC or saline was intravenously infused at a rate of 125 $mg.kg^{-1}.hr^{-1}$ for 15 min, then 25 $mg.kg^{-1}.hr^{-1}$ for 20 min prior to and throughout exercise, which was continued until fatigue. Exercise comprised either intermittent or prolonged exercise.

Study 1: This study investigated whether NAC augmented time to fatigue, affected blood thiols and plasma K^+ regulation during intermittent, high intensity cycling exercise. Eight untrained males completed three 45 s cycling bouts and a fourth bout continued to fatigue, at a workrate of 130% $\dot{V}O_{2peak}$. Arterialised venous blood was analyzed for thiol status, hematology and plasma electrolytes. The modified NAC infusion protocol induced no serious adverse reactions. Exercise decreased the reduced glutathione (GSH; $P<0.005$) and increased oxidized glutathione concentrations (GSSG; $P<0.005$); NAC attenuated both effects ($P<0.05$). NAC increased the rise in plasma $\Delta[K^+]$-to-work ratio ($P<0.05$), indicating impaired K^+ regulation. Time to fatigue was reproducible in preliminary trials (CV 2.4±0.6%) but was not increased with NAC (NAC 102±45s, CON 107±53s). Thus, NAC infusion altered blood thiols during intense intermittent exercise and impaired plasma K^+ regulation, but did not attenuate fatigue.

Study 2: This study investigated whether NAC augmented time to fatigue and affected blood thiols and plasma K^+ regulation during prolonged, submaximal cycling exercise. Seven males completed cycling exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, then to fatigue at 90% $\dot{V}O_{2peak}$. Arterialized venous blood was analyzed for thiol status, hematology and plasma electrolytes. The modified NAC infusion protocol induced no serious adverse reactions. Time to fatigue was reproducible in preliminary trials (CV 7.4±1.2%) and was not augmented by NAC (NAC 14.6±4.5 min; CON 12.8±5.4 min). However, the percentage change in time to fatigue during NAC trials was correlated with $\dot{V}O_{2peak}$ (r=0.78; P<0.05), suggesting that NAC effects on performance may be dependent on training status. The rise in plasma [K^+] at fatigue was attenuated by NAC (P<0.05). The plasma Δ[K^+]-to-work ratio and percentage change in time to fatigue tended to be inversely related (r=-0.71; P<0.07). Thus, NAC effects on performance were dependent upon $\dot{V}O_{2peak}$ and NAC attenuated the rise in plasma [K^+] during exercise.

Study 3 – Part I: This study investigated the effects of NAC on skeletal muscle cysteine, cystine and glutathione, and on time to fatigue during prolonged, submaximal exercise in endurance athletes. Eight males completed cycling exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, then to fatigue at 90% $\dot{V}O_{2peak}$. Arterialized venous blood was analysed for NAC, glutathione status and cysteine concentration. A vastus lateralis biopsy was taken pre-infusion, at 45 min exercise and fatigue and analysed for NAC, total glutathione (TGSH), GSH, cysteine and cystine contents. Time to fatigue at 90% $\dot{V}O_{2peak}$ was reproducible in preliminary trials (CV 5.6±0.6%) and with NAC was enhanced by 26.3±9.1% (NAC 6.4±0.6 vs CON 5.3±0.7 min, P<0.05). NAC increased muscle total and reduced NAC at both 45 min and fatigue (P<0.005). Muscle cysteine and cystine were unchanged during CON, but were elevated above pre-infusion levels with NAC (P<0.001). Muscle TGSH (P<0.05) declined, whereas muscle GSH tended to decline (P=0.06) during exercise. Both were greater with NAC (P<0.05). Neither exercise nor NAC affected whole blood TGSH. Whilst blood GSH was

decreased and GSSG increased with exercise ($P<0.05$), both were unaffected by NAC. In conclusion, NAC improved performance in well-trained individuals, with enhanced muscle cysteine and GSH availability a likely mechanism.

Study 3 – Part II: This study was also designed to investigate the effects of NAC infusion on skeletal muscle Na^+,K^+-ATPase activity and plasma electrolytes during prolonged submaximal endurance exercise, in well trained individuals. Muscle and blood samples were also analysed for maximal *in vitro* Na^+,K^+-ATPase activity (maximal K^+-stimulated 3-*O*-methyfluorescein phosphatase, 3-*O*-MFPase) and plasma K^+ and electrolytes, respectively. Maximal 3-*O*-MFPase activity decreased by 21.6±2.8% at 45 min and by 23.9±2.3% at fatigue when compared to rest ($P<0.05$). This percentage change in maximal 3-*O*-MFPase activity was attenuated by NAC at 45 min ($P<0.05$) but not at fatigue. Furthermore, the Δ3-*O*-MFPase activity to work ratio was attenuated by NAC at 45 min and at fatigue ($P<0.005$). The rise in plasma $[K^+]$ and plasma $\Delta[K^+]$-to-work ratio during exercise were both attenuated by NAC ($P<0.05$). NAC had no effect on any other electrolytes measured. There was no significant correlation between time to fatigue and any of maximal 3-*O*-MFPase, rise in plasma $[K^+]$ and plasma $\Delta[K^+]$-to-work ratio. Hence, NAC attenuated the depression in skeletal muscle Na^+,K^+-ATPase activity and enhanced K^+ regulation during prolonged submaximal exercise in well-trained individuals.

In summary, this thesis demonstrated that a modified NAC infusion protocol did not induce any serious adverse reactions in healthy young volunteers. There was no effect on time to fatigue during high intensity, intermittent exercise. In contrast, NAC effects on time to fatigue were dependent upon $\dot{V}O_{2peak}$ during prolonged, submaximal exercise such that trained subjects had a longer time to fatigue. NAC increased muscle NAC and cysteine contents, which also increased glutathione availability during prolonged, submaximal exercise. NAC had no effect on whole blood glutathione during prolonged, submaximal exercise, but supported whole blood glutathione status during high intensity, intermittent exercise. Contrasting effects of exercise on plasma K^+ regulation

were seen with NAC, which was impaired during high intensity, intermittent exercise in untrained, but enhanced during prolonged, submaximal exercise in well-trained individuals. Enhanced K^+ regulation during prolonged exercise may in part due to NAC attenuating the decrease in Na^+,K^+-ATPase activity. Therefore, improved muscle glutathione and Na^+,K^+-ATPase status may both contribute to improved prolonged exercise performance with NAC.

I, Ivan Medved, declare that the PhD thesis entitled "***The Effects Of N-Acetylcysteine Infusion On Performance, Glutathione And Ion Regulation During Voluntary Exercise In Humans***" is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. 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, this thesis is my own work.

Signature 

Date: 25.7.05

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ABBREVIATIONS USED IN THESIS

ATP	Adenosine 5' triphosphate
Ca^{2+}	Calcium ion
Ca^{2+}-ATPase	Calcium-ATPase
CAT	Catalase
Cl^-	Chloride ion
Cu-Zn SOD	Copper zinc superoxide dismutase
CYS	Cysteine
$\Delta[K^+]$	Change in potassium concentration above rest
$\Delta[K^+]$-to-work	Change in potassium to work ratio
EC-SOD	Extracellular superoxide dismutase
EPR	Electron paramagnetic resonance
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric Iron
F_{max}	Maximum calcium activated force
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCO_3^-	Sodium bicarbonate ion
Hct	Haematocrit

HPLC	High pressure liquid chromatography
Hx	Hypoxanthine
K^+	Potassium ion
LIPOX	Lipid peroxidation
MDA	Malondialdehyde
mM	millimol.litre^{-1}
Mn-SOD	Manganese superoxide
Na^+	Sodium ion
NAC	*N*-acetylcysteine
Na^+,K^+-ATPase	Sodium-Potassium ATPase
$O_2^{-\bullet}$	Superoxide radical
$^\bullet OH$	Hydroxyl radical
P_i	Inorganic phosphate
$RO^\bullet$	Alkoxyl radical
$ROO^\bullet$	Peroxyl radical
ROS	Reactive oxygen species
SOD	Superoxide dismutase
$SQ^\bullet$	Semiquinone radical
SR	Sarcoplasmic reticulum
TBARS	Thiobarbituric reactive substances
TGSH	Total glutathione
$\dot{V}O_{2max}$	Maximal oxygen consumption
$\dot{V}O_{2peak}$	Peak oxygen consumption
XO	Xanthine oxidase
$^1O_2$	Singlet oxygen

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Reactive oxygen species (ROS) are the products of univalent reduction of oxygen. Under normal conditions, ROS accumulation is minimised by endogenous antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione. Although fundamental to the metabolic activity of the cells, ROS may also contribute to the pathogenesis of a wide variety of diseases including Alzheimer's disease, heart disease, tissue ischaemia and asthma (Kerr et al. 1996).

Over the last two decades there has been a growing interest in the role of ROS during muscle fatigue. Numerous studies have demonstrated that ROS accelerates, whereas antioxidants attenuate, muscle fatigue (Barclay and Hansel 1991; Novelli et al. 1991; Reid et al. 1992; Nashawati et al. 1993; Supinski et al. 1993; Diaz et al. 1994; Reid et al. 1994; Travaline et al. 1997; Lands et al. 1999). However, only a few studies have reported positive effects of antioxidant supplementation in humans (Reid et al. 1994; Travaline et al. 1997; Lands et al. 1999). *N*-acetylcysteine (NAC), a non-specific antioxidant, has successfully attenuated muscle fatigue in humans (Reid et al. 1994; Travaline et al. 1997), although the adverse reactions and premedication with antihistamines in these studies make their application to human voluntary exercise difficult. Furthermore, no studies have investigated the effects of prolonged exercise and NAC infusion on muscle total glutathione (TGSH), reduced glutathione (GSH), cysteine (CYS) and cystine, which has further compounded the interpretation of the effects of NAC on voluntary exercise performance.

Recent work using human skeletal muscle obtained by needle biopsies have shown that fatigue induced by prolonged (Leppik et al., 2004; Sandiford et al. 2004) and intense exercise (Fraser et al., 2002; Aughey et al. 2004) and isometric contractions (Fowles et al. 2002) was associated with decreased maximal rate of sodium-potassium adenosine

triphosphatase (Na^+,K^+-ATPase) enzyme activity. These studies implicate depressed Na^+,K^+-ATPase activity as an important site for fatigue during exercise. The mechanism for depressed Na^+,K^+-ATPase activity during exercise remains to be elucidated, but there is growing evidence that Na^+,K^+-ATPase activity within contracting muscle is adversely affected by an accumulation of ROS (Kim and Akera 1987; Kukreja et al. 1990; Sen et al. 1995; Kourie 1998). None of these studies have investigated human skeletal muscle and voluntary exercise.

This thesis will investigate the effects of NAC on voluntary, whole body exercise in healthy human volunteers. The effects of NAC on blood and muscle glutathione status during exercise will be investigated. Finally, the thesis will also investigate whether NAC attenuates the exercise-induced depression in muscle Na^+,K^+-ATPase activity.

CHAPTER 2: LITERATURE REVIEW, AIMS AND HYPOTHESES

This literature review is divided into five sections. Section I will outline the important aspects of muscle contraction and fatigue. Section II will identify the importance of ROS and antioxidants during exercise and their potential roles during fatigue. Since a major part of this thesis is to investigate the effects of NAC on glutathione, the effects of exercise on glutathione will also be reviewed in Section II. Section III reviews the importance of muscle Na^+,K^+-ATPase during exercise, with the effects of ROS highlighted. Finally, Section IV will review the pharmacological manipulation of redox status and their effects on exercise, with special reference to NAC.

SECTION I: MUSCLE CONTRACTION AND FATIGUE

2.1 Brief Overview of Muscle Contraction

After release from the motor nerve terminal, acetylcholine binds to receptors on the motor end plate resulting in depolarisation of the muscle membrane and formation of an action potential (AP). The AP propagates along the sarcolemma and into the t-tubules, where the depolarisation is detected by the dihyropyrodine (DHPR) receptor (Stephenson et al. 1998). The ryanodine receptor (RyR) is located in the terminal cisternae of the SR and is the physiological calcium (Ca^{2+}) release channel (Lamb 2000). A mechanical link is induced between the DHPR and RyR, which results in Ca^{2+} release from the SR into the cytoplasm. Although normal intracellular ATP in resting muscle is ~6 mM, which would open the RyR, this effect is overwhelmed by the inhibitory effects of magnesium (Mg^{2+}; Lamb et al., 1991). Ca^{2+} released from the RyR then binds to the troponin-tropomyosin complex on the actin filaments. Actin then combines with myosin ATPase to split ATP. ATP hydrolysis produces the energy necessary for the myosin filament to slide across the actin filament and muscle shortening. The contraction cycle continues, as long as there is sufficient Ca^{2+} to bind to

troponin and enough ATP to be hydrolysed for energy production (Fitts and Balog, 1996). The ATP-dependent Ca^{2+}-ATPase enzyme removes Ca^{2+} from the cytoplasm to the SR, thus decreasing the cytosolic [Ca^{2+}]. Removal of Ca^{2+} restores the inhibitory action of troponin-tropomyosin and the cross bridge cycle ceases, thus allowing the muscle to relax.

2.2 Fatigue

Exercise-induced muscle fatigue is a multifactorial process and is characterised by an inability to maintain power output or force, is reversible with rest and can occur due to failure at both central and peripheral sites. Fatigue is a gradual and an integral process of physical activity (Bigland-Ritchie 1981), with the mechanisms of fatigue specific to exercise intensity and duration. A brief overview of the various components of the fatigue process is provided below.

2.2.1 Central versus Peripheral fatigue

Factors involved in central fatigue include decreased α-motor neuron excitability, activation of the central nervous system and/or motor unit recruitment (Gandevia 2001; Enoka et al., 1992). Although central fatigue is evident during exercise, it only accounts for a small portion of fatigue (Gandevia, 2001). Bigland-Ritchie and colleagues (1979) demonstrated central fatigue during maximal voluntary isometric contractions of quadriceps muscle. During repeated fatiguing contractions, subjects were unable to achieve maximal voluntary force equal to the maximal electrically stimulated force. Thus, all motor units were not fully recruited and central fatigue was evident.

The twitch interpolation technique, where brief electrical stimulation is superimposed to induce a muscle twitch contraction has also been used to demonstrate central fatigue during high intensity, isometric exercise of human quadriceps and hamstring muscle groups (James et al. 1995). Central fatigue was also evident during isometric

contractions (Fowles et al. 2002), after prolonged cycling for 5 h at 55% $\dot{V}O_{2peak}$ (Lepers et al. 2002) and a 65 km ultra marathon (Millet et al. 2002). Interestingly, there appears to be no literature that has investigated a possible role of ROS in the central fatigue process. However, most research points to fatigue principally occurring beyond the neuromuscular junction (Fitts 1994; Green 1997).

Peripheral fatigue is defined as any fatigue arising from the failure of mechanisms at or beyond the neuromuscular junction (Fitts 1994; Green 1997). Mechanisms underlying peripheral fatigue may include metabolic factors, SR dysfunction, Na^+ and K^+ fluxes, as well as ROS accumulation, as reviewed in the following sections.

2.2.2 Metabolic factors in fatigue

Metabolic contributions to muscle fatigue are dependent upon the exercise intensity and duration. Therefore, the following section separately discusses metabolic aspects of fatigue during intense exercise and prolonged exercise.

2.2.2.1 Metabolic factors in fatigue during intense exercise

With intense, short-term exercise, ATP production rates are unable to match ATP utilization rates, resulting in reduced ATP, accompanied by accumulation of a range of metabolic by-products such as hydrogen ions (H^+), lactate, inorganic phosphate (P_i), adenosine monophosphate (AMP), adenosine 5′ diphosphate (ADP) and inosine 5′ monophosphate (IMP; Green 1997). The accumulation of several of these metabolites has been shown to have adverse effects on exercise performance and lead to fatigue.

During maximal 30s cycling exercise, muscle ATP, CP and glycogen contents decreased by 40%, 70% and 40%, respectively (Gaitanos et al. 1993). Others have also reported similar declines in these substrates during intense exercise (Bogdanis et al. 1995; Hargreaves et al. 1998). Although these studies indicate that ATP was not totally depleted during intense exercise, they obtained average measurements over the muscle

biopsy sample and it is highly likely that reduction in substrates were even more dramatic within some regions of the muscle (McKenna 2003). A recent report demonstrated that human muscle ATP was reduced by 25% in type I fibres but by up to 80% in type II fibres following 25 s of maximal dynamic cycling exercise (Karatzaferi et al. 2001). Several processes involved in excitation and contraction, including reactions catalyzed by the Na^+,K^+-ATPase, Ca^{2+}-ATPase and myosin ATPase enzymes, are critically dependent on the provision of energy provided by the hydrolysis of ATP (Green 1998). Therefore, severe localised ATP depletion may adversely impact on these key regulatory enzymes and impair membrane excitation, Ca^{2+} regulation and force production.

Accumulation of muscle P_i, ADP and H^+ may also be involved in fatigue. With the decline in CP, there is also an accumulation of Pi, which has been shown to be an important component in the fatigue process (Cooke et al. 1988; Wilson et al. 1988) by acting directly on the cross bridges (Pate 1995). However, Pi may also precipitate Ca^{2+} within the SR lumen and thereby reduce Ca^{2+} release (Allen and Westerblad 2001). An increased ADP also decreased Ca^{2+} pump activity in mechanically skinned rat fibers (Macdonald and Stephenson 2001).

During intense exercise, increased H^+ occurs primarily due to the increased rate of glycolysis. Increased muscle acidosis also depressed muscle force at room temperature (Westerblad et al. 1997), but this was not evident or greatly reduced at physiological temperatures (Westerblad et al. 1997). Therefore, acidosis has little direct effect on isometric force production, maximum shortening velocity or the rate of glycogen breakdown in mammalian muscles studied at physiological temperatures (Westerblad et al. 2002).

Although lactate increased by 28-fold during maximal 30s cycling exercise (Gaitanos et al. 1993), its importance in fatigue has been questioned (Sahlin et al. 2002). Furthermore, several studies report that lactate may actually protect against skeletal muscle fatigue (Nielsen et al. 2001; Karelis et al. 2004). Interestingly, in rat hepatocytes lactate prevents lipid peroxidation (LIPOX) by scavenging ROS including the superoxide ($O_2^{-\bullet}$) and hydroxyl radicals ($^{\bullet}OH$). Thus, lactate might be considered as a potential antioxidant agent (Groussard et al. 2000). Whether lactate also demonstrates antioxidant properties in skeletal muscle is unknown.

2.2.2.2 Metabolic factors in fatigue during prolonged exercise

It is well established that fatigue during prolonged exercise may coincide with decreased muscle glycogen and that glycogen supplementation increases exercise time to fatigue (Hermansen et al. 1967; Bjorkman et al. 1984). A decreased muscle glycogen is also likely to affect SR Ca^{2+} release and uptake. Studies in both humans (Booth et al., 1997) and horses (Byrd et al. 1989) have shown decreased SR Ca^{2+} uptake during prolonged exercise, which may be related to decreased glycogen levels. Furthermore, Stephenson et al., (1999) showed that the capacity of skeletal muscle to respond to t-system depolarization was related to glycogen levels in mechanically skinned fibers of the cane toad.

ATP content remains relatively constant during prolonged exercise (McConell et al. 1999) and depressed ATP is therefore unlikely to contribute to fatigue. However, small changes in ATP can result in marked increases in ADP, which has been shown to reduce free energy release during ATP hydrolysis (Sahlin et al. 1998) and limit power output during exercise (Yamashita et al. 1994). Furthermore, any localised ATP depletion may be critical, especially for SR function.

An accumulation of H^+ is unlikely to be an important factor in fatigue during prolonged exercise. Consistent with this, sodium bicarbonate supplementation decreased plasma and muscle H^+, but did not effect performance during intense endurance exercise of ~60 min in endurance trained males (Stephens et al. 2002).

2.2.3 Sarcoplasmic reticulum and fatigue

The importance of SR Ca^{2+} regulation in muscle fatigue is highlighted in a number of recent reviews (Allen et al. 1992; Fitts 1994; Favero 1999; Lamb 2000; Lamb 2002). In addition to the metabolic factors outlined above, several reports also indicate that SR Ca^{2+} release is impaired with fatigue (Hill et al. 2001; Li et al. 2002; Leppik et al., 2004) and Ca^{2+} uptake rates are also decreased in human muscle (Booth et al., 1997; Li et al. 2002; Tupling et al. 2003). One potential mechanism for the decline in SR Ca^{2+} release and uptake is the accumulation of ROS. Although SR Ca^{2+} regulation will not be investigated in this thesis, it is important to recognise that ROS effects on SR are well known in animal models, as briefly reviewed in the next section.

2.2.3.1 Sarcoplasmic reticulum Ca^{2+} release and reactive oxygen species

ROS are defined and their actions detailed in Section II. Davies et al. (1982) were the first to demonstrate decreased SR 'integrity' consequent to ROS following an exhaustive exercise bout in rats. Brotto and Nosek (1996) demonstrated that 5 min exposure to 1 mM hydrogen peroxide (H_2O_2) decreased Ca^{2+} release. However, when Ca^{2+} release was measured in the presence of the Ca^{2+} channel inhibitor ruthenium red, no release of Ca^{2+} was observed with up to 80 mM of H_2O_2, suggesting that H_2O_2 was directly affecting the Ca^{2+} release mechanism (Favero et al., 1995).

H_2O_2 also decreased myofibrillar Ca^{2+} sensitivity and force, which was reversed by the reducing agent dithiothreitol (Andrade et al., 1998). This effect was more prominent in slow twitch fibers and may be glutathione-dependent (Posterino et al., 2003). The

addition of sulfhydryl protecting agents, glutathione or dithiotherritol (DTT) reduced the Ca^{2+} release from the SR (Posterino et al., 2003; Oba et al., 1996; Andrade et al., 1998). The opposing effects of DTT and H_2O_2 on channel gating, are consistent with the suggestion that the redox state of thiol groups in the Ca^{2+} release channel protein can influence channel gating (Boraso and Williams, 1994).

2.2.3.2 Sarcoplasmic reticulum Ca^{2+} uptake and reactive oxygen species

SR Ca^{2+} uptake is decreased with increased ROS concentration (Posterino and Lamb, 1996; Xu, et al., 1997) although the precise molecular mechanism and the site involved in SR Ca^{2+} ATPase inhibition by ROS are unknown (Xu et al., 1997). Numerous studies have reported impaired SR Ca^{2+} ATPase function with oxidative stress (Scherer and Deamer, 1986), H_2O_2 (Grover et al., 2003), and $^{\bullet}OH$ (Xu et al., 1997). The reducing agents, 5 mM DTT or 10 mM glutathione, prevented the decline in Ca^{2+}-ATPase activity (Posterino and Lamb, 1996).

In summary, sulfhydryl oxidation promotes calcium release from the SR by opening release Ca^{2+} channels and inhibiting Ca^{2+} uptake (Khawli and Reid, 1994). During fatiguing exercise, ROS increase, which may impair SR Ca^{2+} release channel opening and Ca^{2+} ATPase activity. By inhibiting oxidant effects in the active muscle, anti-oxidant treatment could enable the SR to maintain calcium homeostasis more effectively, thereby potentially alleviating this component of the fatigue process (Khawli and Reid, 1994). However, the effects of ROS on SR function in human skeletal muscle remain to be elucidated.

2.2.4 Membrane potential, sodium and potassium fluxes and fatigue

Skeletal muscle contraction results in increased intracellular Na^+ and decreased intracellular K^+ concentrations, which are known to effect membrane excitability. The

following section discusses the K^+ and Na^+ fluxes during exercise and their possible role in muscle fatigue.

2.2.4.1 Membrane potential and fatigue

The resting membrane potential (RMP) is approximately -80 mV and is primarily influenced by the K^+ and Na^+ concentration gradients and by K^+ permeability (Sejersted and Sjøgaard 2000). At rest, there is a tendency for K^+ to leak outside and Na^+ to leak into the cell down their concentration gradients, which are counterbalanced by the Na^+,K^+-ATPase enzyme. A single AP was calculated to raise intracellular [Na^+] by 0.0077 mmol/L and decrease intracellular [K^+] by 0.0047 mmol/L (Hodgkin and Horowicz, 1959), but these changes would be magnified during intense muscle contraction (McKenna 1992).

Several studies demonstrate that decreased membrane excitability is linked with fatigue. In isolated rat soleus muscle there was a close correlation between the M-wave area and force during fatiguing stimulation (Overgaard et al. 1997). Furthermore, in humans impairment of AP propagation has been implicated in the fatigue process during intensive cycling exercise (Strojnik and Komi 1998), isometric, single-leg extension exercise at ~60% $\dot{V}O_{2peak}$ (Fowles et al. 2002), maximal voluntary contractions of the adductor pollicis (Bellemare and Garzaniti 1988), isometric contraction of the dorsal interosseous muscle (Fujimoto and Nishizono 1993) and isometric contractions of the knee extensors (Hamada et al. 2003). However, some studies show no relationship between intramuscularly recorded M wave amplitude and fatigue in humans (Bigland-Ritchie et al. 1982) and cellular membrane potential and force development in animal models (Hicks and McComas 1989; Balog et al. 1994).

2.2.4.2 Sodium fluxes and fatigue

It was first noted in 1902, that decreased extracellular [Na^+] eventually led to decreased contractility (Overton 1902). Subsequent studies have confirmed these early findings. A decreased extracellular [Na^+] from 120 to 60 mM decreased force in frog sartorius muscle (Bouclin et al. 1995). Reduced extracellular [Na^+] also depressed force in isolated bundles of rat skeletal muscle fibres (Cairns et al. 1995), rat soleus muscle (Overgaard et al. 1997), mouse skeletal muscle (Cairns et al. 2003; Duty and Allen, 1994) and amphibian muscle (Bezanilla et al. 1972; Nakajima et al. 1975). When extracellular [Na^+] was lowered to 25 mM tetanic force was decreased to 30% of the control level after 60 min in isolated rat soleus muscles (Overgaard et al. 1997). Although numerous studies have sampled isolated skeletal muscle preparations, these results cannot be directly applied to intact muscles however, where extracellular [Na^+] is high (Sejersted and Sjøgaard 2000).

Repetitive electrical stimulation of mouse skeletal muscle resulted in an ~85% increase in intracellular [Na^+], as measured by ion-selective electrodes (Juel, 1986). Intense swimming exercise for ~3 min also increased intracellular [Na^+] in rat plantaris, red gastrocnemius, white gastrocnemius, but not soleus muscle (Lindinger and Heigenhauser, 1987). An increased [Na^+] following electrical stimulation of rat hindlimb was also reported by the same group (Lindinger and Heigenhauser 1988). Exercise also resulted in an increased Na^+ content in human muscles. Exercise to exhaustion comprising 3 cycling bouts for 3 min at 325-395 W, increased muscle Na^+ content from 9.8 to 16.5 mmol/100 g dry wt (Sjøgaard, 1983). Similarly, one legged, dynamic exercise resulted in a 3-fold increase in calculated Na^+ content in human vastus lateralis muscle (Sjøgaard et al., 1985). Moreover, the rise in intracellular [Na^+] can

result in the T-system being unable to support trains of action potential in mechanically skinned rat fibres, thereby reducing tetanic force responses (Nielsen et al., 2004).

Some studies demonstrate that decreased extracellular [Na^+] alone may not affect force, but when combined with increased extracellular [K^+], muscle contractility was affected (Balog et al. 1994). The combination of 9 mM K^+ and 85 mM Na^+ induced a reduction in tetanic force to 30 ± 9% of the control level in isolated rat soleus muscle (Overgaard et al. 1997). The same study also decreased [Na^+] to 71 mM or increased [K^+] to 8 mM and found each had a negligible effect on force in isolated rat soleus muscle, but when these ionic changes were combined force was decreased to 29 ± 7% of control (Overgaard et al. 1997). Therefore, the effects of a decreased [Na^+] are considerably potentiated by increasing extracellular [K^+] (Overgaard et al. 1997) and the combined effects of Na^+ and K^+ on the twitch and tetanic contractions are greater than the sum of their individual effects (Bouclin et al. 1995).

2.2.4.3 Potassium fluxes and fatigue

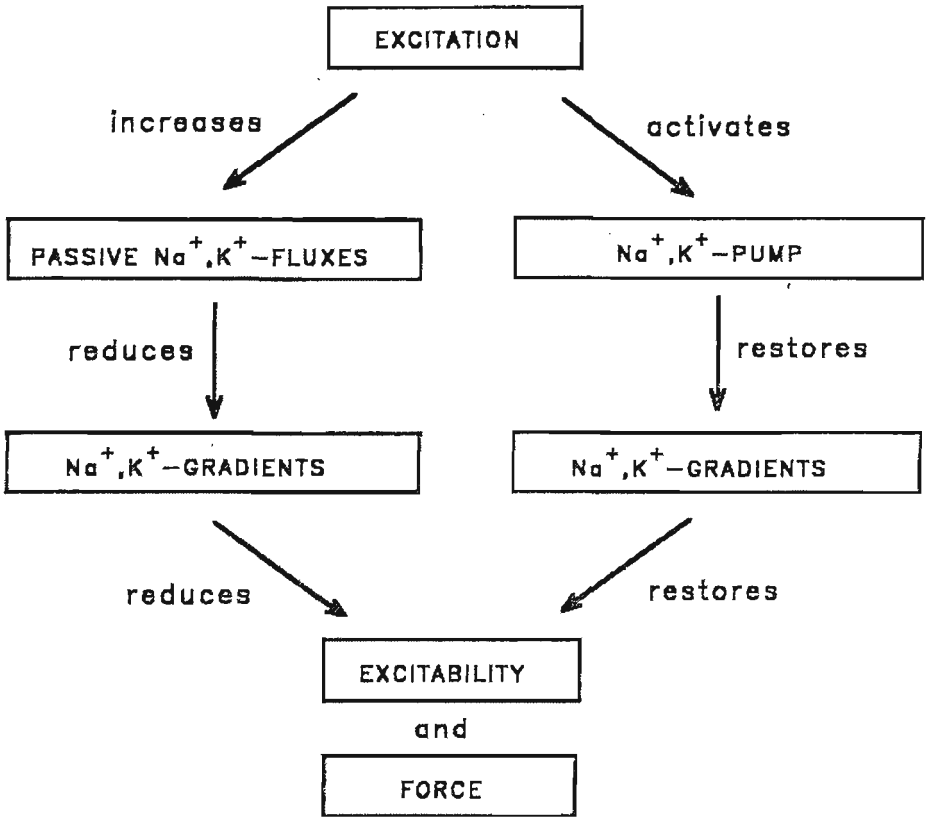
It was known as early as 1936 that K^+ is lost from contracting muscle (Fenn and Cobb 1936), resulting in decreased muscle K^+ content (Costill and Saltin 1975; Sejersted et al. 1982; Sjøgaard 1983; Hallèn et al. 1994; Gullestad et al. 1995; Katz et al. 1985) and increased venous plasma [K^+] (McKenna, 1992; Medbø and Sejersted, 1990; Sejersted, 1985).

The effects of increased extracellular K^+ on muscle force development and fatigue have also been studied. In isolated bundles of fibres and whole soleus muscles from the rat there was a large reduction of force at a [K^+] of 14 mM (Cairns et al. 1995). Similarly, isolated rat soleus muscles exposed to 10 mM K^+, also decreased isometric twitch and tetanic force by 40-69% (Clausen et al. 1993). Numerous other studies also reported

decreased force development with increased K^+ in frog sartorius (Bouclin et al., 1995), mouse soleus (Juel, 1988) and Xenopus toe muscle (Lannergren and Westerblad, 1986). Human studies also suggest that decreased intracellular [K^+] can impair force development. Verburg et al., (1999) studied the effects of two-legged intermittent static knee-extensions at 30% MVC on K^+ and fatigue. They reported a large net K^+ loss during the first minutes of exercise, which disappeared after 20 min but reappeared after 30 min; the decrease in intracellular K^+ was linearly related to the fall of maximal force. One-legged dynamic knee-extension decreased muscle intracellular [K^+] from 165 mM at rest to 129 mM at exhaustion (Sjøgaard et al. 1985). This decrease together with an increased extracellular [K^+] from 4.5 mM at rest to greater than 6.0 mM at exhaustion could also be a cause of muscle fatigue (Sjøgaard et al. 1985).

In summary, excitation-induced changes in the ionic gradients contribute to the development of skeletal muscle fatigue (Figure 2.1). The Na^+,K^+-ATPase enzyme plays a dynamic role in the maintenance of excitability during contractile activity (Nielsen and Clausen 1996) and helps maintain K^+ and Na^+ concentration gradients (Clausen, 2003). However, the transport capacity of the Na^+,K^+-ATPase enzyme in exercising muscle may become rate limiting in the maintenance of electrochemical gradients, membrane excitability and force (Clausen 1996; Nielsen and Overgaard 1996; Figure 2.1). Na^+,K^+-ATPase activity is depressed at the point of fatigue (section 2.19) with one proposed mechanism being an accumulation in ROS (section 2.22). This thesis will investigate the effects of ROS on Na^+,K^+-ATPase activity and hence their importance during exercise are discussed in Sections II and III.

Figure 2.1 The role of Na^+, K^+ and Na^+,K^+-ATPase in the maintenance of excitability and force ion skeletal muscle.



From Nielsen and Overgaard, 1996.

SECTION II: REACTIVE OXYGEN SPECIES, ANTIOXIDANTS AND EXERCISE

2.3 Introduction to reactive oxygen species

During exercise there is an increased formation of ROS, which contribute to muscle fatigue. Several studies indicate that antioxidants not only decrease ROS concentration but also attenuate muscular fatigue. The exact mechanisms for this phenomenon are unknown and remain to be elucidated.

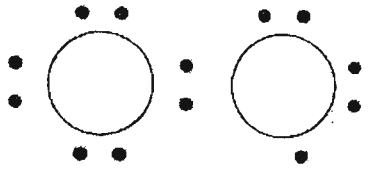
ROS formation during muscle contraction will be reviewed. Although it is difficult to directly measure ROS concentrations, several indirect methods can be used. The importance of endogenous and exogenous antioxidants in decreasing muscle and blood ROS during exercise will be highlighted. Studies indicate that the manipulation of glutathione, a major non-thiol antioxidant, can influence exercise performance, and the effects of acute and chronic exercise on glutathione status are therefore reviewed.

2.4 Overview of reactive oxygen species

An oxygen free radical is defined as "any atom or molecule with an unpaired electron in its outer orbit that can exist independently for a period of time (Jenkins, 1988; Sen, 1995; Figure 2.2). Free radicals are highly reactive compounds, have short-half lives and exist in low steady state concentrations (Table 2.1; Sjødin et al., 1990; Sen, 1995). Oxidation is defined as a loss in electrons by an element or compound, whereas reduction refers to the gain of electrons from an element or compound (Halliwell and Gutteridge, 1989). Reactions occur when there is a transfer of electrons from a suitable donor (i.e. the reductant) to a suitable electron acceptor (i.e. the oxidant). These are termed oxidation-reduction (redox) reactions. Univalent reduction of molecular oxygen refers to the reduction of molecular oxygen by one electron at a time, thus producing oxygen centred free radicals such as $O_2^{-\bullet}$ and $^{\bullet}OH$ (Sen, 1995). Sen (1995) further suggests that these reactive intermediates may escape from the process of complete tetravalent reduction and produce ROS. ROS represents a broader term than free radicals and includes the non-radical derivatives of oxygen which include H_2O_2, singlet oxygen ($^1O_2$) and hydroperoxides (Sen, 1995).

A pro-oxidant (eg. ROS) is any substance that when present in low concentrations compared to those of reducible substrates, significantly increases or promotes oxidation of that substrate (Sjødin et al., 1990; Sen, 1995). To combat the deleterious nature of these pro-oxidants, the body possesses endogenous defences in the form of anti-oxidants. An anti-oxidant is defined as any substance that, when present in low concentration compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Sjødin et al., 1990; Sen, 1995).

Figure 2.2 Model of electron position of reactive oxygen species.



Superoxide (O_2^-)

Hydroxyl radical (•OH)

From Kerr et al., (1996)

Table 2.1 Lifetimes of selected free radicals

Radical	Symbol	Lifespan (s)
Superoxide	$O_2^{-\bullet}$	1×10^{-6}
Hydroxyl	$^{\bullet}OH$	1×10^{-9}
Alkoxyl	$RO^{\bullet}$	1×10^{-6}
Peroxyl	$ROO^{\bullet}$	1×10^{-2}
Singlet oxygen	$^1O_2$	1×10^{-6}

From Yu (1994).

The presence of unpaired electrons in free radicals tends to produce highly reactive species that can interact with numerous biological molecules and initiate a series of damaging free radical reactions (Kanter, 1995). There are three phases in a free radical chain reaction (Halliwell and Gutteridge, 1986; Kanter, 1995). Firstly, there is the initiation process where the free radical is generated. The propagation phase follows, where other free radicals are formed. New free radicals can be formed when a radical gives one electron to, takes one electron from and/or adds on to a non-radical, thus forming a new radical (Halliwell and Chirico, 1993). The final stage of a free radical attack is when the free radical is terminated, and is achieved when two free radicals bond together to form a stable non-radical (Kanter, 1995).

There are a number of possible mechanisms by which anti-oxidants may protect the cells against the harmful effects of ROS (Sen, 1995). These include:

(i) prevention of ROS formation;

(ii) interception of ROS attack by scavenging the reactive metabolites and converting to less reactive molecules;

(iii) enhancing the resistance of sensitive biological targets to ROS attack;

(iv) preventing the transformation of less reactive ROS (eg. $O_2^{-\bullet}$) to more deleterious forms (eg. $^{\bullet}OH$);

(v) facilitating the repair and damage caused by ROS;

(vi) triggering the expression of genes that encode anti-oxidant properties;

(vii) providing a favourable environment for the effective functioning of other anti-oxidants (eg. as a co-factor or by acting to maintain a suitable redox status).

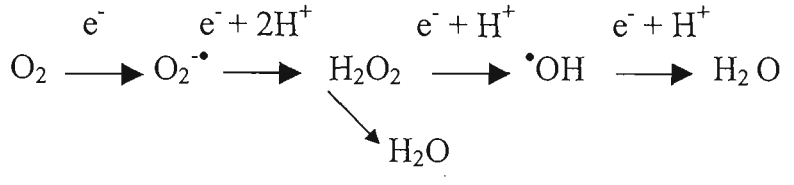
2.5 Formation of reactive oxygen species

2.5.1 Reactive oxygen formation from molecular oxygen

One principal pathway for the production of ROS involves the enzymatically-controlled reduction of molecular oxygen via the cytochrome chain (Kanter, 1995). The rate of whole body oxygen uptake may increase by 20 fold during exercise, whilst oxygen flux through the active peripheral skeletal muscle may increase by ~100 fold (Sen, 1995). In biological systems, the reduction of molecular oxygen to water requires four electrons and can be achieved in two ways (Sjødin et al., 1990). The main pathway is tetravalent reduction of oxygen to water. This proceeds in the mitochondria with no reactive oxygen species generated and accounts for 95-98% of total oxygen consumption (Sjødin et al., 1990). The remaining 2-5% is utilised in an alternative univalent pathway (Figure 2.4), in which ROS are produced (Sjødin et al., 1990). These electrons 'leak' onto

oxygen rather than being transported to the next component in the electron transport train (Boveris and Chance, 1973).

Figure 2.3 The univalent oxygen reduction pathway



O_2, molecular oxygen; $^{\bullet}OH$, hydroxyl radical; $O_2^{-\bullet}$, superoxide anion; H_2O, water; H_2O_2, hydrogen peroxide; e^-, electron; H^+, hydrogen. From Sjødin et al. (1990)

With the acceptance of the first electron, $O_2^{-\bullet}$ is formed (Hess and Manson, 1984), which although it is not highly toxic, can cause free radical chain reactions (Halliwell and Gutteridge, 1989). Furthermore, $O_2^{-\bullet}$ is able to inactivate glutathione peroxidase *in vitro* (Blum and Fridovich, 1985), as well as partially inhibiting catalase *in vitro* (Kono and Fridovich, 1982). If this also occurred *in vivo*, H_2O_2 may increase, as these enzymes are responsible for the dismutation of H_2O_2 (Hess and Manson, 1984; Sen, 1995).

The H_2O_2 molecule readily diffuses through hydrophobic membranes, and can generate highly reactive ROS when interacting with redox active transitional metals (Brotto and Nosek, 1996). For example, H_2O_2 accumulation produces $^{\bullet}OH$ via the Haber-Weiss reaction with a metal chelate (**R1**) or the Fenton reaction (**R2**) as shown below (Hess and Manson, 1984; Halliwell and Gutteridge 1989).

R1 $O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH$

R2 $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$

The hydroxy radical is a very reactive and unstable oxidising species, which reacts with a wide variety of organic compounds and biomembranes (Hess and Manson, 1984). In

fact, $^{\bullet}OH$ is so reactive that it very likely never diffuses more than 1 or 2 molecular diameters without entering a reaction (Pyke et al., 1986). Hess and Manson (1984) suggest that large concentrations of $^{\bullet}OH$ do not exist under physiological conditions. Nevertheless, $^{\bullet}OH$ will react with and alter the structure of enzymes, membranes or nucleic acids (Sjødin et al., 1990). A semiquinone ($SQ^{\bullet}$) radical in the presence of H_2O_2 and a high H^+ concentration may also propagate to form the potent $^{\bullet}OH$ (Jenkins 1988; **R3**).

R3 $\quad H_2O_2 + SQ^{\bullet} + H^+ \rightarrow \text{Quinone} + {}^{\bullet}OH + OH^-$

2.5.2 Xanthine oxidase system

ROS production may also result from the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). XO catalyses the oxidation of hypoxanthine to xanthine as well as the oxidation of xanthine to uric acid, using NAD as an electron acceptor (Sjødin et al., 1990). Under normal conditions 80-90% of XO exists as XDH. However, XDH is converted to an oxidase under metabolic stress, with molecular oxygen replacing NAD^+ as the electron acceptor (Parks et al., 1988; Sjødin et al., 1990). The conversion of XDH to a reversible form of XO occurs through the oxidation of XDH-thiols during periods of ischaemia, oxidative or metabolic stress, or other conditions where NAD^+ is used as an electron acceptor (Parks et al., 1988). With molecular oxygen replacing NAD^+ as an electron acceptor, the superoxide anion is formed (Kuppusamy and Zweier, 1989).

2.5.3 Other reactive oxygen producing mechanisms

The production of ROS is not limited to mitochondrial metabolism and the xanthine oxidase system. The auto-oxidation of haemoglobin to methaemoglobin involves the formation of a ferrous iron-oxygen complex, which slowly decomposes with the release of $O_2^{-\bullet}$ (Misra and Fridovich, 1972; Halliwell and Gutteridge, 1989). The reactions of

adrenaline and noradrenaline and their derivatives can produce $O_2^{-\bullet}$ and H_2O_2 (Halliwell and Gutteridge, 1989). In addition, environmental free radicals can also be taken up into the body from air pollution, UV-radiation and cigarette smoke (Simon-Schnass, 1994). Kukreja and Hess (1992) detail further possible sources of free radicals.

2.6 Measurement techniques for detecting reactive oxygen species

2.6.1 Introduction

Given that ROS are extremely reactive and have short half-lives, their detection is difficult. Oxidative stress is measured either directly or indirectly. Direct measurements include electron paramagnetic resonance (EPR), with the most common indirect markers being LIPOX and changes in glutathione redox status. The following section outlines these methods, with more detailed reviews available elsewhere (Han et al., 2000; Urso and Clarkson, 2003). There are also a number of *in vitro* measurements that can detect specific ROS, including cytochrome *c* reduction, intracellular fluorochrome and salicylate hydroxylation, as detailed by Han et al., (2000). There are numerous ways to measure oxidative stress, each with their advantages and disadvantages. Overcoming the difficulties in measuring ROS in biological systems remains a significant challenge in this field (Han et al., 2000).

2.6.2 Direct measurement of reactive oxygen species

EPR is the most powerful device to directly detect and characterise oxygen free radicals (Sen, 1995), although the use of spin traps is required. EPR measures the energy changes that occur as unpaired electrons align in response to an external magnetic field (Borzone et al., 1994). Several studies have utilised the EPR technique to directly detect free radicals in skeletal muscle and blood, as described below (Davies et al., 1982; Jackson et al., 1985; Borzone et al., 1994; Bailey et al., 2003).

2.6.2.1 Electron paramagnetic resonance

Davies et al., (1982) detected increased ROS concentration in liver and skeletal muscle via EPR in rats following a submaximal treadmill run to exhaustion. Similarly, Jackson et al., (1985) reported an average 70% increase in the amplitude of the major EPR signal in rat hind muscles and an ~30% increase in EPR signal in diaphragm muscle after resistive loading (Borzone et al., 1994).

2.6.2.2 Spin traps

Ashton et al., (1999) used EPR and a spin trap to measure ROS in blood drawn from healthy humans during incremental exercise to exhaustion. They found an increase in the intensity of the alpha-phenyl-tert-butylnitrone adducts, as well as malondialdehyde (MDA) and lipid hydroperoxides during exercise (Figure 2.4). Bailey et al., (2003) applied the EPR spin trapping technique in humans completing single leg knee extensor exercise and provided the first direct evidence of ROS outflow from active skeletal muscle. Together with their use of femoral arterial and venous blood free radical concentrations, these authors demonstrated that muscle contraction was associated with net adduct outflow that increased proportionally with muscle oxygen consumption and cardiac output (Bailey et al., 2003).

2.6.3 Indirect Measurement

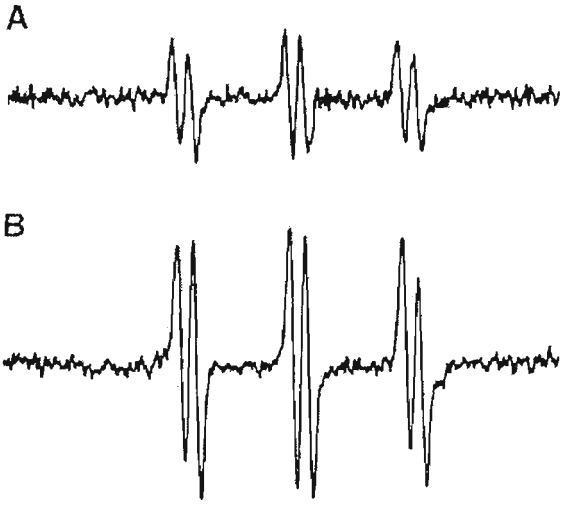
Due to the complexity in directly measuring oxidative stress and/or ROS, most investigators have used indirect measurements. These include markers of lipid peroxidation, changes in glutathione status and acute activation of endogenous antioxidants.

2.6.3.1 Lipid peroxidation

The most frequently used method is the measurement of LIPOX. The process of LIPOX is initiated when a ROS having sufficient energy to abstract a H^+ atom of a methylene

group reacts with polyunsaturated fatty acids (Gutteridge and Halliwell, 1990). Absolute values of LIPOX by-products may vary considerably depending on the assay method used (Sen et al., 1994) and the assessment of oxidative stress by LIPOX data may be considered valid only if other defined oxidative stress indexes are studied (Sen, 1995). Examples of LIPOX by-products that have been measured include pentane, MDA, isoprostanes, conjugated dienes and lipid hydroperoxides.

Figure 2.4 Pre (A) and post-exercise (B) EPR spectra of α-phenyl-tert-butylnitrone adduct in human blood.



From Ashton et al., (1999).

2.6.3.1.1 Pentane

During LIPOX, pentane and ethane are formed during the breakdown of fatty acids (Urso and Clarkson, 2003), allowing its use as an indirect marker of LIPOX. Both Dillard et al. (1978) and Kanter et al., (1993) report increased expired pentane concentrations following human cycling and running exercise, respectively. Transient increases in expired pentane also occur after graded aerobic exercise (Leaf et al., 1997). Although this technique is considered highly sensitive and is non-invasive, this is a difficult technique, which explains its infrequent use (Urso and Clarkson, 2003).

2.6.3.1.2 Malondialdehyde

The most common method used to assess changes in the LIPOX by-product MDA with exercise is measurement of thiobarbituric acid (TBARS; Urso and Clarkson, 2003). Rat treadmill exercise increased TBARS in white, red and mixed vastus (Alessio and Goldfarb, 1988; Goldfarb, 1993; Sen et al., 1994; Kayatekin et al., 2002) and red gastrocnemius muscle (Sen et al., 1994). In humans, cycling for 30 min (Alessio et al., 1997) and 40 min (Laaksonen et al., 1999) also induced a 46-50% increase in plasma TBARS, with similar changes reported after 30 min cycling at the aerobic and anaerobic threshold (Sen et al., 1994). Conversely, there was no increase in plasma TBARS after a $\dot{V}O_{2max}$ test (Sen et al., 1994), a 2.5 hr run (Dufaux et al., 1997), isometric exercise (Alessio et al., 2000) and half marathon (Duthie et al., 1990), whilst supramaximal exercise decreased TBARS by 23% (Groussard et al., 2003).

The use of TBARS as indices of oxidative stress has been widely criticised in the literature. Although this technique is commonly used, it is not very specific because this assay measures aldehyde breakdown products of some, but not all lipid hydroperoxides and may also react with other non-lipid compounds (Haramaki and Packer, 1994). Furthermore, the assay is greatly influenced by the reaction conditions (Han et al., 2000).

2.6.3.1.3 Other markers of lipid peroxidation

Other markers of LIPOX include conjugated dienes, isoprostanes and lipid hydroperoxides. The increase in human blood lipid hydroperoxides is consistent despite varying exercise protocols. Isometric exercise (Alessio et al, 2000), maximum aerobic exercise test to exhaustion (Alessio et al., 2000) and $\dot{V}O_{2max}$ test (Ashton et al., 1999) all increased blood lipid hydroperoxides by between 23-36%. Blood conjugated dienes increased after a marathon run in one study (Liu et al., 1999), but not another (Duthie et

al., 1990). No change in blood conjugated dienes was also reported immediately at the end of a half marathon (Marzatico et al., 1997). Very few studies have used isoprostanes as an indicator of oxidative stress during exercise (Urso and Clarkson, 2003). However, the levels of isoprostanes almost doubled after a 50km ultra-marathon race (Mastaloudis et al., 2001).

2.6.3.2 Other forms of oxidative stress

Apart from LIPOX measurements, oxidative stress can be also used as an indicator of increased ROS generation. Measurements of oxidative stress include changes in glutathione redox status, protein carbonyls, total antioxidant capacity and DNA oxidation.

2.6.3.2.1 Redox status

Change in glutathione status is one of the major biochemical indices of oxidative stress (Ji and Fu, 1992; Sastre et al., 1992). The concentrations and ratios of GSH and GSSG undergo dynamic changes during exercise and are often used as a sensitive measure of tissue oxidative stress (Ji and Fu, 1992). The effects of exercise on muscle and blood glutathione are detailed in sections 2.9 and 2.10.

Since SOD, CAT and GPX specifically quench free radicals, increased activity of each of these enzymes during acute (section 2.11) and chronic (section 2.12) exercise may indicate oxidative stress. Similarly, the use of exogenous antioxidant supplements (section 2.23.1) and ROS-specific antioxidants (section 2.7) during *in vivo* and *in vitro* experiments have been used to provide indices of oxidative stress.

2.6.3.2.2 Other measurements

Apart from redox status, oxidative stress can be also measured by blood protein carbonyls (Alessio et al., 2000), total antioxidant capacity (Cao et al., 1993) and DNA oxidation (Okamura et al., 1997).

2.7 Reactive oxygen species production in skeletal muscle

2.7.1 Resting Muscle

Skeletal muscle produces ROS at low rates under resting conditions and these appear to modulate contractile function. Commoner et al., (1954) provided the first evidence of increased ROS generation in rabbit skeletal muscle, with similar findings in human, rat and mouse skeletal muscle (Jackson et al., 1985). Reid et al., (1992) isolated rat diaphragm fibre bundles and incubated them in a bath medium containing cytochrome *c*, with absorbance of cytochrome *c* used as measurement of $O_2^{-\bullet}$. They found the absorbance of cytochrome *c* was greater in the bath medium exposed to resting muscle, than medium only, indicating $O_2^{-\bullet}$ release by passive muscle.

2.7.2 Contracting muscle

Davies and colleagues (1982) reported a 2- to 3-fold increase in free radical content in rat skeletal muscle and liver following prolonged exercise lasting ~46 min. Thirty min of electrical stimulation at 50 Hz produced an average 70% increase in the amplitude of the EPR spectra in rat hindlimb skeletal muscle (Jackson et al., 1985). Superoxide radical production increased four-fold during repetitive, fatiguing contractions in rat diaphragm (Reid et al., 1992), whilst an increased rate of $^{\bullet}OH$ production was found in contracting rat diaphragm (Diaz et al., 1993) and feline triceps surae (O'Neill et al., 1996). These studies provide strong evidence for increased ROS production in contracting skeletal muscle.

High intensity, exhaustive cycling exercise lasting ~5 min resulted in urate being oxidised to allantoin in muscle and plasma (Hellsten et al., 1997). This is indirectly indicative of increased ROS concentration. The first study to directly demonstrate contracting skeletal muscle and/or associated vasculature generates ROS was conducted by Bailey et al., (2003), who measured femoral arterial and venous blood free radical

concentrations via EPR spectroscopy. Incremental exercise at 75% of maximal work rate increased the venoarterial difference compared to 25% of maximal work rate (387±214 arbitrary units and 85±58 arbitrary units, respectively), with the net adduct outflow significantly correlated with leg oxygen uptake (r^2=0.47) and cardiac output (r^2= 0.47; Bailey et al., 2003).

2.8 Anti-oxidants

2.8.1 Introduction

Evolution of organisms in an oxygen rich atmosphere has led to the development of endogenous physiological defence systems that co-operate to scavenge and de-toxify ROS (Halliwell and Gutteridge, 1989). The three most important endogenous anti-oxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). A second line of defence may be provided by ingestion of exogenous anti-oxidants, primarily obtained as nutrients or nutritional supplements (Sen, 1995). These exogenous anti-oxidants include vitamin E, vitamin C and β-carotene. There are also pharmacological anti-oxidants that may be taken as supplements including NAC and allopurinol.

2.8.2 Superoxide Dismutase

SOD plays a vital role in protecting against the damaging effects of the superoxide radical (McCord and Fridovich, 1969) as identified in **R4**;

$$\textbf{R4} \quad 2O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Up to 92% of total SOD activity is present in the cytosol, with the remainder existing in the mitochondria (Tyler, 1975). However, it has been estimated that 80% of the $O_2^{-\bullet}$ in the mitochondria is reduced by mitochondrial SOD, whilst the remaining 20% escapes to the cytosol (Nohl and Hegner, 1978).

There are three isoenzymes of the enzyme, which differ principally by the nature of the metals in their active centres and by their physical location relative to the cell (Jenkins, 1988). Copper and zinc containing SOD (Cu-Zn SOD) is found predominantly in the cytoplasm (McCord and Fridovich, 1969) and manganese-superoxide dismutase (Mn-SOD) is accommodated by the mitochondria (Weisiger and Fridovich, 1973). The third isoenzyme is located in the extracellular space (EC-SOD; Marklund, 1984). Cu-Zn SOD has a high concentration in erythrocytes and liver, Mn-SOD in heart, liver and kidney and EC-SOD in kidney and lung (Sen et al., 1994). All SOD isoenzymes in skeletal muscle are known to be relatively lower activity compared to these tissues (Nakao et al., 2000; Marklund, 1984). The three isozymes also have different activity, depending on the tissue investigated. Nakao et al., (2000) found that the highest activity of CuZn SOD was found in the liver, whereas the lowest was in the gastrocnemius muscle. Similarly, EC-SOD was the lowest in gastrocnemius muscle and the highest in lung tissue, with Mn-SOD the highest in the heart.

2.8.3 Catalase

Catalase primary function is the dismutation of H_2O_2 (**R5**).

$$\textbf{R5} \quad 2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT activity is mainly in the cytoplasm, with moderate activity in the mitochondrial matrix and is present in most tissues, particularly the liver and erythrocytes (Chance et al., 1979). CAT has a low activity in skeletal muscle, although type I oxidative fibres possess a higher activity than type II glycolytic fibres (Jenkins, 1984). Although it shares a similar function to GPX (see section 2.8.5), CAT has a greater affinity for H_2O_2 at higher concentrations (Chances et al., 1979; Halliwell and Gutteridge, 1989) and Fe^{3+} is a required co-factor (Ji, 1995).

2.8.4 Glutathione

Glutathione (γ-glutamylcysteinylglycine) is a major non-enzymatic anti-oxidant, the most abundant non-protein thiol source in the cell and needs to be continually replenished via metabolic pathways (Ji, 1995). For most tissues, glutathione concentration is in the millimolar range, although there is large variability amongst tissues depending upon their function and oxidative capacity (Halliwell and Gutteridge, 1989). The eye lens has the highest (~10 mM) glutathione content, whilst skeletal muscle glutathione content is relatively low (1-2 mM; Ji, 1995). Furthermore, glutathione content is dependent upon muscle fibre type, with soleus content 2-fold higher than in deep vastus lateralis, and 5-fold higher than superficial vastus lateralis (Ji et al., 1992). When oxidised glutathione (GSSG) undergoes reduction to reduced glutathione (GSH) it ensures the cell is kept in a reduced environment, which is essential for the function of many enzymes and co-factors (Ji, 1995). Under normal conditions, 99% of cellular glutathione is in the reduced state (Deneke and Fanburg, 1989). However, when rapid production of GSSG is occurring, or where glutathione reductase (GR) activity is impaired, there is an accumulation of GSSG and a decline in intracellular GSH (Deneke and Fanburg, 1989).

Although most exercise studies related to thiols have investigated glutathione metabolism, Sen and colleagues used monobromobimane to determine that glutathione only accounts for half of all cell thiols (Sen et al., 1997). The remaining proportion of cell thiols is made up of protein thiols including thioredoxin. The thioredoxin system is comprised of thioredoxin and thioredoxin reductase and helps maintain glutathione and cysteine homeostasis, thus playing an important role in maintaining the redox state of the cell (Sen, 2000).

There is limited data investigating the effects of exercise on protein thiols. Although plasma protein thiol concentrations decreased after a marathon race and 30 min of treadmill exercise, the role of protein thiols during exercise remain poorly understood (Inaymama et al., 1996). As protein thiols are oxidised during sample processing (Sen and Packer, 2000), this may help explain the relatively few studies

2.8.5 Glutathione Peroxidase

The role of reduced glutathione is to act as a substrate for GPX, which then removes H_2O_2 at low concentrations (**R6**; Deneke and Fanburg, 1989). GPX is a versatile hydroperoxide remover in the cell and thus plays an important role in the prevention of lipid peroxidation (LIPOX; Li, 1995). GPX activity is highest in the liver, moderate in erythrocytes, heart, kidney, brain and lung and a low activity in skeletal muscle and plasma (Chance et al., 1979).

$$\textbf{R6} \quad 2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

SOD plays an important role in relation to the glutathione cycle, preventing the inactivation of GPX by removing $O_2^{-\bullet}$, which can inactivate GPX *in vitro* (Blum and Fridovich, 1985). The NADPH dependant enzyme, GR (**R7**) also helps regenerate glutathione.

$$\textbf{R7} \quad GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Although not classified as an anti-oxidant enzyme, GR has a subcellular distribution similar to that of GPX and is essential to normal anti-oxidant function (Ji and Leeuwenburgh, 1995).

2.8.6 Other antioxidants

There are also a number of other antioxidants that provide protection against oxidative stress, including α-tocopherol, ascorbic acid and β-carotene.

2.8.6.1 α-tocopherol

Vitamin E (α-tocopherol) is a major lipid soluble antioxidant in cell membranes and protects against LIPOX by acting directly with a variety of ROS, including $^1O_2$ and $O_2^{-\bullet}$, to form the relatively innocuous tocopherol radical (Clarkson and Thompson, 2000). Vitamin E content is ~60-70 $nmol.g^{-1}$ in liver, heart, lung and adipose tissue, with approximately half this content found in skeletal muscle (Ji, 1995). It is difficult to deplete Vitamin E as it quenches a radical from ROS and is converted to a Vitamin E radical, which is then reduced back to Vitamin E by ascorbate and/or GSH (Ji, 1995).

2.8.6.2 Ascorbic acid

Vitamin C (ascorbic acid) is a water-soluble antioxidant (Clarkson and Thompson, 2000) and its actions are two fold. Firstly, Vitamin C can scavenge $^1O_2$, $^\bullet OH$ and $O_2^{-\bullet}$. Secondly, it helps recycle Vitamin E (Powers and Sen, 2000). However, high concentrations of Vitamin C may act as a pro-oxidant when interacting with transition metal ions (Yu, 1994). Consequently, megadoses of Vitamin C are unwarranted.

2.8.6.3 β-carotene

Carotenoids (eg. β-carotene) are lipid-soluble antioxidants located primarily in the tissue membranes and similar to Vitamin C, can also act as a prooxidant (Powers and Sen, 2000). β-Carotene is an effective quencher of $^1O_2$ (Burton and Ingold, 1984) and also reduces indices of LIPOX (Kellog and Fridovich, 1975) and breath pentane (Allard et al., 1994). β-Carotene can also decrease $O_2^{-\bullet}$ and peroxyl radicals (Yu, 1994).

In addition to Vitamin C, Vitamin E and β-carotene, other compounds also exhibit antioxidant properties including α-lipoic acid, uric acid, bilirubin and ubiquinone, as detailed elsewhere (Kanter, 1995; Kanter and Williams, 1995; Packer, 1997; Sen and Packer, 2000; Sen, 2001; Sen, 2001).

2.9 Effect of exercise on muscle glutathione

There is no consensus in the literature on the effects of acute exercise on muscle glutathione in animal and human models. Furthermore, there are limited data in human muscle, which necessitates further investigation.

2.9.1 Animal muscle

A large number of studies have been investigated the effects of exercise on glutathione in animals. Although consistent findings are found on GSSG, there is no agreement regarding the effects of exercise on TGSH and GSH (Table 2.2).

2.9.1.1 Total glutathione

There are conflicting reports on the effects of exercise on muscle total glutathione (TGSH) as summarized in Table 2.2. Treadmill running increased rat deep vastus lateralis (DVL) TGSH (Ji and Fu, 1992), with this increase also proportional to exercise intensity (Ji et al., 1992). In these studies there was no change in soleus or superficial vastus lateralis (SVL) indicating a fiber specific effect. In contrast, exercise lasting up to 120 min resulted in decreased TGSH in rat red gastrocnemius, mixed vastus and quadriceps (Lew et al., 1985; Pyke et al., 1986; Sen et al., 1994; Khanna et al., 1999). To further highlight the conflicting literature several studies report no change in muscle TGSH after various exercise protocols (Ji et al., 1992; Leeuwenburgh and Ji, 1996; McArdle et al., 1999; Gul et al., 2002) as highlighted in Table 2.2.

2.9.1.2 Reduced glutathione

Numerous studies have investigated exercise effects on muscle GSH, again with conflicting findings (Table 2.2). In rats, running decreased GSH by up to 39% in mixed vastus (Lew et al., 1985) and fast vastus muscles (Liu et al., 2000). In contrast, Sen et al., (1994) reported no change in red gastrocnemius muscle, but an ~160% increase in

mixed vastus muscle. Similarly, swimming exercise in mice decreased adductor muscle GSH (Di Simplicio et al., 1997).

Muscle GSH increased proportionally with exercise intensity in deep vastus lateralis, but not in soleus muscle (Ji et al., 1992), which may indicate that the muscle takes up GSH with the exercise induced stress. Since the soleus muscle has a higher GSH concentration and antioxidant enzyme activity (Sen et al., 1992; Leeuwenburgh et al., 1994; Leeuwenburgh et al, 1997), it may also be more protected from ROS-mediated damage when compared to DVL muscle (Ji et al., 1992).

2.9.1.3 Oxidised glutathione

The consensus in the literature is that exercise results in an increased muscle GSSG (Table 2.2). High intensity exercise increased GSSG in rat mixed vastus (Lew et al,. 1985) and gastrocnemius muscle (Lang et al., 1987), with these effects dependent upon exercise intensity (Ji et al., 1992). Although no effect of exercise was observed in GSSG in SVL, soleus and DVL muscles at low intensities, at increased intensities, exercise increased GSSG in DVL and soleus muscles (Ji et al., 1992). A similar fibre dependent effect was observed as running increased GSSG in rat DVL (Bejma et al., 2000), red gastrocnemius and mixed vastus (Sen et al., 1994), but not in fast vastus muscle (Liu et al., 2000). These studies indicate that the increased GSSG is dependent on both exercise intensity and muscle fibre type.

2.9.2 Human Muscle

There are limited reports investigating the effects of exercise on glutathione in human muscle and it is therefore difficult to draw conclusions. This necessitates further research to elucidate the role of glutathione during exercise in human muscle.

2.9.2.1 Total glutathione

The limited number of studies that have investigated the effects of exercise on TGSH all demonstrated no change during either cycling and running exercise (Table 2.3). From

these limited studies, however, it is difficult to draw any definitive conclusions on the small number of studies completed and very little is known about skeletal muscle glutathione metabolism in humans (Svensson et al., 2002).

Table 2.2 The effects of treadmill exercise on skeletal muscle total (TGSH), reduced (GSH) and oxidised (GSSG) glutathione contents in rat.

Source	Muscle	Speed (m/min)	Grade (%)	Duration (min)	TGSH (% change)	GSH (% change)	GSSG (% change)
1	mixed vastus	24	8.5	90-120	↓ 20	↓~21	↑ 62
2	quadriceps	24	8.5		↓ 60		
3	gastroc	26	15	~35	nc		↑ 50
4	DVL	20	0	76	↑ 21	↑ 18	↑ 60
5	soleus	15-25	0	56-60	nc	nc	nc
		25	5	55	nc	nc	nc
		25	10	51	nc	nc	↑~36
	SVL	15-25	0	56-60	nc	nc	nc
		25	5	55	nc	nc	nc
		25	10	51	nc	nc	nc
	DVL	15-25	0	56-60	nc	nc	nc
		25	5	55	↑~18	↑~20	↑~50
		25	10	51	↑~16	↑~20	↑~45
6	red gastroc	30	0	~110	↓~22	nc	↑~54
	mixed vastus	30	0	~110	↓~15	↑~160	↑ 160
7	soleus	20	5			nc	↑~20
	DVL	20	5			nc	↑~70
8	soleus	20	0	15		↑~45	↓~5
	soleus	20	0	15		↓~100	nc
	white gastroc	20	0	15		↑~100	↑~200
	white gastroc	20	0	15		↓~257	nc
9	DVL	25	5	~55	nc	nc	↑ 31

Reference: 1, Lew et al., (1985); 2, Pyke et al., (1986); 3, Lang et al., (1987); 4, Ji and Fu (1992); 5, Ji et al., (1992); 6, Sen et al., (1994); 7, Leeuwenburgh and Ji (1996); 8, Ohkuwa et al., (1997); 9, Bejma and Ji (1999). Muscles: gastroc, gastrocnemius; SVL, superficial vastus lateralis; DVL, deep vastus lateralis; ↑, increase; ↓, decrease; nc, no change; blank, not reported.

2.9.2.3 Oxidised glutathione

Two studies demonstrated no change in GSSG during exercise (Sahlin et al., 1992; Rabinovich et al., 2001; Table 2.3). In contrast, Cooper et al. (1986) found a 189% increase in quadriceps GSSG following a marathon run. This may indicate that the effects of exercise on GSSG are intensity dependent as both studies were conducted at relatively low exercise intensities (Sahlin et al., 1992; Rabinovich et al., 2001). This is especially true for the study conducted by Rabinovich et al. (2001) who matched the work rate of healthy controls to COPD patients, who had very low exercise capacity, as demonstrated by a peak exercise work rate of 48 W.

Due to the limited amount of data in humans further studies are warranted. The varying exercise intensity, mode, duration and subjects utilised in these studies, makes direct comparisons difficult and further work is required to elucidate the effects of acute exercise on human muscle GSH.

Table 2.3 The effects of exercise on vastus lateralis total glutathione (TGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) contents in humans.

Source	n	Mode	Intensity ($\%\dot{V}O_{2peak}$)	Duration (min)	TGSH (% change)	GSH (% change)	GSSG (% change)
1	10	running				nc	↑189%
2	7	cycling	90%	~20	nc		
3	5	cycling	40%	~11	nc	nc	nc
4	7	cycling	60%	~80	nc		nc
5	15	cycling	55-85%	50	nc	↓ 12%	

Reference: 1, Cooper et al., (1986); 2, Hellsten et al., (2001); 3, Rabinovich et al., (2001); 4, Sahlin et al., (1992); 5, Svensson et al. (2002). ↑, increase; ↓, decrease; nc, no change; blank, not reported.

2.10 Effect of exercise on blood glutathione

Although there are a vast number of studies investigating blood glutathione homeostasis in both animal and humans, there are many conflicting findings and interpretations, making it difficult to fully understand the possible mechanisms by which exercise is able to alter blood glutathione homeostasis. The different models used to induce oxidative stress and varying species further compound this interpretation. Thus the ability to compare results across a broad range of studies is difficult. Importantly, there are two separate pools of glutathione in blood, with the majority found in the erythrocyte with a concentration of ~2 mM, whereas the plasma has a much lower concentration of only ~0.05 mM (Ji and Leeuwenburgh, 1995). Consequently, some studies have been unable to detect plasma glutathione during exercise in both humans (Gohil et al., 1988; Dufaux et al., 1997) and animals (Mills et al., 1996).

2.10.1 Animal studies

There are conflicting reports on the effects of exercise on blood glutathione during exercise in animals, as summarised in Table 2.4.

2.10.1.1 Total glutathione

No change in blood TGSH was reported when rats ran at 1.8km/hr for ~110 min (Sen et al., 1994) and to fatigue for an unknown duration (Khanna et al., 1999). Conversely, swimming exercise increased blood TGSH in mice (DiSimplico et al., 1997), but decreased hemolysate TGSH in horses (Marlin et al., 2002).

2.10.1.2 Reduced glutathione

Some studies report an increased GSH (Lew et al., 1985; Sen et al., 1994; DiSimplicio et al., 1997). Together with a decreased liver GSH (Lew et al., 1985), this is consistent with a mechanism in which the liver may be releasing reduced glutathione into the plasma, thereby enhancing GSH delivery to skeletal muscle (Lew et al., 1985).

However, some studies have found that exercise decreased blood GSH (Vina et al., 2000; Hargreaves et al., 2002; Marlin et al., 2002). These effects may be intensity dependent as there was no change in plasma GSH during either walking or slow running, but a 66% decrease during fast running (Gambelunghe et al., 2001).

2.10.1.3 Oxidised glutathione

In contrast to TGSH and GSH, most studies report that blood GSSG increases during exercise in various animal models (Table 2.4). Several studies have reported increased GSSG ranging from 18-200% during exercise (Sastre et al., 1992; Sen et al., 1994; Mills et al., 1996; DiSimplicio et al., 1997; Vina et al., 2000). A rise in GSSG would indicate increased glutathione oxidation and is also indicative of increased oxidative stress (Sastre et al., 1992). However, some studies report no change in GSSG during exercise (Ohkuwa et al., 1997; Leeuwenburgh and Ji, 1996).

2.10.2 Human studies

Numerous studies have investigated the effects of exercise on blood glutathione in humans but their results are conflicting and interpretation is therefore difficult (Table 2.5).

2.10.2.1 Total glutathione

The effects of exercise on human blood TGSH are summarised in Table 2.5. TGSH increased in some studies (Duthie et al., 1990; Sahlin et al., 1991; Sahlin et al., 1992; Ji et al., 1993; Sen et al., 1994), but not in others (Sen et al., 1994; Tessier et al., 1995; Margaritis et al., 1997). Since the exercise protocols and subjects utilised in these studies were similar, it is difficult to elucidate the reasons for these discrepancies.

2.10.2.2 Reduced glutathione

Although the reported effects of exercise on blood GSH are extensive, there is little agreement (Table 2.5). Some studies report no change (Camus et al., 1994; Sen et al.,

1994; Margaritis et al., 1997) or a decreased blood GSH during exercise (Inayama et al., 2002; Weiss et al., 2002; Groussard et al., 2003). In contrast, others have reported increased blood GSH during exercise (Ji et al., 1993; Subudhi et al., 2003), although when subjects were supplemented with a CHO drink, the blood GSH increase was prevented (Ji et al., 1993).

The reasons for these discrepancies may also be related to increasing exercise intensity. Although Gohil et al. (1988) reported no change in blood GSH during incremental cycling exercise to fatigue, cycling at 65% $\dot{V}O_{2peak}$ in moderately trained subjects resulted in a 60% decrease in whole blood GSH. These differences may be related to the differing lactate concentrations that were encountered in the two protocols. During high intensity exercise, lactate rose by 625% compared to the 100% increase in submaximal exercise. ROS scavenging properties of lactate have been demonstrated *in vitro* (Groussard et al., 2000) and this may have prevented the decrease in GSH. Conversely, some studies have demonstrated no change in blood GSH in trained subjects, which may be caused by increased GR activity (Evelo et al., 1992).

2.10.2.3 Oxidised glutathione

The majority of studies report either an increased or unchanged blood GSSG during exercise (Table 2.5). An increased GSSG during exercise may be due to increased oxygen flux through the blood and provides an opportunity for methaemoglobin formation, which is accompanied by $O_2^{-\bullet}$ production (Gohil et al., 1988). In contrast, several studies demonstrate no change in GSSG (Ji et al., 1993; Margaritis et al., 1997; Weiss et al., 2002). Although no change in blood GSSG following graded cycling exercise to fatigue was found in moderately trained individuals (Gohil et al., 1988), there was a 72% increase in trained subjects using a similar exercise protocol (Sastre et al., 1992). This may indicate a training dependent effect, as highlighted by a recent

study (Subudhi et al., 2003). In this study, trained and untrained subjects completing exercise to exhaustion which lasted ~20 min, with blood GSSG at exhaustion, increased in untrained, but not trained individuals. An increased GR activity as a result of training (section 2.12.4) could explain the mechanism underlying the rise in GSSG in untrained individuals. Therefore, it is important to consider the training status of the individual when interpreting the results of exhaustive exercise.

As demonstrated, there are conflicting reports regarding the effects of exercise on blood glutathione, most likely attributed to a difference in the subjects' characteristics and/or exercise intensity. However, other factors may contribute to the conflicting reports including methodological concerns, sample timing and tissue sampled. Biologically, it is most likely that blood GSH decreases during exhaustive exercise to combat the rapid increase in ROS production, as GSH is used as a substrate for GPX, which is known to increase during exercise. Blood GSH is likely to decrease during prolonged exercise due to a diminished hepatic GSH reserve and when GSH use exceeds GSH uptake (Sen et al., 1992). Consequent to decreased GSH, blood GSSG would increase during exercise.

Table 2.4 Effects of exercise on blood total (TGSH), reduced (GSH) and oxidised glutathione (GSSG) concentrations in animals.

Source	Species	Tissue	Mode	Intensity	Duration (min)	TGSH (% change)	GSH (% change)	GSSG (% change)
DiSimplico et al., (1997)	mice	blood	swim		3.5	↑ 12%	↑ 10%	↑ 21%
Khanna et al., (1999)	rat	blood	run	1.8 km/h, 10% grade		nc		
Sastre et al., (1992)	rat	blood	run	24 m/min, 0% grade			nc	↑ 200%
Sen et al., (1994)	rat	blood	run	1.8 km/hr, 0% grade	~110	nc	↑~130%	↑~150%
Hargreaves et al., (2002)	horse	blood	run	80 km endurance race			↓ 59%	
Marlin et al., (2002)	horse	haemolysates	run	140 km endurance race		↓ 15%	↓ ~16%	↓ ~18%
Mills et al., (1996)	horse	haemolysates	run	simulate intense racing	~25		nc	↑~64%
Gambelunghe et al, (2001)	rat	plasma	walk	0.8 m/min, 0% grade	45		nc	
	rat	plasma	run	4 m/min, 0% grade	45		nc	
	rat	plasma	run	8 m/min, 0% grade	45		↓ 66%	
Leeuwenburgh and Ji (1996)	rat	plasma	run	20 m/min, 5% grade			↑ ~18	nc
Lew et al., (1985)	rat	plasma	run	24.1 m/min, 8.5% grade	90-120	↑ 68%	↑ ~48%	↑ 751%
Ohkuwa et al., (1997)	young rat	plasma	run	20 m/min, 0% grade	15		↑ ~100%	nc
Pyke et al., (1986)	rat	plasma	run	24.1, 8.5 % grade		↓ 60%		
Sen et al., (1994)	rat	plasma	run	60m/min, 0% grade	~110	↑ ~60%	↑ ~300%	↑ ~300%
Leeuwenburgh and Ji (1996)	rat	plasma	swim		326	nc	↓ ~11%	nc

↑, increase; ↓, decrease; nc, no change; blank, not reported.

Table 2.5 The effects of exercise on blood total glutathione (TGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) concentration in humans.

Source	Mode	Intensity	Duration (min)	TGSH (% change)	GSH (% change)	GSSG (% change)
Submaximal Intensity						
Whole Blood						
1	cycle	65% $\dot{V}O_{2peak}$	90		↓ 60%	↑100%
2	cycle	40%, 70%, 100% $\dot{V}O_{2peak}$	~20	↑15%		
3	cycle	60% $\dot{V}O_{2peak}$	~80	↑24%		
4	cycle	65% $\dot{V}O_{2peak}$	90		↓55%	↑28%
5	cycle	70% $\dot{V}O_{2peak}$	~120	↑~33%	↑~40%	nc
6	cycle	aerobic threshold	30	nc	nc	↑~45%
	cycle	anaerobic threshold	30	↑~22%	nc	↑~100%
7	cycle	65% $\dot{V}O_{2peak}$	20	↓12%		↑33%
8	run		30	nc	nc	nc
9	run	60% $\dot{V}O_{2peak}$	35	nc	nc	nc
			35	nc	nc	nc
10	run		150		↓~64%	↑~68%
	run			↑~50%	↓~50%	nc
11	run	anaerobic threshold	60	nc	↓20%	nc
12	triathlon			nc	nc	nc
Plasma						
13	cycle	65% $\dot{V}O_{2peak}$	90		nd	nd
14	run		150		nd	nd
15	cycle	40%, 70% 100% $\dot{V}O_{2peak}$	~20	↑ 90%		
16	cycle	60% $\dot{V}O_{2peak}$	40		↓ ~13%	↑ ~50%

Table 2.5 (continued)

Source	Mode	Intensity	Duration (min)	TGSH (% change)	GSH (% change)	GSSG (% change)
High intensity						
Whole blood						
17	cycle	graded exercise to max			nc	nc
18	cycle	graded exercise			nc	↑72%
19	cycle	graded exercise		nc	nc	↑100%
20	cycle	graded exercise to max	~10		↓~5%	↓~62%
21	cycle	Wingate test	0.5		↓14%	
Plasma						
22	cycle	graded exercise to max			nd	nd

1, Gohill et al., (1988); 2, Sahlin et al., (1991); 3, Sahlin et al., (1992); 4, Vigue et al., (1993); 5, Ji et al. (1993); 6, Sen et al., (1994); 7, Tessier et al., (1995); 8, Marin et al., (1990); 9, Camus et al (1994); 10, DuFaux et al. (1997); 11, Weiss et al., (2002); 12, Margaritis et al., (1997); 13, Gohill et al., (1988); 14, DuFaux et al. (1997); 15, Sahlin et al., (1991); 16, Laaksonen et al., (1999); 17, Gohill et al., (1988); 18, Sastre et al., (1992); 19, Sen et al., (1994); 20, Heunks et al., (1999); 21, Groussard et al., (2003); 22, Gohill et al., (1988). ↑, increase; ↓, decrease; nc, no change; nd, not detected; blank, not reported.

2.11 Endogenous antioxidants and acute exercise

SOD, CAT and GPX provide the first line of defence against ROS generated during exercise and it is therefore expected that exercise may have a direct impact on these enzymes (Ji and Leeuwenburgh, 1995). Numerous studies demonstrate an increased SOD, CAT and GPX activity in blood and skeletal muscle during exercise, although there is some evidence to the contrary. Moreover, it is likely that the effects are dependent upon exercise intensity, duration and muscle fibre type.

2.11.1 Superoxide Dismutase

Acute exercise increased muscle SOD activity in rats during both short (Ji and Fu, 1992; Radak et al., 1999; McArdle et al., 2001) and prolonged exercise (Scherer and Deamer, 1986; Lawler et al., 1993; Somani et al., 1995), which may be related to increased mRNA levels of Mn-SOD and CuZn-SOD (Hollander et al., 2001). The increased SOD activity is indicative of $O_2^{-\bullet}$ production, but the mechanism for SOD activation remains unclear due to the short half-life of $O_2^{-\bullet}$ (Ji and Leeuwenburgh, 1995).

Conflicting findings have been reported in humans. Decreased blood SOD activity was reported after sprint (Groussard et al., 2003) and endurance exercise (Tauler et al., 2002), with others reporting no change (Tauler et al., 1999; Miyazaki et al., 2001). Conversely, increased blood SOD activity has also been reported (Koska et al., 2000). Only one study has reported increased SOD activity in vastus lateralis muscle, which peaked 3 d following acute exercise (Khassaf et al., 2001). The reasons for these discrepancies are unclear and it is difficult to compare results from various studies due to the different exercise intensities and durations. Furthermore, intra-assay inconsistencies could also explain the different findings as there is a 10-fold difference in sensitivity between the seven assay methods used to determine SOD activity (Oyanagui, 1984).

2.11.2 Catalase

The effects of acute exercise on CAT activity are conflicting, with some authors reporting no change (Ono et al., 1990; Ohishi et al., 1998), decreased (Schauer et al., 1990; Vani et al., 1990; Smolka et al., 2000) or increased activity (Ji et al., 1992; Ji, 1993; Somani et al., 1995). It is expected that CAT activity would increase during exercise, due to increased H_2O_2 production (Reid et al., 1992). However, CAT activity is in the cytoplasm, whereas the main source of H_2O_2 is from the mitochondria (Chance et al., 1979). Therefore, mitochondrial GPX may be more effective in detoxifying H_2O_2. The effects of exercise and CAT activity are tissue specific (Ji, 1993). In rats run to exhaustion, CAT activity was not changed in heart and liver, but elevated in skeletal muscle. Moreover, exercise at moderate intensities increased CAT activity in rat DVL, but not SVL (Ji et al., 1992), which may be more pronounced in untrained and selenium deficient rats (Ji et al., 1988).

There are limited data regarding the effects of acute exercise on CAT activity in human tissue. Repeated bouts of continuous jumping had no effect on skeletal muscle or erythrocyte CAT in either jump trained or untrained subjects (Ørtenblad et al. 1997). Similarly, incremental exercise to exhaustion did not change erythrocyte CAT activity (Miyazaki et al., 2001). However, an increase in erythrocyte CAT activity was reported during a duathlon race (Tauler et al., 1999). The data regarding acute exercise effects on human CAT activity are extremely limited. Given the important role of CAT in detoxifying H_2O_2, further research is required.

2.11.3 Glutathione Peroxidase

Early work conducted by Ji et al. (1988) demonstrated that GPX activity was significantly correlated to endurance time in trained and untrained rats. Subsequent work by this group demonstrated that GPX activity is elevated in rat DVL, but not SVL

(Ji and Fu, 1992) and these effects were intensity dependent (Ji et al., 1992). Fibre type specific effects have also been reported by others (Lawler et al., 1993). Interestingly, a fibre specific effect was only present in young rats and not old rats, suggesting either a diminished response of mechanisms that would increase antioxidant protection and/or less ROS formation with acute exercise in old rats (Lawler et al., 1993).

In humans, several studies demonstrated an increased blood GPX activity during an acute bout of submaximal exercise (Kanaley and Ji, 1991; Atalay et al., 1997; Laaksonen et al., 1999; Tauler et al., 1999; Koska et al., 2000; Tauler et al., 2003) although two studies reported no change (Rokitzki et al., 1994; Miyazaki et al., 2001). Short-term "supramaximal" anaerobic exercise also resulted in no change in blood GPX activity (Groussard et al., 2003). There is no clear explanation for these discrepancies (Ji and Leeuwenburgh, 1995) and further research is required to determine the effects of exercise on GPX activity in humans.

2.11.4 Glutathione Reductase

Although GR is not directly involved in removing ROS, it is responsible for supplying GSH to maintain GPX catalytic function and to maintain a reduced intracellular milieu during exercise (Ji and Leeuwenburgh, 1995). The majority of research demonstrates increased GR activity following an acute exercise bout in both animals (Spodaryk et al., 1985; Ji and Fu, 1992; Ji et al., 1992) and humans (Ohno et al., 1988). Spodaryk et al. (1985) reported a 38% increase in GR activity in rats run to exhaustion. The extensive work conducted by Ji and colleagues confirm these earlier findings and reported increased GR activity after acute exercise in rat DVL (Ji and Fu, 1992) and unspecified skeletal muscle (Ji, 1993), which was intensity dependent (Ji et al., 1992).

The majority of antioxidant studies have been limited to measurements of enzyme activities, with very few studies examining antioxidant gene expression. To gain more

definitive information about the regulatory mechanisms involved in increased antioxidant enzyme activity, measurements of mRNA level and enzyme protein content are required. Whilst endogenous antioxidant enzyme activity is increased during exercise in response to increased ROS accumulation, the mechanism for this increase remains unresolved although rapid up-regulation of gene expression of antioxidant enzymes in response to acute oxidative stress is unlikely. Oh-Ishi et al., (1997) observed that rat soleus SOD activity was significantly increased with chronic training, but enzyme protein content and mRNA levels were unchanged. Similarly, significant increases in skeletal muscle SOD enzyme content were not accompanied by concomitant changes in mRNA expression (Nakao et al., 2000).

Therefore, the levels of antioxidant enzymes appear to be regulated by posttranslational mechanism(s) during physical exercise, as increases in protein expression have not been reported. A potential regulatory site may be related to nuclear protein binding as SOD gene expression involves activation of nuclear factor κB and activator protein binding sites which are activated by ROS (Hollander et al., 1999).

However, the factors that regulate acute changes of antioxidant enzyme activities in erythrocytes are attributable to direct interactions on the enzymatic proteins or associated regulatory factors because erythrocytes do not have the machinery to synthesize proteins (Tauler et al., 1999). The molecular and genetic links between exercise, antioxidant enzyme mRNA and protein expression is in its embryonic stage and resolution of the mechanisms for increased antioxidant enzyme activity provides an exciting challenge for future research.

In summary, the majority of studies report increased endogenous antioxidant enzyme activity during exercise in both skeletal muscle and blood. Although there are conflicting reports, this is most likely due to the different training status, exercise

protocols and fibre type investigated. The activation of endogenous antioxidants may reflect an increased ROS production during exercise, although the precise mechanisms has not been identified. However, since mammalian tissue do not demonstrate a rapid up-regulation of gene expression of antioxidant enzymes in response to acute exercise (Harris, 1992), increased production of ROS remains a likely mechanism for increased antioxidant enzyme activity (Ji and Leeuwenburgh, 1995). Further research is required to identify the precise mechanism involved in the upregulation of endogenous antioxidant enzyme activity, particularly in human skeletal muscle.

2.12 Effects of chronic training on endogenous skeletal muscle antioxidants

A major problem when reporting the effects of training on antioxidant enzymes is that investigators have employed different training protocols, thus making it difficult to compare results, as adaptations are intensity- and duration-dependent (Powers et al., 1994). The initial training status of the subject also needs to be considered, as there was a linear relationship between endogenous antioxidant activity in human vastus lateralis and $\dot{V}O_{2max}$ (Jenkins et al., 1984). A difference in fibre composition also has a profound effect, as several studies have demonstrated a fibre-specific effect (Ji et al., 1992; Powers et al., 1994; Powers et al., 1999). Finally, aged skeletal muscle has limited capacity to further increase its antioxidant potential (Leeuwenburgh et al., 1994). Therefore, numerous factors may influence the effects of training on endogenous antioxidant adaptations.

2.12.1 Superoxide dismutase

Run training increased SOD activity in soleus (Criswell et al., 1993; Powers et al., 1994; Oh-ishi et al., 1997), DVL (Leeuwenburgh et al., 1997; Gore et al., 1998; Hollander et al., 2000), red gastrocnemius (Powers et al., 1994) and diaphragm (Oh-ishi et al., 1997; Vrabas et al., 1999) muscle. Conversely, several investigators report no

change (Alessio and Goldfarb, 1988; Laughlin et al., 1990; Criswell et al., 1993; Leeuwenburgh et al., 1994; Atalay et al., 1996), with similar discrepancies also found in humans (Hellsten et al., 1996; Tiidus et al., 1996).

Several animal studies have reported the effects of training on the different isoenzymes of SOD. It is generally accepted that CuZn-SOD does not increase after training (Higuchi et al., 1985; Ji et al., 1988; Hollander et al., 2000), although Oh-ishi et al. (1997) showed increased rat diaphragm Cu-Zn-SOD after 9 weeks of treadmill training. Increased SOD activity following training is primarily due to increased Mn-SOD, in both soleus and gastrocnemius muscles (Higuchi et al., 1985; Ji et al., 1988; Hollander et al., 2000; Nakao et al., 2000) and this would support mitochondrial respiration as a major source of ROS (Longo et al., 1996). Interestingly, increased SOD activity after training occurred despite no change in protein content or mRNA (Oh-ishi et al., 1997), suggesting that training induced adaptations are caused by post-translational mechanisms (Ji, 1999).

In humans, blood SOD activity is positively correlated to training (Ørtenblad et al., 1997) and $\dot{V}O_{2peak}$ (Jenkins et al., 1984). Similarly, muscle SOD activity is correlated to age, which may reflect an adaptation to increased generation of ROS in senescent muscle (Tonkonogi et al., 2000). An increased muscle SOD activity would allow the muscle to eliminate $O_2^{-\bullet}$ more effectively (Halliwell and Gutteridge, 1989) and thus potentially reduce the amount of oxidative stress in the exercising muscle. However, no change in SOD activity has been reported after high intensity (Hellsten et al., 1996) and submaximal training (Tiidus et al., 1996; Tonkonogi et al., 2000). The reason for this discrepancy is unclear, but may be related to the different isoenzymes of SOD as none of these studies have investigated the effects of training on the different isoenzymes of SOD.

2.12.2 Catalase

There is no consensus in the literature regarding the effects of training on skeletal muscle CAT activity. Several reports demonstrate no change in rat skeletal muscle (Ji, 1993; Leeuwenburgh et al., 1994; Powers et al., 1994; Tiidus et al., 1996; Plant et al., 2003), whereas others have reported a decreased muscle CAT activity after exercise training (Alessio and Goldfarb, 1988; Laughlin et al., 1990). Conversely, increased skeletal muscle CAT activity has also been demonstrated by others (Tiidus and Houston, 1994; Smolka et al., 2000). The only human study reported no change after 8 weeks of cycling exercise (Tiidus et al., 1996). The reasons for these discrepancies are not known and further research is required to evaluate the effects of chronic training on CAT activity, particularly in humans.

2.12.3 Glutathione peroxidase

There is a general consensus in the literature that training increases GPX activity as reviewed by (Powers et al., 1999) although these changes are directly related to exercise duration (Powers et al., 1994). An increased GPX activity facilitates the removal of hydrogen and organic peroxide during exercise and alleviates potential oxidative stress in skeletal muscle (Ji and Leeuwenburgh, 1995). Muscles showing a greater training adaptation typically have relatively low endogenous GPX activity (Ji and Leeuwenburgh, 1995). Although training increased cytosolic GPX, a greater increase was seen in the mitochondrial GPX fraction (Ji et al., 1988), thus allowing the removal of hydroperoxides from both the mitochondria and cytosol (Powers et al., 1999).

In humans, the effects of training on muscle GPX are intensity dependent as there is no change in GPX activity after submaximal training (Tiidus et al., 1996; Tonkonogi et al., 2000), but an increase following high intensity training (Hellsten et al., 1996; Ørtenblad et al., 1997). This suggests that short-term aerobic training increased the capacity for

flux through the citric acid cycle without necessarily increasing the capacity to handle potential ROS generated by the enhanced electron flux (Tiidus et al., 1996), although there is reduced antioxidant protection per individual mitochondrion (Tonkonogi et al., 2000). Similarly, high intensity exercise promotes a greater increase in GPX activity compared to moderate intensity exercise (Criswell et al., 1993; Powers et al., 1994) in rats.

2.12.4 Glutathione reductase

Although animal studies demonstrate no consistency on the effects of GR activity following training (Leeuwenburgh et al., 1994; Sen et al., 1992; Smolka et al., 2000; Atalay et al., 1996), consistency is shown in human studies. GR activity increased by 35% after 7 wk of high intensity intermittent cycle training (Hellsten et al., 1996) and was higher in jump trained than untrained subjects (Ørtenblad et al., 1997). Given that GR is primarily involved in the regeneration of GSH from GSSG, this would help minimise the rise in GSSG and assist in the maintenance of glutathione redox status.

In summary, there is a general consensus in the literature that chronic exercise training increases skeletal muscle endogenous antioxidant activity. The increased detoxifying capacity of the antioxidant enzymes is an important adaptive response to regular exercise and provides support for the argument that exercise increases ROS generation (Ji, 1993).

2.13 Reactive oxygen species, antioxidants and fatigue

The specific involvement of ROS in muscle fatigue has been established using ROS selective antioxidants as mechanistic probes (Reid, 1999). Antioxidants have only been proven to be effective in volitional exercise or simulation protocols that utilise low frequency stimulus patterns (Reid 2001). No effect of ROS has been identified in high

frequency fatigue (Reid, et al., 1992; Reid et al., 1994) or acute recovery (Shindoh et al., 1990; Reid et al., 1992; Reid et al., 1994).

Barclay and Hansel (1991) examined the effect of free radicals on fatigue in mouse soleus muscle stimulated in vitro at 70 Hz for 60 min. Addition of purine (0.29 mg/ml) and xanthine oxidase (0.01 U/ml) to produce free radicals increased the rate of fatigue two-fold. In the same study, canine gastrocnemius muscle was isometrically contracted at 4 Hz, with 10 μM dimethyl sulfoxide (DMSO), 1 mM allopurinol and 200 μM desferoxamine all resulting in a significantly slower rate of fatigue over 30 min compared to saline, presumably by inhibiting increased ROS.

In an *in vitro* study conducted by Reid et al., (1992), rat diaphragm bundles were stimulated to contract isometrically with the force production being measured at low and high frequencies. Selective anti-oxidants SOD (5×10^2 U/ml), CAT (1.8×10^4 U/ml), and DMSO (6.4 mM) were used to evaluate the role of the reactive oxygen intermediates $O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$ in acute fatigue, respectively. They found that ROS contributed to low frequency, but not high frequency fatigue of diaphragm fibre bundles since, ROS removal by selective anti-oxidants slowed the decline in force development. Furthermore, antioxidant protection became more evident as fatigue progressed, culminating in a 20% elevation of sustainable force when the protocol was terminated (Reid et al., 1992). In a companion study, Reid et al., (1992) used a similar preparation where the active fibre bundles were fatigued, reflected by a progressive decline in tetanic stress over a 1 hr period. The absorbance of reduced cytochrome *c* was greater in active than in passive muscle, an increase that was alleviated by the addition of SOD (10^3 U/ml). This suggests that the magnitude of fatigue was inversely related to the amount $O_2^{-\bullet}$ released.

The effect of two ROS scavengers (SOD and DMSO) on fatigue during prolonged electrically stimulated contraction of an *in vivo* canine diaphragm preparation were also studied (Supinski et al., 1997). In the control group, force declined to 24% of its initial value, whereas, in SOD and DMSO treated groups, force decreased to only 50 and 47% of baseline force, respectively. These data suggest that diaphragm fatigue resulting from repetitive low-frequency stimulation is associated with LIPOX within this muscle and that pre-treatment with ROS scavengers prevents LIPOX and reduces the rate of development of fatigue (Supinski et al., 1997). Ischaemia for 3 h, followed by 1 h of reperfusion, also produced a downward shift of the force-frequency relationship in canine diaphragm and markedly increased diaphragm fatigability, with fatigue prevented by DMSO (Supinski et al., 1993; Supinski et al., 1997). Furthermore, in saline treated animals, TBARS concentration was significantly higher when compared to diaphragm strips that were treated with SOD and DMSO (Supinski et al., 1997). These results indicate that the administration of selective free radical scavengers SOD and DMSO attenuates the rate of fatigue and can decrease the concentration of ROS as measured by TBAR concentration.

H_2O_2 can lead to the formation of $^{\bullet}OH$, which contributes to low frequency fatigue in rat diaphragm (Diaz et al., 1993). The effects of $O_2^{-\bullet}$, $^{\bullet}OH$ and H_2O_2 were studied on maximum calcium-activated force (F_{max}) in chemically skinned rat diaphragm fibers (Callahan et al., 2001). The xanthine/xanthine oxidase system was used to produce $O_2^{-\bullet}$ radicals, whilst 1 mM $FeCl_2$, 1 mM ascorbate and 1 mM H_2O_2 was used for $^{\bullet}OH$ production. Both $O_2^{-\bullet}$ and $^{\bullet}OH$ significantly decreased F_{max} by 14.5% and 43.9%, respectively, whilst there was no effect of 10 or 1,000 μmol H_2O_2 on F_{max}. Furthermore, peroxynitrate, which can be formed in the presence of $^{\bullet}OH$, also reduced F_{max} in rat single skinned diaphragm muscle fibres (Supinski et al., 1999). In contrast,

administration of oxypurinol, a xanthine oxidase inhibitor, did not reduce rat diaphragm fatigue following loaded breathing (Supinski et al., 1999), nor was xanthine oxidase generated-ROS responsible for fatigue in rat diaphragm during hypoxia or hyperoxia (Heunks et al., 2001).

Infusion of ferric chloride and iron ADP, a ROS-generating solution, in an *in situ* canine diaphragm preparation, significantly decreased diaphragm tension compared to saline (Nashawati et al., 1993). This decrease was prevented by concomitant administration of SOD (Nashawati et al., 1993). In rat costal diaphragm bundles, twitch and low-frequency tension at 10 min post fatigue was depressed with oxidative stress induced by xanthine oxidase and hypoxanthine (Lawler et al., 1997). This was mediated directly or indirectly through H_2O_2 and exacerbated by acidosis (Lawler et al., 1997).

The existing data clearly establishes a role for ROS-mediated oxidative stress in muscle fatigue (Reid, 1999). Furthermore, positive effects of anti-oxidants *in vivo* also indicate that oxidative stress may limit endurance during whole body exercise (Cazzulani et al., 1991; Novelli et al., 1991) as reviewed in section 2.23.1.

SECTION III:

MUSCLE NA^+,K^+-ATPASE, FATIGUE AND REACTIVE OXYGEN SPECIES

The Na^+,K^+-ATPase enzyme has been implicated as an important potential site contributing to muscle fatigue and is also susceptible to ROS. These are reviewed in the following section.

2.14 Structure and function of the Na^+,K^+-ATPase enzyme

The Na^+,K^+-ATPase enzyme actively transports 3 Na^+ out of the cell and 2 K^+ into the cell thereby opposing the K^+ and Na^+ fluxes across the cell membrane and is therefore

crucial in the maintenance of these concentration gradients and the muscle membrane potential (Clausen 1986).

The Na^+,K^+-ATPase is a transmembraneous protein expressed in all excitable cells and comprises an α and β subunit (Clausen 1986; McKenna 1999). The formation of the αβ complex is the minimal functioning unit. The α subunit is referred to as the catalytic subunit and contains binding sites for ligands and the inhibitors ouabain and digoxin (Jorgensen et al. 2003). The β subunit function is less clear but is required to facilitate assembly and membrane insertion (Geering 1990; Geering 1991; Hundal et al. 1994).

Seven isoforms of human Na^+,K^+-ATPase have been identified. The α subunit has four isoforms (α_1, α_2, α_3, α_4), whilst three β subunits are expressed (β_1, β_2, β_3). Our laboratory has recently reported expression of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms at both gene transcript and protein levels in human vastus lateralis muscle (Murphy et al. 2004).

2.15 Quantification of Na^+,K^+-ATPase

The best available method to quantify Na^+,K^+-ATPase content is via ^{3}[H]-ouabain binding, first performed by Hansen (1979). As reviewed by Clausen (2003), ^{3}[H]-ouabain binds stoichiometrically to specific receptors on the α-subunit of the Na^+,K^+-ATPase, allowing accurate quantification of the Na^+,K^+-ATPase content in intact skeletal muscle, biopsies and membranes. Furthermore, ^{3}[H]-ouabain bindings measures all functional Na^+,K^+-ATPase, as incubation in a buffer containing a saturating concentration of ouabain allows each Na^+,K^+-ATPase to tightly and specifically bind to one molecule of ^{3}[H]-ouabain (Clausen 1990). The ^{3}[H]-ouabain binding method is a robust measurement with values mainly ranging from 240 to 340 pmol/g wet wt in resting human skeletal muscle (Table 2.6). In rats, muscle fibre type may influence Na^+,K^+-ATPase content, being 20-30% higher in rat EDL muscle than in soleus muscle

(Clausen et al., 1982). Chin and Green (1993) reported lower Na^+,K^+-ATPase content in white vastus (238 pmol/g wet wt) compared to soleus (359 pmol/g wet wt), EDL (365 pmol/g wet wt) and red vastus lateralis (403 pmol/g wet wt). However, a fibre dependent effect has not been identified in human skeletal muscle (Benders et al., 1992; Madsen et al., 1994; Fraser et al., 2002).

Table 2.6 Resting human skeletal muscle Na^+,K^+-ATPase content, as determined by the ^{3}[H] ouabain binding site method.

Source	**n**	**Age (yr)**	**Gender (m/f)**	$\dot{V}O_{2peak}$ **(ml.kg^{-1}.min^{-1})**	**^{3}[H] ouabain (pmol^{-1}.g^{-1}.wet wt)**
Green et al., (1993)	9	NR	m		339
McKenna et al., (1993)	6	19	m	51.1	333
Madsen et al., (1994)	39	30	m	54.9	307
Evertsen et al.., (1997)	20	NR	11m/9f		343
Green et al., (1999)	9	19-27	m		326
Green et al., (1999)	7	21		44.4	278
Green et al., (1999)	9	20		45.3	289
Medbø et al., (2001)	8	19	m	58	425
Fowles et al., (2002)	14	23	7m/7f		~240
Fraser et al., (2002)	8	26	m	44.4	311
Fraser et al., (2002)	8	27	7m/1 f	43.8	302
Fraser et al., (2002)	8	26	m	67.6	357
McKenna et al., (2003)	8	38	5f/3m *	18.8	279
McKenna et al., (2003)	8	37	5f/3m	35.6	250
Aughey et al. (2004)	6	25	m	69.5	324
Leppik et al.(2004)	8	28	7m/1 f	53.0	332
				min	240
				max	425
				mean±SEM	320±11

* subjects were recipients of lung transplants; blank, not reported.

2.16 Measurement of Na^+,K^+-ATPase activity

To fully quantitate muscle Na^+/K^+ exchange capacity it is necessary to measure Na^+,K^+-ATPase content and maximal activity. Na^+,K^+-ATPase activity can be measured via radiolabelled ion fluxes (Clausen et al., 1987), but this method cannot be used in human muscle obtained by needle biopsy. Other *in vitro* measurements may be employed to measure Na^+,K^+-ATPase activity including the accumulation of inorganic phosphate (Bonting, 1971). However, it is not possible to detect Na^+,K^+-ATPase activity by Pi liberation due to the low sample obtained by needle biopsies and the high total ATPase activity relative to Na^+,K^+-ATPase activity in either human or rat skeletal muscle (Benders et al., 1992; Fraser and McKenna, 1998; Fowles et al., 2004). Consequently, the activity of related K^+ dependent phosphatases, including p-nitrophenylphosphatase activity (pNppase; Hundal et al., 1994) or 3-*O*-methylfluorescein phosphatase activity (3-*O*-MFPase; Kjeldsen 1988; Fraser and McKenna 1998) may be used. The K^+-stimulated 3-*O*-MFPase *in vitro* assay has a 2-3 times higher sensitivity, thus requiring 50-100 times less tissue, than the K^+ stimulated pNpp assay (Nørgaard, 1986). Furthermore, our laboratory has demonstrated that this assay is specific for Na^+,K^+-ATPase through abolition of 3-*O*-MFPase activity by ouabain (Fraser and McKenna 1998). The 3-*O*-MFPase activity has also been optimised to measure Na^+,K^+-ATPase activity in human muscle obtained by needle biopsies (Fraser and McKenna 1998).

There is still limited data utilizing the 3-*O*-MFPase assay to determine Na^+,K^+-ATPase activity in human skeletal muscle (Table 2.7). However, results from our laboratory (Fraser and McKenna, 1998; Fraser et al., 2002; McKenna et al., 2003; Aughey et al., 2004; Leppik et al., 2004) are in agreement with others (Fowles et al., 2002). Human skeletal muscle resting Na^+,K^+-ATPase activity ranges from 168 to 292 $nmol.min.^{-1}.g^{-1}$ wet wt.

Table 2.7 Maximal Na^+,K^+-ATPase activity in resting human skeletal muscle as determined by the *in vitro* 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity assay.

Source	n	Age (yr)	Gender (m/f)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	3-*O*-MFPase activity *	3-*O*-MFPase activity **
1	7	30	m		292	1745
2	14	23	7m/7f			1350
3	8	26	m	44.4	207	1199
	8	27	7m/1 f	43.8	229	1295
	8	26	m	67.6	249	1359
4	8	37	5f/3m	35.6	168	1219
5	6	25	m	69.5	265	1600
6	8	28	7m/1 f	53.0	288	1713
7	10	20	7m/3f	42.1	~234	~1200

Reference: 1, Fraser and McKenna (1988); 2, Fowles et al., (2002); 3, Fraser et al., (2002); 4, McKenna et al., (2003); 5, Aughey et al. (2004); 6, Leppik et al. (2004); 7, Sandiford et al., (2004). * ($nmol.min.^{-1}.g^{-1}$ wet wt); **($pmol.min^{-1}.mg^{-1}$.protein). Blank, not reported.

2.17 Activation of Na^+,K^+-ATPase enzyme

The Na^+,K^+-ATPase enzyme activity is low at rest (Clausen, 2003). However, during periods of contractile activity there is a rapid activation of the Na^+,K^+-ATPase enzyme, induced by electrical, ionic and hormonal factors (Clausen, 1986; Clausen, 2003). The following section provides a brief overview of Na^+,K^+-ATPase enzyme activation.

2.17.1 Electrical Activation

Electrical stimulation may increase Na^+,K^+-ATPase enzyme activity by 10-to-20 fold above resting activity (Nielsen, 1998). Stimulation of isolated rat soleus muscle at 2 Hz increased the rate of ^{3}[H]ouabain binding by approximately 120% within 1 min,

indicating an early specific activation of the Na^+,K^+-ATPase enzyme (Everts and Clausen, 1994). The activation of Na^+-K^+-ATPase was proportional to the stimulation frequency, with maximal theoretical activation nearly achieved at 120 Hz. Other studies also report very large increases in Na^+,K^+-ATPase activity as a result of electrical stimulation in mouse (Juel, 1986) and rat skeletal muscle (Nielsen and Clausen, 1996; McKenna et al., 2003).

2.17.2 Sodium and potassium activation

Sejersted and Hallèn (1985) demonstrated that Na^+,K^+-ATPase activity increased linearly with increased intracellular [Na^+]. However, Na^+,K^+-ATPase activity can be increased in the absence of increased [Na^+], indicating the presence of other factors (Everts and Clausen, 1994). Although increased extracellular [K^+] also activated the Na^+-K^+-ATPase enzyme, the contribution during exercise is almost non-existent, as the extracellular site is already saturated (Sejersted and Hallèn, 1985).

2.17.3 Hormonal Activation

Numerous hormones, including catecholamines, insulin, aldosterone, thyroid hormones, calcitonin gene related peptide and insulin like growth factor also activate the Na^+,K^+-ATPase enzyme, although these effects are small relative to those of membrane excitation (see Clausen (2003) for extensive review).

An increase in catecholamines increased resting pump activity in isolated rat soleus and extensor digitorium muscle but these effects were not additive to stimulation (Everts et al., 1988). Although insulin also increased Na^+,K^+-ATPase activity in rat soleus muscle (Clausen and Everts 1989), these effects were not as pronounced as catecholamine activation and also had a slower time course (Everts and Clausen, 1994).

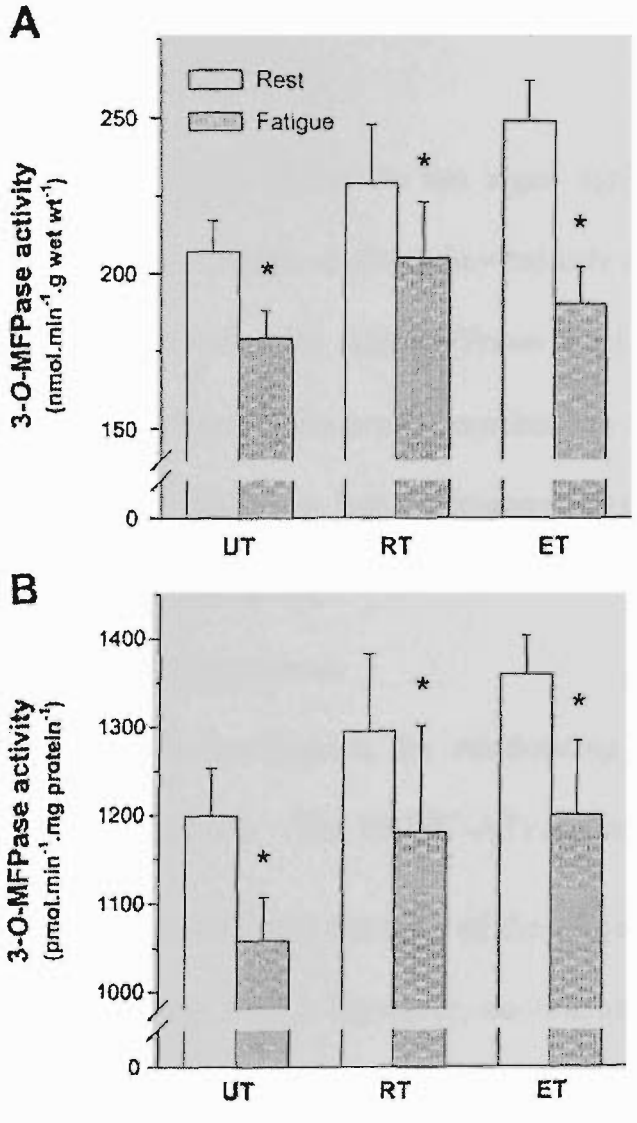
2.18 Na^+,K^+-ATPase and muscle contractility

Clausen et al., (1993) demonstrated in isolated rat soleus muscle that exposure to 12.5 mM K^+ decreased force by 96%. The inhibitory effect of high [K^+] on contractility in skeletal muscle could be counterbalanced by stimulation of active electrogenic Na^+-K^+ transport, the ensuing increase in the clearance of extracellular K^+ and in the transmembrane electrochemical gradient for Na^+ (Clausen et al., 1993). The addition of salbutamol, adrenaline and insulin produced 57-71%, 61-71% and 38-47% recovery of force within 10-20 min, respectively (Clausen et al., 1993). Furthermore, the effects of salbutamol and insulin on force recovery were additive. Finally, the force recovery induced by salbutamol, adrenaline and insulin was suppressed by pre-exposure of the muscle to the Na^+,K^+-ATPase inhibitor ouabain.

2.19 Depressed human Na^+,K^+-ATPase pump activity with fatigue

Skeletal muscle Na^+,K^+-ATPase activity increased markedly above rest as a consequence of contractile activity (Everts and Clausen, 1994; McKenna et al., 2003). This is also evident in human muscle with the rapid post-exercise decline in plasma [K^+] (Sejersted and Sjøgaard, 2000). However, the maximal Na^+,K^+-ATPase activity is depressed at the point of fatigue during dynamic exercise. Fraser et al. (2002) found maximal 3-*O*-MFPase activity was depressed at fatigue by ~14% following 50 maximal isokinetic contractions in untrained, endurance trained and resistance trained individuals (Figure 2.5). There were no differences between groups, suggesting that depressed maximal Na^+,K^+-ATPase activity may be an obligatory response and an important determinant of fatigue.

Figure 2.5 Depressed skeletal muscle maximal *in vitro* 3-*O*-MFPase activity with fatigue induced by 50 maximal knee extensions in untrained (UT), resistance trained (RT) and endurance trained (ET) subjects. *A*: activity expressed per gram weight wet. *B*: activity expressed per milligram protein. * Main effect of exercise, fatigue < rest.



From Fraser et al., 2002.

Decrements in maximal Na^+,K^+-ATPase activity have been found during various exercise intensities and duration. Isometric, single-leg extension contractions at ~60% of maximal voluntary contraction for 30 min resulted in a 38% decrease in maximal Na^+,K^+-ATPase activity (Fowles et al., 2002). Incremental cycling exercise decreased 3-*O*-MFPase activity by ~12% in well-trained individuals, both before and after 20 nights sleeping in simulated altitude conditions (Aughey et al., 2004). Cycling exercise

at ~75% $\dot{V}O_{2peak}$ until fatigue decreased muscle maximal 3-*O*-MFPase activity by ~7% at 10 min, ~11% at 45 min and ~13% at fatigue (Leppik et al., 2004). The reduction in Na^+,K^+-ATPase activity was associated with loss of excitability as indicated by decreased M-wave amplitude by some authors (Fowles et al., 2002) but not others (Sandiford et al., 2004).

It is important to note that these studies do not argue against an increased Na^+,K^+-ATPase activity as a result of exercise. Rather, they indicate a reduction in the maximal attainable Na^+,K^+-ATPase activity with fatigue (Fraser et al., 2002). This may be an important factor in muscle fatigue. The precise mechanism for the depressed Na^+-K^+-ATPase activity at fatigue is unknown but the accumulation of ROS is an attractive possibility, as discussed in section 2.22.

2.20 Na^+,K^+-ATPase and performance.

Relatively few studies have investigated the relationship between Na^+,K^+-ATPase function and muscle performance. The Na^+,K^+-ATPase content correlated with the $\dot{V}O_{2max}$, 20-min treadmill run time and the rank of the subjects' performance as cross-country skiers (Evertsen et al., 1997). However, no relationship was found between Na^+,K^+-ATPase content and exercise time to exhaustion at 86% of $\dot{V}O_{2max}$ (Madsen et al., 1994), repeated sprint performance (McKenna et al., 1993), maximal squat lifting (Medbø et al., 2001) or 10 km running time (Overgaard et al., 2002).

2.21 Na^+,K^+-ATPase and training

2.21.1 Na^+-K^+-ATPase activity

There are limited data regarding the effects of exercise training on skeletal muscle Na^+,K^+-ATPase activity, in both animal models and humans. A 165% increase in Na^+,K^+-ATPase enzyme activity was found after 6 weeks of treadmill training (Knochel et al., 1985). This was measured in isolated membranes from canine gracilis muscle

(Knochel et al., 1985) and the method to determine Na^+-K^+-ATPase activity utilised in this study has been criticized due to poor membrane recovery (Hansen and Clausen, 1988). Little is known about exercise training on human skeletal muscle Na^+,K^+-ATPase enzyme activity. The maximal *in vitro* 3-*O*-MFPase activity in resting muscle tended to be 20% higher in the endurance trained compared to untrained individuals (Fraser et al., 2002). Furthermore, maximal Na^+,K^+-ATPase activity increased by 41% after 6 days cycle training for 2 h per day at 60-65% $\dot{V}O_{2peak}$ (Green et al., 2004).

2.21.2 Na^+,K^+-ATPase content

The first study to investigate the effects of exercise training on Na^+,K^+-ATPase content demonstrated a 46% increase in rat hindlimb muscle after 6 weeks of swim training (Kjeldsen et al., 1986). A similar change was seen in Na^+,K^+-ATPase content with training in soleus and plantaris muscles in chronic heart failure rats (Helwig et al., 2003). Leivseth et al., (1992) demonstrated a 25% increase in Na^+,K^+-ATPase guinea pig skeletal muscle after 3 weeks running training. An almost identical increase was observed in thoroughbred middle gluteal muscle after 10 days running training at 55% $\dot{V}O_{2max}$ (McCutcheon et al., 1999). Other equine studies also demonstrated an increased Na^+,K^+-ATPase content after 5 months sprint training (Suwannachot et al., 1999). Veeneklaas and colleagues (2002) also demonstrated a gender effect. After a 15 days of training, bovine semitendinous Na^+-K^+-ATPase content increased by ~30% in females, compared to only ~16% in males.

Chronic electrical stimulation of animal muscle also resulted in increased skeletal muscle Na^+,K^+-ATPase content. Green et al., (1992) demonstrated a 40% increase in rabbit EDL Na^+,K^+-ATPase content after 4 d of stimulation at 10 Hz. Similarly, 10 Hz stimulation of rabbit tibialis anterior muscle increased Na^+,K^+-ATPase content by 60% after 6 d and by 113% after 20 days (Hicks and McComas, 1989).

The first study in humans found no change in Na^+,K^+-ATPase content after 10 weeks of moderate physical activity in army conscripts (Kjeldsen et al., 1990). However, several investigators have subsequently reported an increased Na^+,K^+-ATPase content after physical training. Untrained individuals completing 7 weeks of sprint cycling exercise demonstrated a 16% increase in vastus lateralis Na^+,K^+-ATPase content (McKenna et al., 1993), which is identical to the change reported after 5 months of training in elite junior cross-country skiers (Evertsen et al., 1997). Furthermore, 6 day cycling training at 60% $\dot{V}O_{2max}$ (Green et al., 1993), 6 weeks training at 78% maximum heart rate in moderately endurance trained individuals (Madsen et al., 1994) and 3 months resistance training in elite alpine skiers (Medbø et al., 2001) all increased Na^+,K^+-ATPase content by 13 to 15%. Green et al., (1999) showed that 11 weeks of prolonged or resistance training each increased vastus lateralis Na^+,K^+-ATPase content by 29 and 17% respectively in healthy untrained individuals. Therefore, despite the vastly different training protocols and initial training status, the increases in Na^+,K^+-ATPase content are all within the range of 13 to 29%, indicating that increases in this enzyme are tightly regulated.

Other investigators have also compared the Na^+,K^+-ATPase content in trained versus untrained individuals. Endurance trained individuals had a 17% higher vastus lateralis Na^+,K^+-ATPase content than untrained individuals (Fraser et al., 2002). Similarly, long-term swim, run and strength trained individuals had a 30, 32 and 40% higher Na^+,K^+-ATPase content than untrained individuals (Klitgaard and Clausen, 1989). Interestingly, following a 100 km ultra marathon lasting 10.7 hours, there was a 13% increase in Na^+,K^+-ATPase content, suggesting that long-lasting exercise promoted Na^+,K^+-ATPase synthesis (Overgaard et al., 2002). Furthermore, all six of the Na^+,K^+-ATPase gene transcripts expressed in human skeletal muscle were elevated after ~6

minutes of fatiguing, isokinetic, one-legged knee extensor exercise (Murphy et al., 2004).

Chronic exercise training and electrical stimulation of animal and human muscle results in increased skeletal muscle Na^+,K^+-ATPase content. Furthermore, the increased content seems to be independent of the mode, intensity and duration of the training protocol. Na^+,K^+-ATPase upregulation after training would most likely reduce muscle K^+ loss and Na^+ gain, thereby minimising fatigue and enhancing muscle performance (McKenna, 1999). Increased ROS adversely affect Na^+,K^+-ATPase activity, which may therefore have important implications in fatigue, as discussed in the next section.

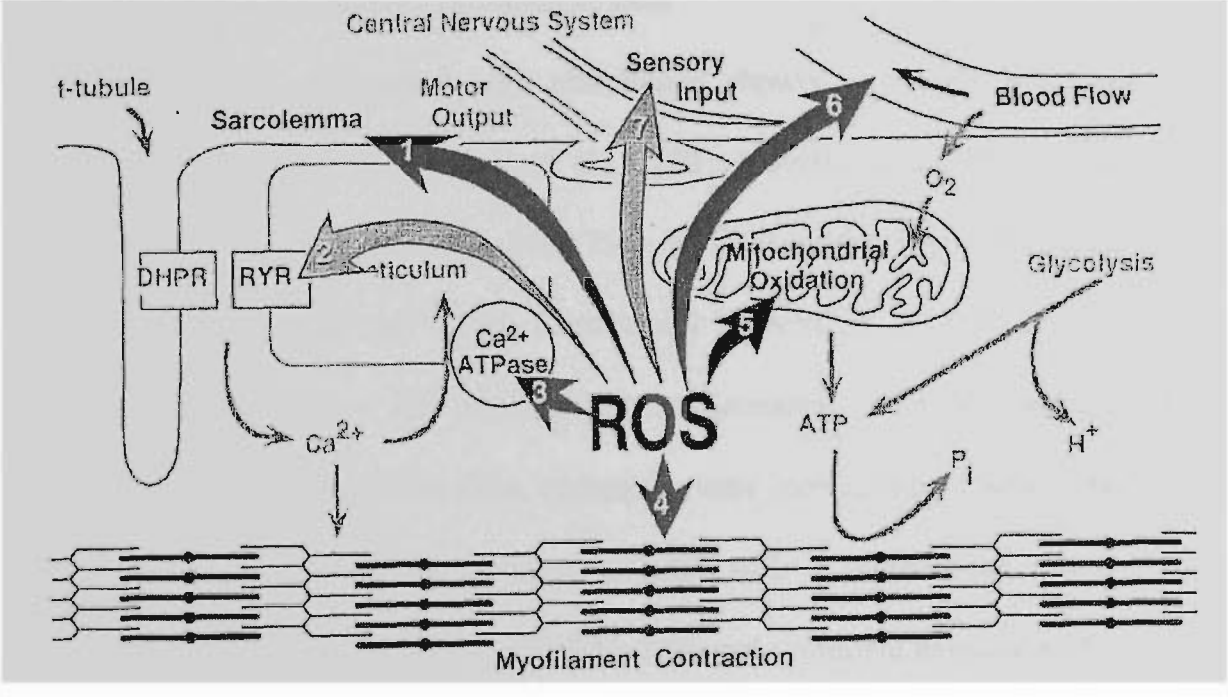
2.22 Na^+,K^+-ATPase and reactive oxygen species

Studies investigating the effects of ROS on Na^+,K^+-ATPase have largely examined cardiac muscle. Na^+,K^+-ATPase activity was inhibited by H_2O_2 and dependent on both concentration and time of incubation, with 10 mM H_2O_2 inhibiting 80% of Na^+,K^+-ATPase activity at 90 min in canine myocardium sarcolemmal vesicles (Kukreja et al., 1990). With the addition of 5 mM H_2O_2 and ferrous iron (Fe^{2+}), thus producing $^{\bullet}OH$, there was an approximate 40% inhibition of the maximum binding of ouabain, which was attenuated by the $^{\bullet}OH$ scavenger mannitol (Kukreja et al., 1990). Singlet oxygen also inhibited canine cardiac sarcolemmal Na^+,K^+-ATPase activity in a dose-dependent manner, which was attenuated by histidine (Vinnikova et al., 1992). Kim and Akera (1987) also found that SOD (100 U/mol), CAT (150 U/ml), DMSO (50 mM), histidine (10 mM) and allopurinol (1 mM) each attenuated the decrease in ischaemia-reperfusion induced reduction in Na^+,K^+-ATPase activity in guinea pig sarcolemma.

In rat skeletal muscle, *in vitro* Na^+,K^+-ATPase activity was depressed by ~70% following incubation in a ROS generating system which was not specified (Clough, 1994). Tert-butyl hydroperoxide (TBOOH) activated the Na^+,K^+-ATPase enzyme in

skeletal muscle derived L6 cells (Sen et al., 1995), but when the concentration of TBOOH was increased to 75 and 200 mM there was a significant inhibition of the Na^+,K^+-ATPase enzyme, suggesting that this enzyme is under redox modulation. Thus, the Na^+,K^+-ATPase enzyme and SR function are adversely affected by ROS and have a likely role in muscle fatigue (Figure 2.6). However the data is limited in skeletal muscle and the effects in human skeletal muscle remain to be elucidated.

Figure 2.6 Potential sites of ROS on skeletal muscle fatigue. Diagram illustrates aspects of muscle contraction that ROS may inhibit (solid arrows) or exaggerate (shaded arrows)



1, sarcolemmal function; 2, calcium release from the SR; 3, calcium re-uptake by the SR Ca^{2+}-ATPase; 4, myofilament contraction; 5, mitochondrial oxidation; 6, vascular control; 7, afferent feedback to the CNS. DHPR, dihydropyridine-sensitive voltage sensor; RYR, ryanodine-sensitive SR calcium release channel; Ca^{2+}-ATPase, SR calcium-dependent ATPase; Ca^{2+} calcium; ATP, Adenosine 5′ triphosphate; P_i, inorganic phosphate; H^+, hydrogen ion; O_2, molecular oxygen.

From Reid, 2000.

SECTION IV: MANIPULATION OF REDOX STATUS AND EXERCISE

2.23 Pharmacological manipulation of redox status and exercise performance

Positive results have been found using pharmacological antioxidants as experimental probes (Reid, 1999). Although nutritional antioxidants including vitamins A, C and/or E result in decreased markers of ROS formation (Dillard et al., 1978; Di Mascio et al., 1991; Kanter et al., 1993; Viguie et al., 1993; Ashton et al., 1999), the consensus in the literature is that vitamin supplementation has no ergogenic effect, as reviewed elsewhere (Evans, 2000; Sen, 2001).

2.23.1 Exogenous glutathione supplementation

Mice that were supplemented with glutathione showed increased endurance during exhaustive swimming (Cazzulani et al., 1991; Novelli et al., 1991). Glutathione administered as a single dose at 500, 750, or 1000 mg/kg i.p, increased swimming endurance by 102, 120 and 140%, respectively (Novelli et al., 1991). Although 250 mg/kg as a single dose did not improve performance, when the same dose was administered over 7 successive days, swimming time increased by 104% (Novelli et al., 1991). Three months supplementation (20g/d) with a whey-based cysteine donor (Immunocal) increased lymphocyte GSH by 35% and volitional exercise performance in humans (Lands et al., 1999). Both peak power and 30 s work capacity were increased by 13% with Immunocal, with no changes observed in the placebo group (Lands et al., 1999).

These studies demonstrating that exogenous glutathione supplementation may increase performance, failed to investigate direct mechanism(s). Hence, it cannot be concluded that exogenous glutathione attenuated oxidative stress (Sen and Packer, 2000).

Therefore, further research is required to establish whether glutathione alters redox status during exercise and improves volitional performance, particularly in humans.

2.23.2 Induced glutathione deficiency and exercise performance

The physiological role of glutathione is clearly evident when tissues deprived of glutathione, hence with a reduced antioxidant capacity, are subjected to an oxidative challenge (Ji and Leeuwenburgh, 1995). Methods employed to deplete tissue glutathione levels include diethylmaleate (DEM), buthioninie SR-sulfoximine (BSO) and food deprivation.

DEM depleted intracellular glutathione (Deneke and Fanburg, 1989) and decreased swimming exercise performance in rats (Kramer et al., 1993), although no mechanisms were studied. However, Morales and colleagues (1993) injected rats with DEM (1 mg/kg) and then applied resistive breathing until they were unable to maintain 70% of their maximal airway opening pressure. Maximum tetanic tension (P_o) and twitch tension (P_t) were significantly depressed, suggesting that glutathione may play an important role in protecting the diaphragm against oxidative stress (Morales et al., 1993).

BSO also decreased glutathione in various tissues (Griffith and Meister, 1979). Rats intraperitoneally injected with BSO had an ~80% decrease in TGSH in various tissues including red gastrocnemius muscle, which resulted in an ~50% reduced endurance time (Sen et al., 1994). This suggests a critical role of endogenous GSH in the circumvention of exercise-induced oxidative stress and as a determinant of exercise performance (Sen et al., 1994). Chronic BSO treatment also exacerbated the exercise-induced declines in liver and kidney GSH, as well as decreased muscle GSH by 25% (Leeuwenburgh and Ji, 1995). Therefore, glutathione homeostasis is essential for the prooxidant-antioxidant balance during prolonged physical exercise (Leeuwenburgh and Ji, 1995).

Food deprivation also decreased skeletal muscle GSH (Tateishi et al., 1977; Cho et al., 1981; Lauterburg et al., 1984). Consistent with these studies, food deprivation decreased liver glutathione content and GSH:GSSG ratio in skeletal muscle (Leeuwenburgh and Ji, 1996). Interestingly, MDA levels were also higher in starved rats suggesting increased ROS formation. However, there was no exacerbation of the exercise-induced effects on redox status with food deprivation (Leeuwenburgh and Ji, 1996).

2.23.3 Selenium deficiency

Selenium deficient tissues are more vulnerable to oxidative stress (Ji et al., 1988). Therefore, it is surprising that few studies have investigated selenium deficiency, glutathione metabolism and exercise performance. Although GPX activity is dependent upon selenium, there is no evidence that selenium deficiency impairs exercise performance. Furthermore, skeletal muscle antioxidant enzymes are capable of adapting to selenium deficiency and exercise to minimise oxidative damage caused by ROS (Ji et al., 1988).

Brady et al. (1979) reported increased oxidative stress with selenium deficiency in rats swum to exhaustion. Selenium deficiency in rats had no effect on performance, despite an 80% decrease in liver and skeletal muscle GPX (Lang et al., 1987). Hence, residual GPX activity may be sufficient to prevent the possible impairment of exercise endurance capacity (Lang et al., 1987). In contrast, selenium supplementation increased plasma GPX activity in healthy humans but did not increase maximal aerobic power and capacity (Tessier et al., 1995).

2.24 *N*-acetylcysteine as an antioxidant

N-acetylcysteine has been used extensively in clinical toxicology and its major use is in the treatment of acetaminophen (paracetamol) overdose (Flanagan and Meredith, 1991). NAC has been shown to reduce a number of different oxidants that are produced during

metabolism at varying rates (Aruoma et al., 1989). NAC is a powerful scavenger of $^{\bullet}OH$ and at low concentrations, NAC can be a powerful scavenger of hypochlorus acid. NAC is a poor scavenger of $O_2^{-\bullet}$, with a rate constant of $< 10^3\ M^{-1}s^{-1}$ at pH 7.4 (Aruoma et al., 1989).

2.25 *N*-acetylcysteine pharmacokinetics

The pharmacokinetics of NAC were determined in healthy humans, who received either intravenous (600 mg) or oral NAC (600 mg) in four different tablets (Borgstrom et al., 1986; Table 2.8). Intravenous (i.v.) NAC infusion resulted in a 20-fold greater peak plasma concentration (C_{max}) of NAC compared to oral NAC (~300 $\mu mol.L^{-1}$ vs ~16 $\mu mol.L^{-1}$). Of the orally administered NAC, the fast dissolving effervescent tablet had the highest C_{max} (16.9 $\mu mol.L^{-1}$) and the slow dissolving tablet the lowest C_{max} (4.7 $\mu mol.L^{-1}$). The bioavailability for the oral NAC varied between only 6 and 10%, with the fast dissolving tablet having the highest bioavailability (Borgstrom et al., 1986).

Olsson et al. (1988) administered i.v. and oral NAC at a lower dose than Borgstrom et al. (1986), resulting in much lower C_{max}, with the highest [NAC] reported at 162 $\mu mol.L^{-1}$ (Table 2.8). Following a 7 day washout period, the same subjects were administered with 400 mg oral NAC as effervescent tablets, with results indicating a C_{max} of ~10 $\mu mol.L^{-1}$. From these studies and others listed in Table 2.8 it is evident that i.v. administration results in a 17-60 fold higher plasma [NAC] and has increased bioavailability compared to oral doses.

2.25.1 Reduced vs Total

Olsson and colleagues (1988) also compared the pharmacokinetics of reduced and total NAC following i.v. and oral administration. After the oral dose, the bioavailability of reduced NAC was 4.0% compared to 9.1% for total NAC. The lower bioavailability of reduced NAC compared with total NAC indicate that NAC is rapidly oxidised before it

reaches the general circulation, probably in the gastrointestinal tract (Olsson et al., 1988; Holdiness, 1991). The low bioavailability of NAC is probably not due to incomplete absorption, but rather extensive first pass metabolism in the gut wall and liver (Olsson et al., 1988; Flanagan and Meredith, 1991; Holdiness, 1991).

2.26 Adverse reactions with *N*-acetylcysteine

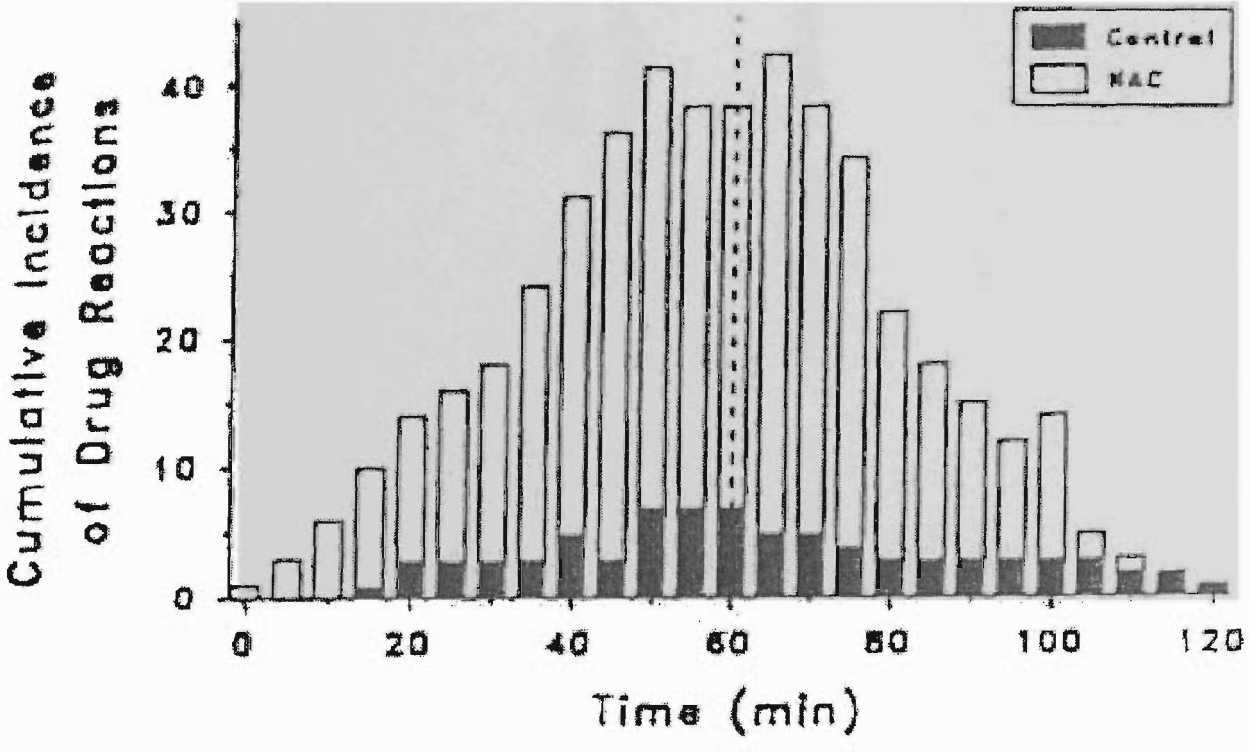
The most common side effects associated with NAC are vomiting and diarrhoea (Holdiness, 1991). Following NAC overdose, adverse reactions observed are similar but much more severe, and fatalities have occurred. The treatment of NAC toxicity is directed at the reversal of anaphylactic reactions and/or controlling nausea and vomiting (Flanagan and Meredith, 1991). Other side effects have also been reported with NAC including dyspepsia, pruritus and dysphoria (Table 2.8). These have been treated with anti-histamines, corticosteroids, salbutamol and infusion cessation.

For NAC treatment of paracetamol overdose, adverse reactions were said to occur in 9% of patients (Flanagan and Meredith, 1991). However, the side effects experienced by patients may be confused with the effects of paracetamol intoxication (Holdiness, 1991). NAC administered during varying treatment programs of 10 days to 6 months and in varying daily doses of 300 to 600 mg have been well tolerated (Boman et al., 1983) and without major side effects (Bonanomi and Gazzaniga, 1980; Ferrari, 1980; Saraiva et al., 1980; Cotgreave et al., 1987; De Vries and De Flora, 1993).

When NAC is given to healthy volunteers in small doses (eg. 600 mg) no side effects have been reported (Table 2.8). However, when subjects were infused with an NAC bolus dose of 150 mg/kg (Reid et al. 1994), numerous adverse reactions were reported (Table 2.8). However, at a similar dose and when subjects were orally pre-medicated with 25 mg of diphenhydramine and 150 mg rantidine to decrease the incidence of anaphylactoid side effects of NAC, no significant adverse reactions were reported

(Travaline et al., 1997). A reduction in the loading dose of NAC may reduce the risk of adverse reactions while still maintaining efficacy (Flanagan and Meredith, 1991). Similarly, Reid et al., (1994) demonstrated that side effects were time, and thus presumably dose, dependent during i.v. bolus infusion in healthy volunteers (Figure 2.7). Therefore, although a low oral NAC dose may be effective in reducing the incidence of adverse reactions, the low bioavailability would allow limited effects during exercise. Conversely, increasing the dose would also increase the incidence of adverse reactions, thus possibly precluding voluntary exercise.

Figure 2.7 Time course of reactions to intravenous NAC infusion (150 mg/kg) in healthy humans.



0 = start of NAC infusion; 60 = start of electrical stimulation. From Reid et al., 1994

Table 2.8. The incidence of adverse reactions following NAC administration in healthy and disease individuals.

Reference	n	Subjects	Route	Dosage	Duration	Adverse reactions
Borgstrom et al. (1986)	10	healthy	i.v.	600mg	5 min	
Olsson et al. (1988)	6	healthy	i.v.	200mg	1 min	
Reid et al., (1994)	10	healthy	i.v.	150 mg/kg	60 min	conjunctival irritation (11); dysphoria (8)[f]; cough (7); light-headiness (6); palmar erythema (6); facial erythema (5); dyspepsia (5); nausea (4); pruritus (3)
Travaline et al., (1997)	4	healthy	i.v.	150 mg/kg	60 min	flushing (2); pruritus (2); nausea (2)
Mant et al., (1984)	127	paracetamol overdose	i.v.	clinical dose†	single dose	Pruritus (29); nausea (8); angiodema [‡] (8); hypotension (6); bronchospasm (5); flushing (3); tachycardia (3)
Dawson et al., (1989)	29	paracetamol overdose	i.v.	clinical dose†	single dose	Rash (26); pruritus (16); angiodema (9); nausea (9); bronchospasm (8); hypotension (3); hypertension (2); nausea (2)
Sen et al., (1994)	9	healthy	oral	4 x 200 mg /d	2 d (800mg on day of test)	none
Borgstrom et al. (1986)	40	healthy	oral	600mg	single dose	
Bridgeman et al. (1994)	6	healthy	oral	600mg	5 d	none

Cotgreave et al., (1987)	6	healthy	oral	2 x 200 mg / d	2 wk	none
DeCaro et al. (1989)	12	healthy	oral	200mg	single dose	none
	12	healthy	oral	600mg	single dose	none
Maddock (1980)	20	healthy	oral	400mg	single dose	
	10	healthy	oral	3 x 200 mg	1d	
Olsson et al. (1988)	6	healthy	oral	400mg	single dose	
Bridgeman et al. (1994)	6	COPD	oral	600mg	5 d	none
DeVries et al., (1993)	372	cancer	oral	600 mg / d	2 yr	none in 79%; detectable in 14%; poorly tolerated in 3%, unbearable in 3%
Boman et al., (1983)	98	chronic bronchitis	oral	2 x 200 mg / d	6 months	21-GI symptoms * (21); diarrhoea * (7); nausea * (5); stomach pain # (2); oedema # (1); dyspnoea # (1)
Rodenstein et al., (1980)	10	respiratory disease	oral	100 mg	single dose	none
Saraiva et al., (1980)	744	respiratory disease	oral	20 mg / kg /d	1-119 months	none

Key:

side effects caused withdrawal from the study; * side effects not causing withdrawal from the study; ** actual side effects none stated. Report the most common side effects were headache and dyspepsia; † clinical dose is initial 150mg/kg NAC followed by varying doses depending on the severity of overdose; ‡ no life threatening laryngeal involvement reported; GI = gastro-intestinal tract, blank, not reported.

2.27 *N*-acetylcysteine effects on cysteine and glutathione

Cysteine supplementation is undesirable as moderate doses are toxic (Meister, 1991), which would potentially preclude voluntary exercise. To overcome the toxic effects of cysteine, analogous forms of cysteine have been used (Ji and Leeuwenburgh, 1995).

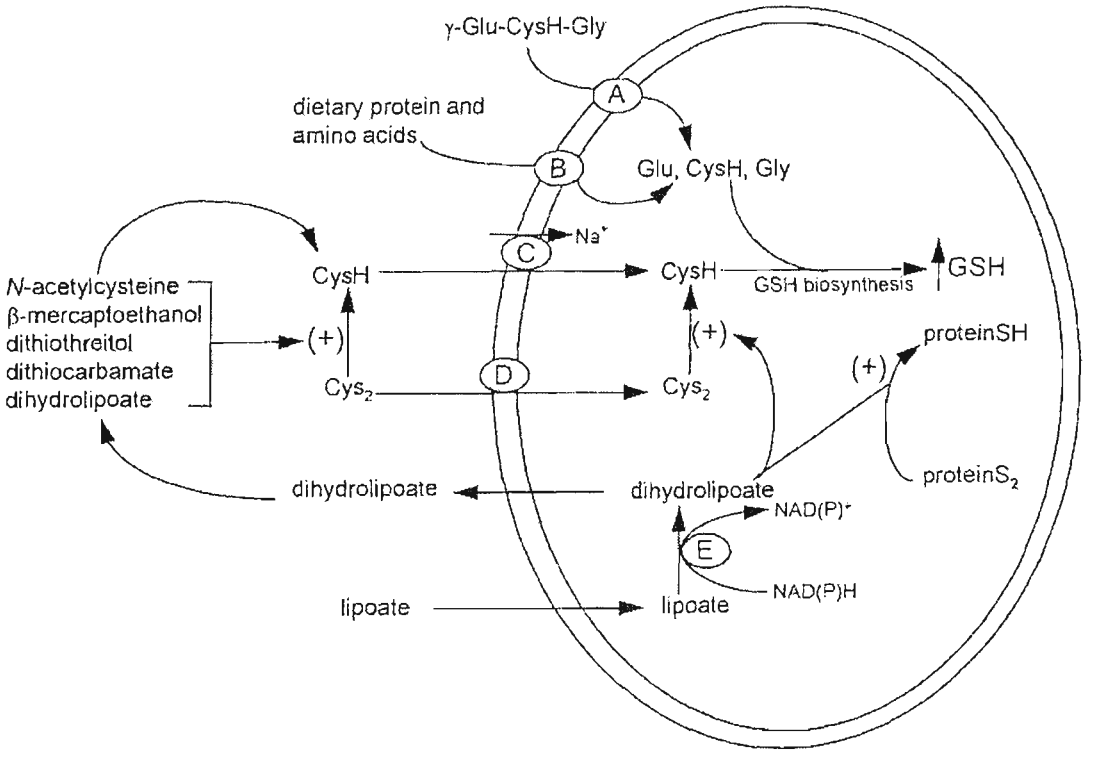
Oral NAC supplementation increased plasma cysteine and glutathione, with no further accumulation of cysteine or glutathione after multiple doses (Bridgeman et al., 1994). The increase in plasma cysteine and glutathione is an important factor when considering NAC's anti-oxidant capacity. The major mode of action for NAC to act as an anti-oxidant is by increasing circulating cysteine and glutathione levels (Issels et al., 1988) and the availability of cysteine is rate limiting in the biosynthesis of glutathione (Cotgreave, 1997). Glutathione is a major anti-oxidant that de-toxifies H_2O_2 and is also rapidly oxidised during exercise. The administration of NAC could potentially aid the biosynthesis of glutathione and increase the antioxidant potential of muscle. Since NAC is also able to increase circulating glutathione levels (Olsson et al., 1988; De Caro et al., 1989; Holdiness, 1991; Bridgeman et al., 1994), NAC may act as a direct precursor to glutathione biosynthesis (Figure 2.8).

Few studies have investigated the effects of NAC supplementation on glutathione during an acute exercise bout. Rats supplemented with NAC, glutathione and vitamin C for 20 d prior to an acute exercise bout had an attenuated increase in blood GSSG (Sastre et al., 1992). Similarly, in rats exercised to exhaustion, intraperitoneal-injected NAC attenuated the exercise-induced increases in blood and lung GSSG/TGSH, although this was not observed in liver, red gastrocnemius muscle, heart or plasma (Sen et al., 1994).

Sen et al. (1994) tested the effect of orally administered NAC (200 mg/d, 2 d) on exercise associated blood glutathione oxidation in subjects performing two identical

maximal cycle ergometer exercise tests. Nine men took four NAC tablets (200 mg) per day (two 50 mg tablets, twice per day) for two days. NAC increased the ROS scavenging capacity of the plasma, as measured by TBARS, indicating that the antioxidant properties of the drug may augment the net antioxidative capacity of the plasma. Blood glutathione oxidation with exercise, reflected by an increase in blood GSSG and GSSG/TGSH ratio, was significantly attenuated by NAC (Sen et al., 1994). These results indicate that NAC supplementation may spare exercise-induced blood glutathione oxidation and the thiol redox status perturbation.

Figure 2.8 Improvement of cysteine availability is an effective strategy for enhancing amounts of intracellular glutathione.



A, γ-Glu-CysH-Gly, γ-glutamyltransferase; B Glu, glutamine; CysH, cysteine; Gly, glycine; C, ASC amino acid transport system; D, x_c^- amino acid transport system; Cys_2, cystine; Na^+, sodium; GSH, reduced glutathione; $proteinS_2$, protein disulfides; proteinSH, protein sulfhydryls.

From Sen and Packer, 2000.

2.28 *N*-acetylcysteine effects on skeletal muscle fatigue

2.28.1 Animal studies

NAC effects on muscle fatigue have been investigated in various animal models. The rate of fatigue development induced in rabbit diaphragm strips by rhythmically stimulating strips *in situ* at 20 Hz for 20 min was reduced by NAC (1.5 ml/kg, 100 mg/ml followed by 0.1ml/kg), compared to saline treated animals (Shindoh et al., 1990). Similarly, Diaz et al. (1994) demonstrated that NAC (4 mg/ml) attenuated fatigue in *in vitro* rat diaphragm preparations. Intravenous NAC (150 mg/kg) together with mannitol (0.5 g/kg) and SOD (2000 IU/kg) also attenuated fatigue in rabbit diaphragm (Jiang et al., 2001). Environmental conditions may also be important as the effects of NAC were magnified at 37°C compared to 26°C (Diaz et al., 1994) and NAC attenuated rat diaphragm fatigue in hypoxic, but not hyperoxic, conditions (Heunks et al., 2001).

NAC reduced peak twitch stress, shortened time to peak twitch stress and shifted the stress-frequency curve down and to the right in unfatigued muscle of rat diaphragm fiber bundles (Khawli and Reid, 1994). During 30 Hz stimulated contractions fiber bundles incubated in 0.1-10 mM NAC exhibited a dose-dependent decrease in relative stresses developed, with no change in maximal tetanic stress and also inhibited acute fatigue.

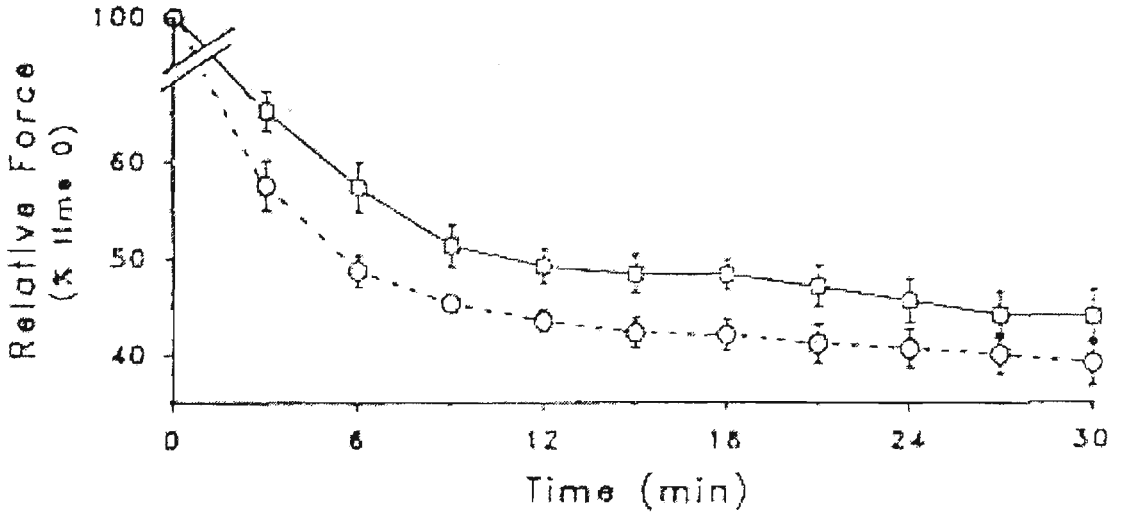
Supinski and colleagues (1997) compared the inspiratory volume and pressure generation over time in animals pre-treated with either saline or NAC (150 mg/kg) and then loaded until respiratory arrest. NAC-treated rats tolerated loading better than the saline-treated group, maintained higher inspiratory pressures, sustained higher inspired volumes and demonstrated increased time that animals could tolerate loading before the development of respiratory arrest. These studies demonstrate that NAC attenuates fatigue in animal models.

2.28.2 Human studies

The pioneering work of Reid et al. (1994) demonstrated that NAC infusion attenuated fatigue in human skeletal muscle. Healthy volunteers received 150 mg/kg for 1 h immediately before low-and high-frequency electrical stimulation of tibialis anterior muscle, to determine the effects of NAC on isometric force development. To blunt the adverse reactions of NAC, subjects were intravenously administered diphenhydramine as required. Low frequency stimulation comprised 10 Hz stimuli for 30 min, whilst high frequency comprised 40 Hz for 180 s. During repetitive low-frequency stimulation the force fell progressively, with 15% higher forces maintained during NAC trials compared to control (Figure 2.9). There was no effect of NAC on muscle force during high frequency stimulation (Reid et al., 1994). However, because stimulation frequencies greater than 50 Hz are rarely seen with voluntary contractions, low frequency protocols are more physiologically relevant (Jones, 1996). There was also no effect on post-fatigue recovery (Reid et al., 1994), consistent with animal studies (Diaz et al., 1994).

Pre-administration of intravenous NAC also attenuated the development of diaphragm fatigue in normal subjects breathing against high inspiratory loads, with time to fatigue increased by ~50% with NAC pre-treatment (Travaline et al., 1997). These studies demonstrating the efficacy of NAC in enhancing muscle performance suggest an important role of ROS in human skeletal muscle fatigue. However, no studies have investigated the effects of NAC on voluntary, whole body exercise performance in healthy humans and such studies are clearly required.

Figure 2.9 Intravenous *N*-acetylcysteine infusion inhibits low frequency fatigue during electrical stimulation of human tibialis anterior muscle.



NAC (solid line), control (dashed line)

From Reid et al., 1994.

SECTION V: AIMS AND HYPOTHESES

The general aim of this thesis was to investigate the effects of intravenous *N*-acetylcysteine (NAC) infusion on time to fatigue, glutathione and potassium (K^+) regulation during voluntary fatiguing exercise in healthy humans. Specific aims and hypotheses for each study are outlined below.

Study 1

The aim was to investigate the effects of a modified intravenous NAC infusion protocol on blood and plasma NAC, glutathione, cysteine, electrolytes and performance during voluntary high intensity, intermittent exercise comprising three 45s bouts and a fourth bout continued to fatigue, each at 135% $\dot{V}O_{2peak}$, in healthy humans.

Specific hypotheses tested were that intravenous infusion of NAC will:

1. Be free of serious adverse reactions.

2. Attenuate the decrease in whole blood reduced glutathione and alleviate the increase in whole blood oxidised glutathione during exercise.

3. Enhance plasma K^+ regulation, as evidenced by a reduced rise in the plasma $\Delta[K^+]$-to-work ratio during exercise.

4. Increase exercise performance, as measured by a longer time to fatigue.

Study 2

The aim was to investigate the effects of intravenous NAC infusion on the same variables as in Study 1, during prolonged, submaximal exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, followed by 90% $\dot{V}O_{2peak}$ to fatigue, in healthy humans.

Specific hypotheses tested were that intravenous infusion of NAC will:

1. Be free of serious adverse reactions, despite the increased infusion duration.

2. Attenuate the decrease in whole blood reduced glutathione and alleviates the increase in whole blood oxidised glutathione during exercise.

3. Enhance plasma K^+ regulation, as evidenced by a decreased rise in plasma $[K^+]$ during exercise.

4. Increase exercise performance, as measured by a longer time to fatigue.

Study 3- Part I

The aim was to investigate the effects of intravenous NAC infusion on muscle, blood and plasma NAC, glutathione, cysteine and on performance during prolonged, submaximal exercise, comprising 45 min at 70% $\dot{V}O_{2peak}$, followed by 90% $\dot{V}O_{2peak}$ to fatigue, in well-trained humans.

Specific hypotheses tested were that intravenous infusion of NAC will:

1. Increase NAC in both muscle and red blood cells.

2. Attenuate the decline in muscle and whole blood total glutathione during exercise.

3. Attenuate the decline in muscle and whole blood reduced glutathione during exercise.

4. Alleviate the rise in muscle and whole blood oxidised glutathione during exercise.

5. Increase exercise performance, as measured by a longer time to fatigue.

Study 3 – Part II

The aim was to investigate the effects of intravenous NAC infusion on skeletal muscle maximal Na^+,K^+-ATPase activity and plasma K^+ regulation during prolonged submaximal exercise in well-trained individuals.

Specific hypotheses tested were that intravenous infusion of NAC will:

1. Attenuate the decline in skeletal muscle maximal Na^+,K^+-ATPase activity during exercise.

2. Enhance plasma K^+ regulation, as evidenced by a decreased rise in plasma $[K^+]$ and $\Delta[K^+]$-to-work ratio during exercise.

CHAPTER 3: STUDY 1

N-ACETYLCYSTEINE INFUSION ALTERS BLOOD REDOX STATUS BUT NOT TIME TO FATIGUE DURING INTENSE EXERCISE IN HUMANS.

3.1. Introduction

Reactive oxygen species (ROS) including the superoxide, hydrogen peroxide and hydroxyl radicals are produced in contracting skeletal muscle (Davies et al. 1982; Jackson et al. 1985; Reid et al. 1992) and their presence is obligatory for optimal muscle contractile function (Reid et al. 1993). However, despite being scavenged by endogenous antioxidant enzymes (Barclay and Hansel 1991), ROS concentrations in skeletal muscle increase during contraction, which may compromise contractile function.

Numerous studies utilizing isolated animal muscle preparations have demonstrated that ROS accelerates, whereas antioxidants attenuate, muscle fatigue. Studies inducing increased ROS demonstrated accelerated fatigue in diaphragm and limb muscles (Diaz et al. 1993; Nashawati et al. 1993), which was inversely related to the amount of ROS produced (Reid et al. 1992). Antioxidant compounds have reduced fatigue in diverse experimental preparations, including canine diaphragm (Supinski et al. 1997) and gastrocnemius muscle (Barclay and Hansel 1991), rat diaphragm bundles (Reid et al. 1992), human diaphragm (Travaline et al. 1997), voluntary exercise in mice (Novelli et al. 1991) and humans (Lands et al. 1999), as well as electrical stimulation of limb muscles in humans (Reid et al. 1994). Whilst most studies in humans utilized oral vitamins A, C and/or E as antioxidants and typically found no exercise performance enhancement (Oostenbrug et al. 1997; Ashton et al. 1999; Akova et al. 2001), positive effects have been found using pharmacological antioxidants (Reid 1999). In a landmark study, Reid and colleagues (1994) found the decline in force during fatiguing electrical

stimulation of tibialis anterior muscle in humans was attenuated by intravenous infusion of the antioxidant compound, *N*-acetylcysteine (NAC). However, numerous serious adverse reactions were reported, including conjunctival irritation, dysphoria, vomiting, diarrhoea, nausea and loss of co-ordination, thus precluding NAC infusion during voluntary exercise (Reid et al. 1994). These adverse reactions may have been related to a high peak NAC concentration ([NAC]) resulting from the large bolus NAC dose, but this cannot be determined since [NAC] was not reported. Based on pharmacokinetic data (Prescott et al. 1989), estimates of their peak [NAC] are approximately 500 $mg.l^{-1}$, which would have declined rapidly during the experimental period (Reid et al. 1994). Therefore, their muscle stimulation experiments (Reid et al. 1994) were performed both at unknown and uncontrolled NAC concentrations. Interpretation of their data is further confounded since subjects were pre-treated with the anti-histamine drug diphenhydramine, to blunt the adverse reactions to NAC. The effects of diphenhydramine on muscle function were not reported. Others (Sen et al. 1994) have given oral NAC, but this approach is limited due to the low bioavailability of oral NAC (Holdiness 1991) and blood [NAC] was again not reported (Sen et al. 1994). Thus, the effects of NAC infusion on human muscle function are worthy of further exploration.

The first aim of this study was to develop a modified NAC infusion protocol designed to obviate serious adverse reactions, thus allowing whole body exercise. Specifically, this study aimed to: (i) avoid an excessive peak [NAC], (ii) attain a stable [NAC] during the exercise period and (iii) include measurements of blood and plasma [NAC] for the first time during exercise. On the basis of this modified NAC infusion protocol, the second aim was to investigate the effects of intravenous NAC on fatigue during voluntary whole body exercise in healthy individuals.

Changes in glutathione redox state are often used as a sensitive measure of tissue oxidative stress (Ji and Fu 1992). Exercise results in an increase in oxidized (GSSG) and decline in reduced glutathione (GSH) concentrations in blood (Gohil et al. 1988). Thus, the third aim was to investigate the effects of NAC infusion on blood GSH and GSSG during exercise. Finally, this study investigated the effects of NAC infusion on potassium (K^+) regulation during intense exercise, since impaired K^+ regulation has been linked with muscle fatigue (Nielsen and Clausen 2000; Sejersted and Sjøgaard 2000; Fraser et al. 2002). Results from our laboratory have recently found that intense fatiguing muscle contractions impaired skeletal muscle Na^+,K^+-ATPase activity (Fraser et al. 2002). Although the mechanisms for this effect are unknown, an increase in ROS was suggested as a possibility (Fowles et al. 2002; Fraser et al. 2002). The muscle Na^+,K^+-ATPase activity was inversely correlated to an index of plasma K^+ regulation during exercise, the ratio of rise in plasma K^+ per work done (Fraser et al. 2002). Consequently, the final aim was to investigate NAC effects on plasma K^+ regulation during exercise.

The three hypotheses tested were that NAC infusion would: (1) attenuate the decrease in GSH and rise in GSSG during exercise; (2) reduce the plasma $\Delta[K^+]$-to-work ratio during exercise; and (3) enhance time to fatigue during voluntary, high intensity intermittent exercise.

3.2. Methods

3.2.1. Subjects

Eight male subjects (age, 22.5±2.4 years; body mass, 77.81±10.30 kg; height, 177.6±1.6 cm mean±SD) volunteered for the study after being informed of all risks and giving written informed consent (Appendix 2). Subjects refrained from vigorous activity and avoided ingesting caffeine, alcohol, or other drugs in the 24 h before their visits to the

laboratory. Ethical approval was obtained from the Victoria University of Technology Human Research Ethics Committee (Appendix 3).

3.2.2. Overview of trials

Subjects attended the laboratory on six separate occasions, separated by a 5-7 d period. All exercise trials were completed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands). Subjects first completed an incremental exercise test to determine their peak oxygen consumption ($\dot{V}O_{2peak}$). Subjects then completed a total of five high intensity, intermittent exercise trials. The first trial was for familiarization purposes, the second and third trials were to determine the within-subject variability of the time to fatigue, whilst the final two trials were the *N*-acetylcysteine (NAC; Parvolex™, Faulding Pharmaceuticals) or saline (CON) trials. The last two trials were conducted in a double blind, randomized, counterbalanced design to determine the effects of NAC or CON infusion on exercise performance and blood redox status. For ethical reasons, the attending medical practitioner was non-blinded.

3.2.3. Peak Oxygen Consumption

Subjects were seated at a comfortable seat height, which was kept constant for all trials. Subjects performed four 4 min submaximal workloads at 60, 90, 120 and 150W, cycling at a pedal cadence of 80rpm. Following a 10 min rest period, with water consumed *ad libitum*, subjects re-commenced cycling at 150W, with increments of 25W each min until fatigue, defined as the inability to maintain pedal cadence above 60rpm. The highest $\dot{V}O_{2peak}$ over a 30s period was defined as $\dot{V}O_{2peak}$. A regression equation of $\dot{V}O_2$ versus power output was derived from the four sub maximal workloads and used to determine the power output corresponding to 130% $\dot{V}O_{2peak}$. All equipment and

calibration procedures for $\dot{V}O_2$ measurements were as previously described (Li et al. 2002).

3.2.4. Intermittent, High Intensity Exercise Protocol

The intermittent sprint test comprised four exercise bouts (EB) at a power output corresponding to 130% $\dot{V}O_{2peak}$. The first three EB lasted 45s, separated by a 135s passive recovery period on the ergometer (work:rest ratio 1:3), whereas the last EB was continued to volitional fatigue, defined as an inability to maintain pedal cadence above 60 rpm. High intensity exercise was chosen since this results in a markedly enhanced rate of production of ROS (Sjødin et al. 1990).

3.2.5. Experimental trials

The two experimental trials investigated the effects of intravenous NAC during voluntary, high-intensity, intermittent exercise. On arrival to the laboratory, subjects were weighed and then whilst supine on a couch, a 20G catheter was inserted into a dorsal hand vein for subsequent arterialised venous blood sampling. A 22G catheter was then inserted into a superficial median forearm vein for infusion of either NAC or saline. Subjects sat on a chair of comparable height immediately adjacent to the cycle ergometer for 10 min with their hand sheathed in a waterproof glove, placed in a 45°C water bath. After an initial control blood sample, the infusion of NAC or saline was commenced and continued until the cessation of the exercise trial. After 25 min subjects were transferred from the chair to the cycle ergometer, where they remained for a further 10 min before the onset of the exercise period to normalize any postural changes in plasma volume. After a total of 35 min of pre-infusion, subjects began to exercise, with blood sampled at the beginning and final seconds of each EB. Blood samples were also taken at 1, 2, 5, 10 and 30 min into the recovery period. Expired gases were measured 2 min prior to exercise and continued until the cessation of the exercise trial.

3.2.6. N-acetylcysteine Infusion

The NAC infusion protocol was modified from previous rapid, non-steady state bolus infusion protocols (Prescott et al. 1989; Reid et al. 1994). An initial loading dose of 125 $mg.kg^{-1}.hr^{-1}$ for 15 min was used to achieve the peak plasma [NAC], followed by a constant infusion of 25 $mg.kg^{-1}.hr^{-1}$ continued until the end of exercise (Appendix 4). These modifications aimed to avoid the initial high dose and thus minimize the many adverse reactions previously reported (Reid et al. 1994). This study also aimed to induce a constant plasma [NAC] prior to commencement of exercise and finally, to maintain this concentration throughout the exercise trial. An anaesthesia infusion pump was used for all infusions (Graseby 3400, Graseby Medical Ltd., Watford, UK).

3.2.7. Blood processing

Two blood samples were drawn in rapid succession at each sample point. The first 1 ml sample was taken using a blood gas syringe containing lithium heparin (RapidLyte, Chiron Diagnostics, MA, USA), air bubbles immediately expelled and the syringe placed on ice for immediate plasma gas, pH and electrolyte analyses (Ciba Corning 865pH/Blood Gas Analyzer, Bayer, MA, USA). A second 5 ml sample was used for measurement of reduced and total thiols in blood and plasma, haemoglobin concentration ([Hb]), haematocrit (Hct) and plasma concentrations of K^+ ($[K^+]$), sodium ($[Na^+]$), chloride ($[Cl^-]$) and calcium ($[Ca^{2+}]$). Samples were also processed and analyzed for metabolite concentrations and fluid shifts. Due to insufficient blood sample volume, not all variables were measured in some samples, as noted in results.

3.2.7.1. Blood and Plasma Thiols

A 3 ml aliquot of blood was initially separated into two equal portions, for reduced and total thiol analysis. For analyses of reduced thiols in each of blood and plasma, 1.5 ml of blood was pipetted into a heparinised tube containing 150 µl of 30 mM

monobromobimane (mBrB) in phosphate buffered saline. The remaining blood was pipetted into a second heparinised tube for subsequent analyses of total thiols in each of blood and plasma. Samples were then prepared for later analysis of reduced and total thiols in each of blood and plasma. First, 300 µl was pipetted into a 2 ml eppendorf tube and placed on ice for subsequent analysis of blood thiols. Then the remaining blood was immediately centrifuged for 2 min at 1,000 *g* (3K 15 refrigerated centrifuge, Sigma Laborzencentrifugen, Germany) with the plasma extracted for subsequent analyses of plasma thiols.

3.2.8. Blood Analyses

Concentrations of the thiol compounds *N*-acetylcysteine, glutathione, and cysteine were determined concurrently by high pressure liquid chromatography analysis (HPLC; Waters Associates, MA, USA), with fluorescence detection (Hitachi, Tokyo, Japan). Whole blood was measured for total NAC ($[NAC]_{tb}$), reduced NAC ($[NAC]_{rb}$), total glutathione ($[TGSH]_{tb}$), reduced glutathione ($[GSH]_{rb}$), total cysteine ($[CYS]_{tb}$) and reduced cysteine ($[CYS]_{rb}$). Plasma was analysed for all of these variables and are denoted by tp and rp for total and reduced concentrations, respectively.

3.2.8.1. Reduced Thiols

After thawing at room temperature, 100 µl of the sample was added into 100 µl of distilled water. Plasma and blood proteins were then precipitated with addition of 25 µl 50% (w,v) sulphosalicylic acid and immediately vortexed. Samples were then centrifuged at 1,000 g for 5 min and the supernatant injected for HPLC analysis.

3.2.8.2. Total thiols

After thawing at room temperature, 100 µl of the sample was pipetted into an eppendorf tube. To reduce oxidized thiols, 200 µl of 4 mM dithiothreitol (DTT) was added, immediately vortexed and left at room temperature for 10 min. Free thiols were then

derivatised by adding 50 µl of 20mM mBrB, vortexed and placed in the dark for a further 10 min. Plasma and blood proteins were then precipitated with 25 µl 50% (w,v) sulphosalicylic acid and immediately vortexed. Samples were centrifuged at 1,000 g for 5 min and the supernatant injected for HPLC analysis. Total and reduced thiols were analyzed in the same manner via HPLC.

The HPLC mobile phase was 18:82 methanol:20 mM KH_2PO_4 at pH 2.9 and 5 mM octanesulphonic acid running through a 150 by 3.9 mm NovapakC_{18} column (Waters Associates, MA, USA) at 1 ml.min^{-1}, with fluorescence detection at 400 nm excitation and 475 nm emission. This gives baseline separation of thiol compounds from each other and the reagent peaks, with a quantitation limit of approximately 100 nM and coefficient of determination of <5% for each. Total concentrations of the thiols (ie oxidized + reduced) were determined in a similar manner except that the oxidized thiols were reduced with DTT before a second derivitisation with mBrB was performed. After precipitation of plasma protein the supernatant was injected into the HPLC as for analysis of reduced concentrations.

3.2.8.3. Oxidized thiols

Oxidized thiol concentrations were calculated by subtraction of the reduced concentration from the total concentration. Due to insufficient blood sample volume being obtained, the calculated oxidized glutathione ([cGSSG]) could not be measured at EB1, fatigue and early recovery. Cystine concentrations were calculated by subtraction of the reduced [CYS] from total [CYS].

3.2.9. Calculations

Fluid shifts and K^+. The decline in plasma volume (ΔPV) from rest with exercise was calculated from changes in [Hb] and Hct (Harrison 1985), with [Hb] and Hct measured in triplicate using an automated analyzer (Sysmex, K-800, Kobe, Japan). The ratio of

the rise in plasma $[K^+]$ ($\Delta[K^+]$) per work output was calculated for each EB ($\Delta[K]^+$-to-work ratio; $nmol.l^{-1}.J^{-1}$), and used to represent the net plasma K^+ accumulation per EB (McKenna et al. 1993).

Red blood cell NAC, cysteine and cystine: Red blood cell NAC ($[NAC]_{rbc}$) and cysteine ($[CYS]_{rbc}$) and cystine concentrations were calculated using an equation (Buono and Yeager 1986) modified to exclude the correction of Hct for trapped plasma:

$$[NAC]_{rbc} = ([NAC]_{wb} - ([NAC]_p \times (1\text{-}Hct))) \times Hct^{-1}$$

where rbc, wb and p represent erythrocytes, whole blood and plasma, respectively.

3.2.10. Statistical analyses

Anthropometric data are presented as mean ± SD, with all other data reported as mean ± SEM. Single comparisons were analyzed using a paired Student t-test. For NAC, a one-way analysis of variance (ANOVA) with repeated measures was used. All other blood analyses were analyzed using a two-way (treatment, time) ANOVA with repeated measures on both factors. Significance was accepted at $P<0.05$. Post-hoc analyses used the Newman-Kuels test. Individual coefficients of variation (CV) were calculated for all subjects within the exercise protocol and averaged to obtain an overall CV (Jeukendrup et al. 1996).

3.3. Results

3.3.1. Exercise performance variability and effects of NAC

3.3.1.1. Incremental exercise

The $\dot{V}O_{2peak}$ was 43.0 ± 6.0 $ml.kg^{-1}.min^{-1}$ and the work rate corresponding to $130\,\dot{V}O_{2peak}$ was 327 ± 41W.

3.3.1.2. Intermittent exercise

Excellent reproducibility was seen in the time to fatigue in the fourth EB during the two variability trials, with a CV of 2.4±0.6% (range 0.6 to 5.5%, Table 3.1). No differences

were seen in time to fatigue (NAC 103±15 s, CON 106±19 s) or total work (NAC 33.2 ± 4.6 kJ, CON 34.1 ± 5.7 kJ) during the final EB.

Table 3.1. Individual time to fatigue during pre-experimental high intensity, intermittent exercise trials. Each trial comprised three 45s bouts, followed by a final bout to fatigue, each at 130% $\dot{V}O_{2peak}$. Coefficient of variance (CV) was calculated from variability trial #1 and #2.

	Fatigue time (s)			CV (%)
Subject	**Familiarization trial**	**Variability trial#1**	**Variability trial#2**	
1	89	91	92	0.8
2	206	208	215	2.3
3	119	126	125	0.6
4	51	56	59	3.7
5	71	79	77	1.8
6	75	89	87	1.6
7	55	53	49	5.5
8	110	120	125	2.9
Mean±SEM	97±18	103±18	104±19	2.4±0.6

3.3.2. N-acetylcysteine and adverse reactions

Total NAC content infused was 3.45 ±0.16 g. In contrast to previous studies, no severe or even moderate adverse reactions requiring treatment or causing discomfort were observed in any of the subjects using our modified infusion protocol (Table 3.2).

Table 3.2. Adverse reactions during experimental trials. Lack of severe or moderate adverse reactions with *N*-acetylcysteine (NAC) or saline (CON) infusion, before and during high intensity, intermittent exercise. n=8.

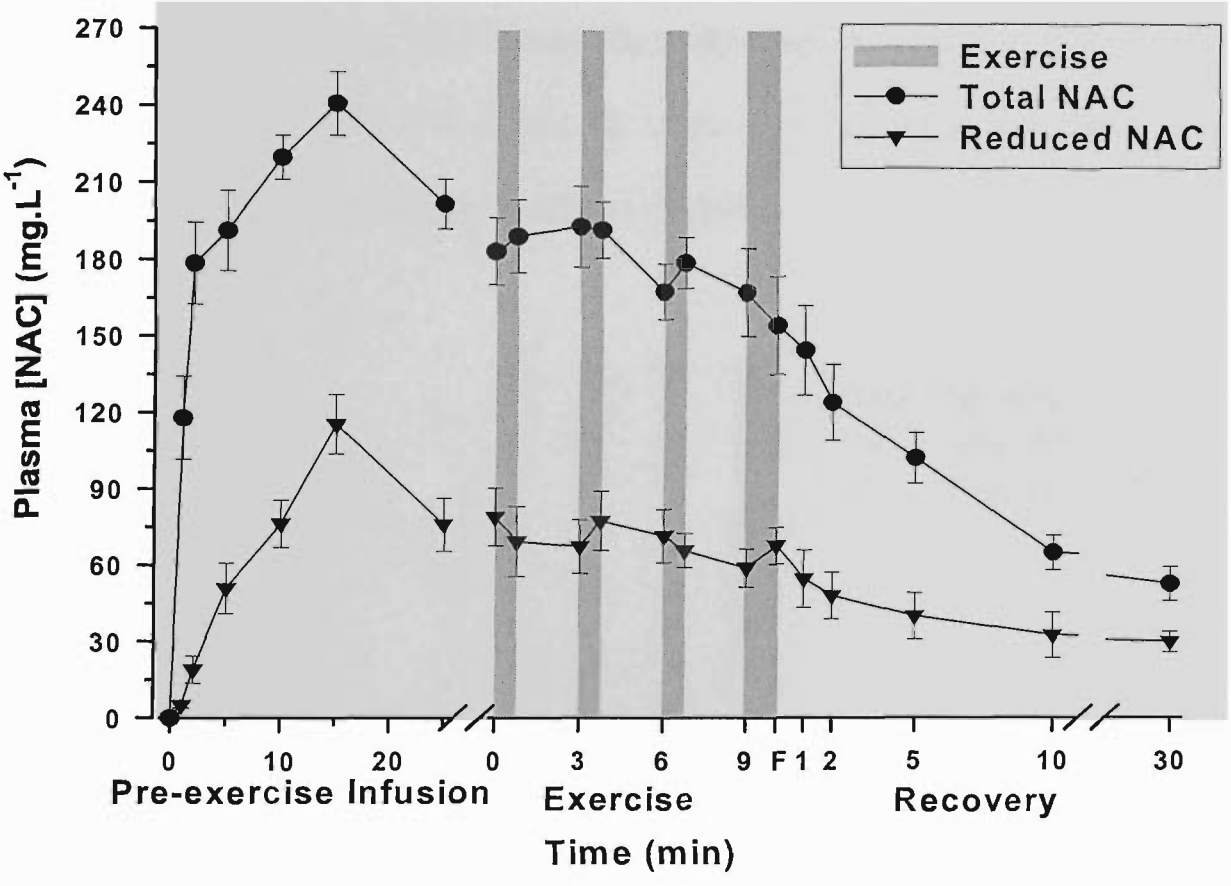
	Reaction Frequency and Severity							
	None		Mild		Moderate		Severe	
Reaction	**NAC**	**CON**	**NAC**	**CON**	**NAC**	**CON**	**NAC**	**CON**
Vomiting	7	7	1	1	0	0	0	0
Erythema	6	5	2	3	0	0	0	0
Swelling	1	8	7	0	0	0	0	0
Flushing	7	8	1	0	0	0	0	0
Rash	6	7	2	1	0	0	0	0
Coughing	8	7	0	1	0	0	0	0
Altered moods	6	8	2	0	0	0	0	0

3.3.3. Plasma NAC:

During the 15 min loading infusion phase $[NAC]_{tp}$ increased progressively until a peak at 15 min ($P<0.05$; Figure 3.1). During the maintenance infusion phase, $[NAC]_{tp}$ then decreased immediately prior to exercise ($P<0.05$), with no further changes during exercise. In recovery, $[NAC]_{tp}$ had decreased from fatigue at 30 min ($P<0.05$), but remained elevated above pre-infusion ($P<0.05$).

A similar pattern was found for $[NAC]_{rp}$, which peaked at the end of the loading phase ($P<0.05$), then declined slightly during the maintenance phase ($P<0.05$), with no subsequent differences during exercise. No differences were observed in $[NAC]_{rp}$ at 30 min recovery compared to fatigue levels, but $[NAC]_{rp}$ remained elevated above pre-infusion levels ($P<0.05$).

Figure 3.1. Plasma total (•) and reduced (▼) *N*-acetylcysteine concentration ([NAC]) concentration prior to, during and after high-intensity, intermittent exercise. Shaded bars represent exercise bout (EB) at 130% $\dot{V}O_{2peak}$; EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). Mean ± SEM: n=8 except EB1 where n=7. Significant time main effect (P<0.005); all times greater than pre-infusion (P<0.05; asteriks not shown for clarity).

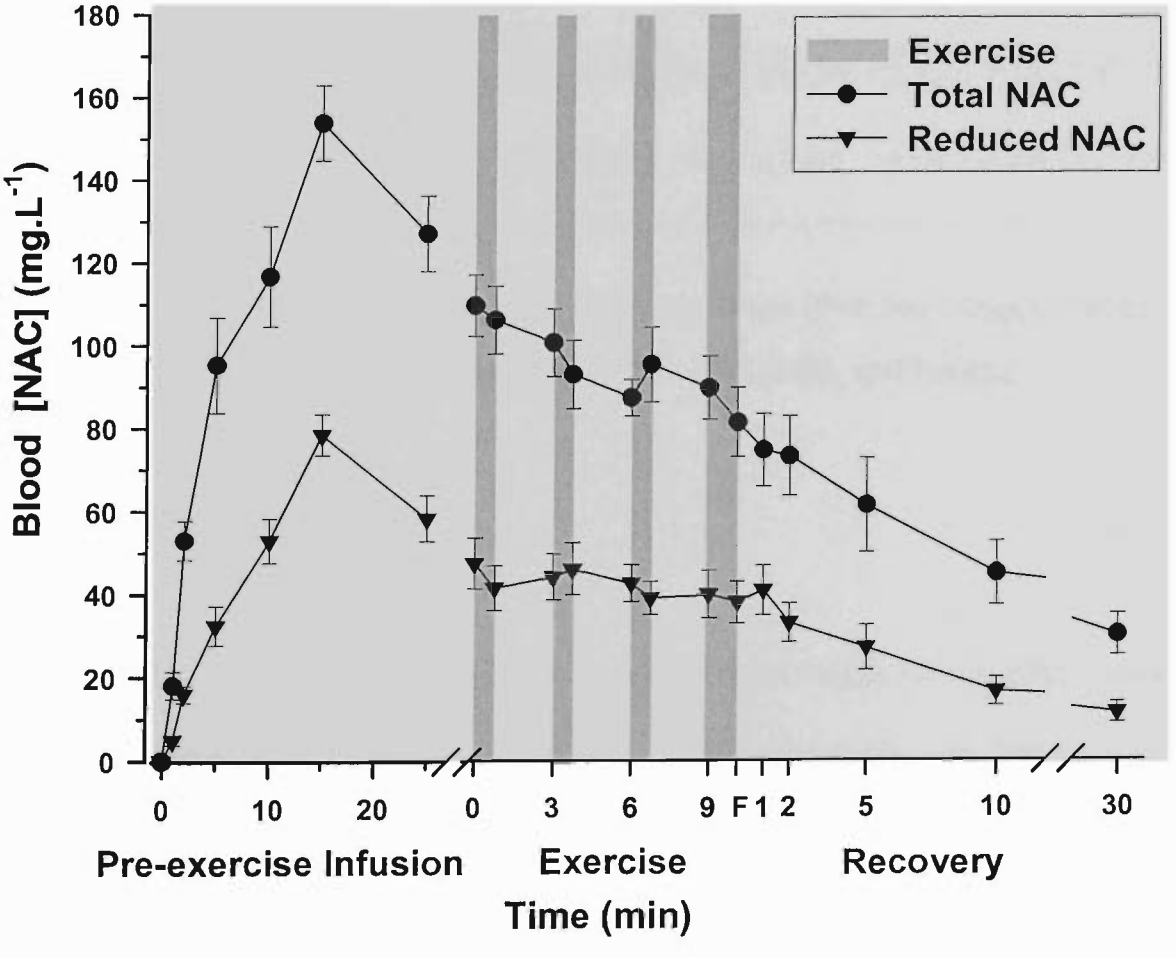


3.3.4. Whole Blood NAC:

During the loading infusion phase, $[NAC]_{tb}$ increased progressively until a peak at 15 min (P<0.05) and then decreased during the maintenance infusion phase (P<0.05; Figure 3.2). No significant changes were seen in $[NAC]_{tb}$ during exercise, but $[NAC]_{tb}$ decreased from fatigue at 30 min after infusion cessation (P<0.05), but remained

elevated above pre-infusion levels ($P<0.05$). Similarly, $[NAC]_{rb}$ peaked at the end of the loading phase ($P<0.05$), declined slightly during the maintenance phase ($P<0.05$), with no differences during exercise and until 30 min recovery, where $[NAC]_{rb}$ remained elevated above pre-infusion levels ($P<0.05$).

Figure 3.2. Blood total (•) and reduced (▼) *N*-acetylcysteine concentration prior to, during and after high-intensity, intermittent exercise. Shaded bars represent exercise bout (EB) at 130% $\dot{V}O_{2peak}$. EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). Mean ± SEM: n=8 except EB where n=7. Significant time main effect ($P<0.005$); all times greater than pre-infusion ($P<0.05$).



3.3.5. Red Blood Cell NAC

Total $[NAC]_{rbc}$ increased from pre-infusion levels at pre-EB1 ($P<0.001$, Table 3.3) was then unchanged during exercise, decreased from fatigue at 30 min recovery ($P<0.005$), but remained elevated above pre-infusion levels ($P<0.05$). Similarly, reduced $[NAC]_{rbc}$ increased at pre-EB1 ($P<0.001$), with no further changes during exercise, decreased from fatigue at 30 min recovery ($P<0.005$), but remained elevated above pre-infusion levels ($P<0.005$, Table 3.3).

Table 3.3 Calculated red blood cell *N*-acetylcysteine during high intensity, intermittent exercise prior to, during and following *N*-acetylcysteine (NAC) infusion. Units are $mg.l^{-1}$.

	Pre-EB1	EB1	EB2	EB3	Fatigue	30min Recovery
Total	29.77 ± 4.87	44.95 ± 4.92	33.44 ± 14.61	39.60 ± 8.20	47.12 ± 7.19	13.17 ± 2.80
Reduced	16.21 ± 4.78	13.26 ± 6.34	23.59 ± 8.68	18.37 ± 5.64	16.94 ± 6.19	3.84 ± 0.93

EB, Exercise Bout; All times significantly different to rest ($P<0.001$). Mean ± SEM, n=6 for Pre-Infusion, EB3 and 30min recovery. n=5 for EB1, EB2, and Fatigue.

3.3.6. Whole Blood Glutathione

3.3.6.1. Total

Whole blood [TGSH] was elevated above pre-infusion levels at pre-EB2, remained elevated thereafter during exercise and until 1 min recovery ($P<0.05$, Figure 3.3). No differences were found in [TGSH] between treatments at any time.

3.3.6.2. Reduced

Whole blood [GSH] was not significantly changed during the pre-exercise infusion, but at EB1 had declined from pre-infusion levels ($P<0.05$) and remained lower during the exercise and recovery periods ($P<0.05$, Figure 3.4). Whilst no differences were found in [GSH] between NAC and CON during the pre-infusion period, [GSH] was higher in NAC than in CON at EB1, subsequent exercise ($P<0.005$) and throughout recovery ($P<0.05$).

3.3.6.3. Oxidized

The [cGSSG] was unchanged during pre-infusion, increased during exercise and remained elevated above pre-infusion levels at 30 min recovery ($P<0.05$, Figure 3.5). No differences between NAC and CON were found in [cGSSG] from rest through to pre-EB1. However, from pre-EB2 through to 30 min recovery, [cGSSG] was lower in NAC compared to CON ($P<0.05$, Figure 3.5).

3.3.6.4. GSH to TGSH ratio

No differences in the GSH:TGSH ratio were found before or during the 35 min pre-infusion period in either trial. Exercise decreased the GSH:TGSH ratio ($P<0.005$), which was also attenuated by NAC ($P<0.005$, data not shown). At 30 min recovery, GSH:TGSH ratio remained lower than pre-infusion levels in both trials ($P<0.05$), but was still higher in NAC compared to CON.

3.3.7. Plasma Glutathione

Plasma glutathione levels were too low to be reliably detected.

Figure 3.3. Effect of NAC (•) and CON (■) infusion on blood total glutathione during high intensity, intermittent exercise prior to, during and after high-intensity, intermittent exercise.. Shaded bars represent exercise bout at 130% $\dot{V}O_{2peak}$. EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). Mean ± SEM: n=8 except EB1 where n=7 and 1-10 min recovery where n=6. * Significant time main effect (P<0.005).

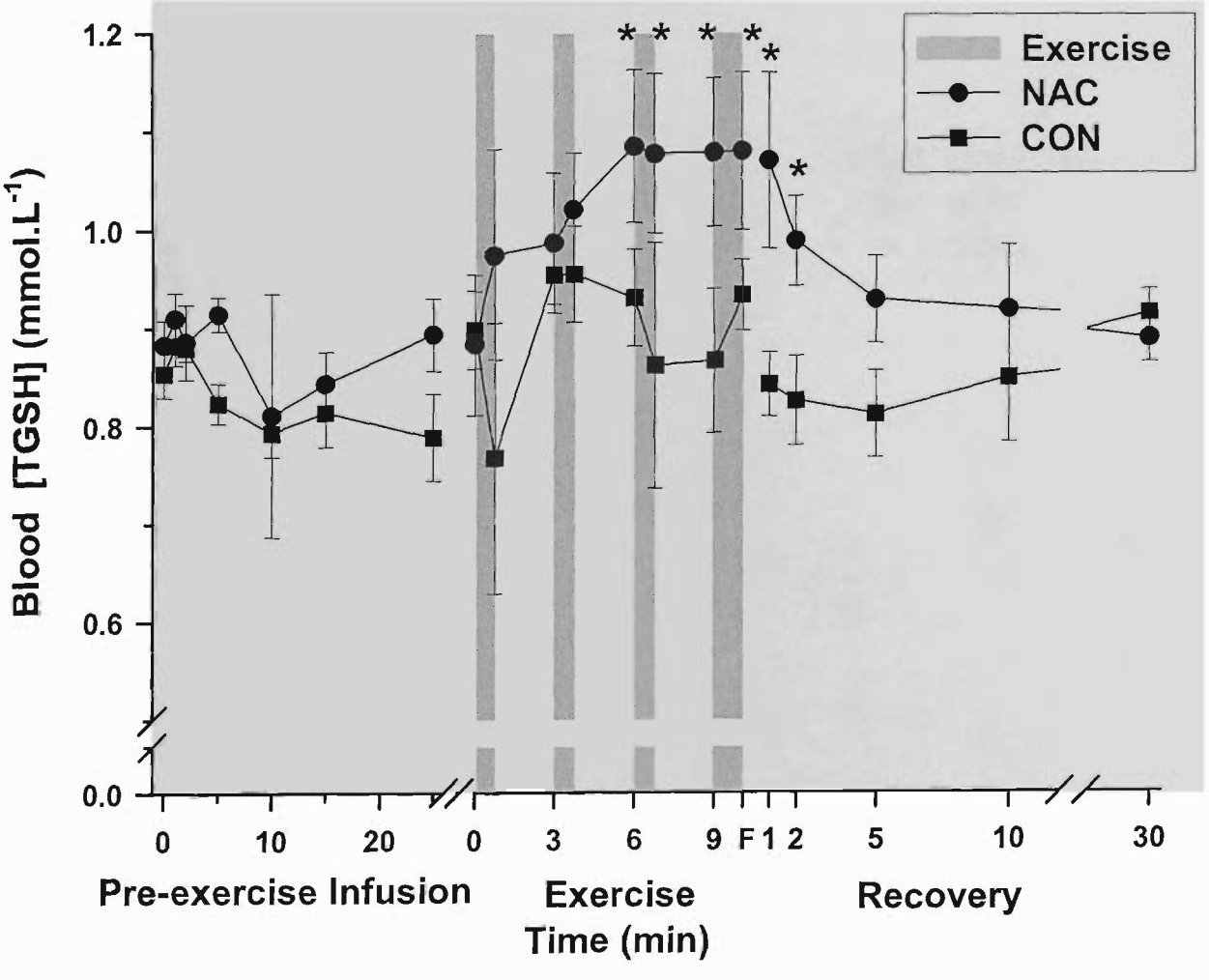


Figure 3.4. Effect of NAC (•) and CON (▪) infusion on blood reduced glutathione during high intensity, intermittent exercise prior to, during and after high-intensity, intermittent exercise. Shaded bars represent exercise bout at 130% $\dot{V}O_{2peak}$. EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). Mean ± SEM: n=8 except EB1 where n=7 and 1-10 min recovery where n=6. * Significant time main effect ($P<0.005$); † Significant NAC > CON ($P<0.05$).

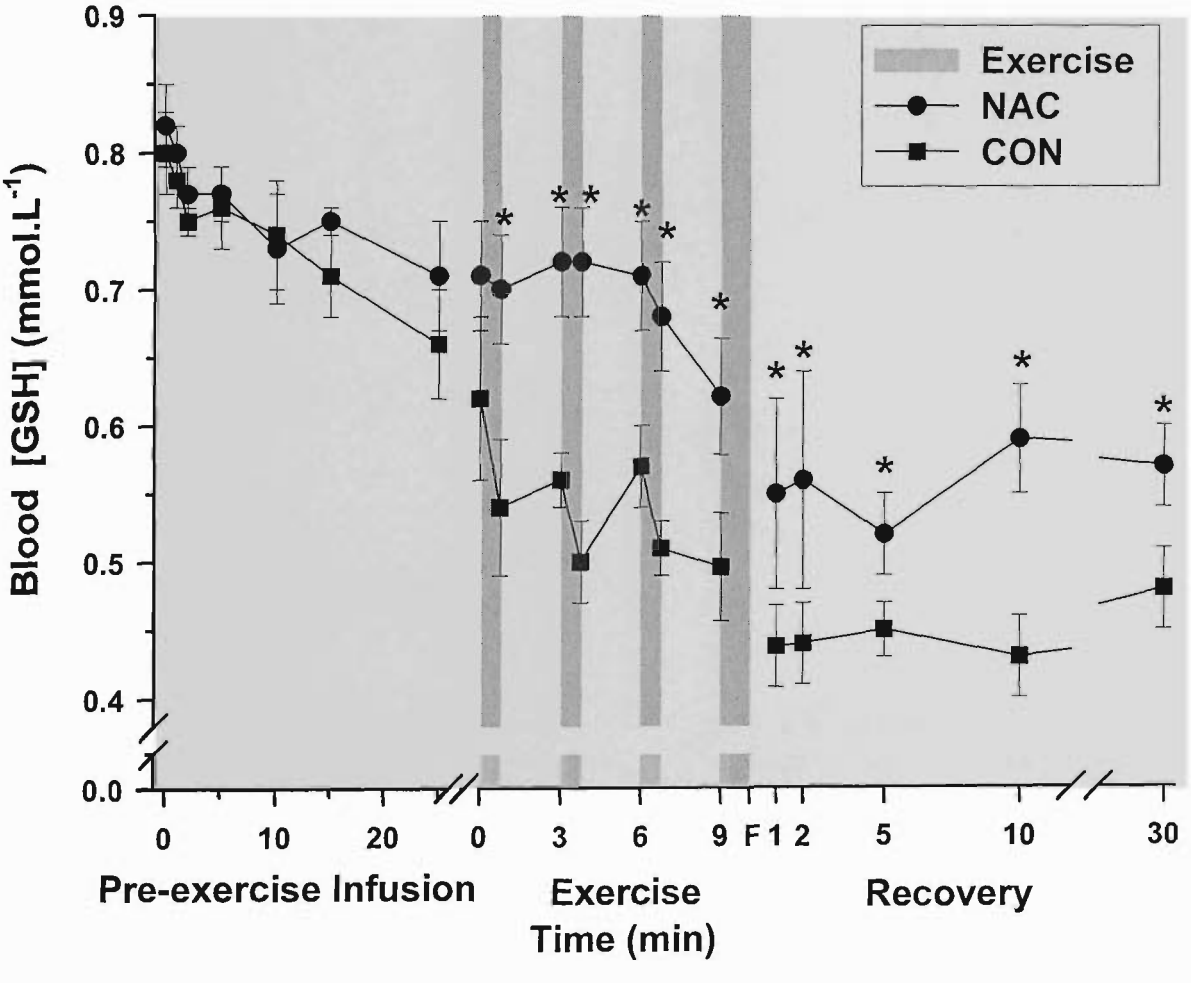
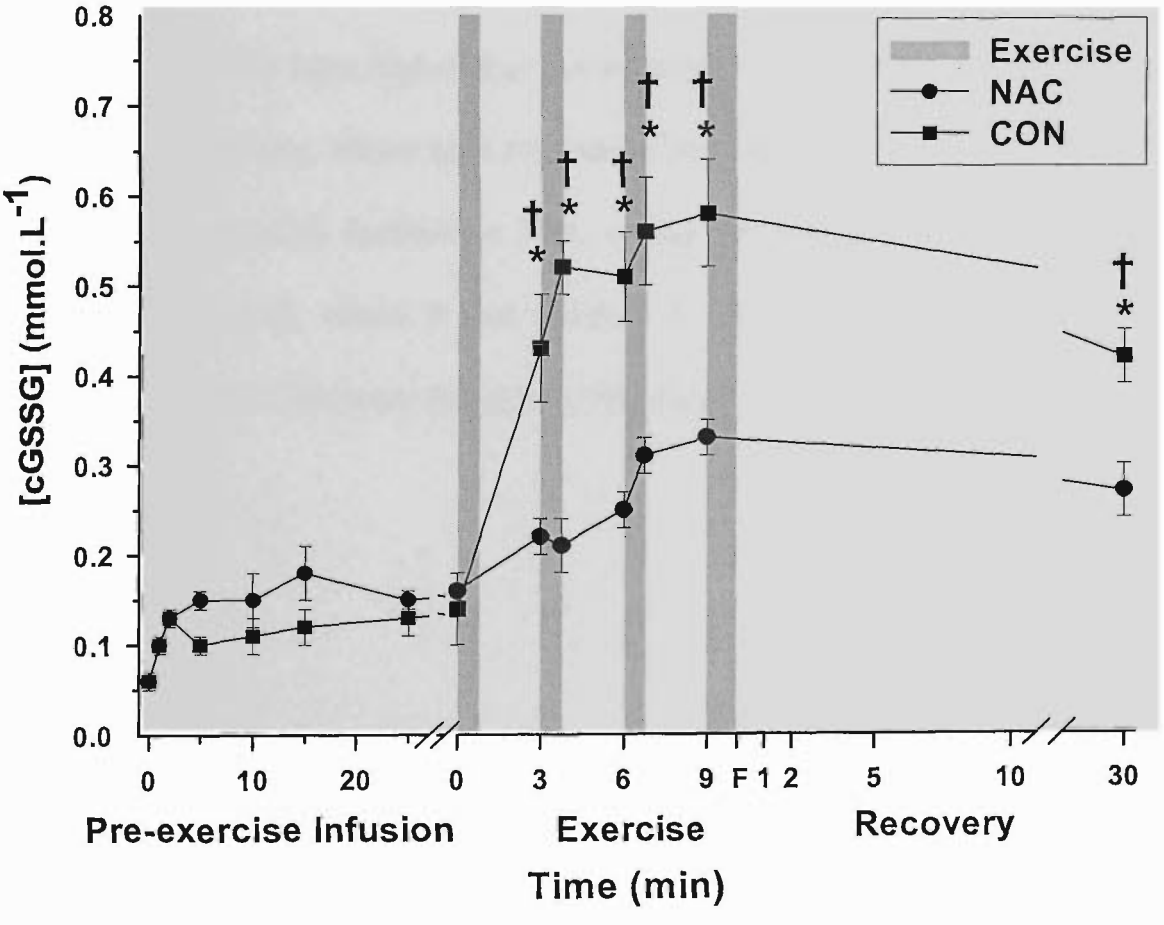


Figure 3.5. Effect of NAC (•) and CON (▪) infusion on calculated blood oxidized glutathione during high intensity, intermittent exercise prior to, during and after high-intensity, intermittent exercise. [cGSSG] is defined as [TGSH] – [GSH]. Shaded bars represent exercise bout at 130% $\dot{V}O_{2peak}$. EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). Mean ± SEM: n=8 except EB1, EB2 where n=7. * Significant time main effect (P<0.005); † Significant NAC < CON (P<0.05).



3.3.8. Cysteine and Cystine

Prior to infusion, no differences in cysteine ([CYS], Table 3.4) or cystine (Table 3.5) were found between NAC and CON, in whole blood, plasma or red blood cells, either in total or reduced forms. No change in [CYS] or cystine from pre-infusion levels occurred in CON at any time (Tables 3.4 and 3.5). However, NAC increased [CYS] and cystine in whole blood, plasma and red blood cells, in both total and reduced forms, compared to pre-infusion levels ($P<0.05$). Hence, [CYS] and cystine were higher in NAC than CON at all times during exercise and recovery ($P<0.05$, Tables 3.4 and 3.5).

3.3.9. Fluid shifts

Both [Hb] and Hct were higher than pre-infusion levels at EB2, during exercise and until 30 min recovery, where each returned to pre-infusion levels ($P<0.05$; Table 3.6). Plasma volume (ΔPV) declined at EB1, during exercise and until 30 min recovery ($P<0.05$, Table 3.6), where it had returned to pre-infusion levels. No differences between NAC and CON were found for [Hb], Hct or ΔPV.

Table 3.4. Cysteine concentrations ($\mu mol.l^{-1}$) in whole blood, plasma and red blood cells during high intensity, intermittent exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-EB1	EB1	EB2	EB3	Fatigue	30min Recovery
$[CYS]_{tb}$	NAC	51.0±8.4	161.3±19.9*‡	157.9±17.6*‡	158.0±19.0*‡	165.3±18.4*‡	284.4±17.9*‡	122.3±13.6*‡
	CON	49.1±7.6	51.6±11.6*	55.6±13.1*	51.1±12.0*	52.6±14.8*	56.6±9.6*	43.6±9.1*
$[CYS]_{rb}$	NAC	7.6±1.9	42.9±4.4*‡	40.0±3.4*‡	39.9±2.4*‡	37.1±2.0*‡	38.8±.3.0*‡	22.15±3.0*‡
	CON	7.1±2.7	10.4±3.4*	11.3±2.4*	11.2±3.1*	11.6±4.3*	11.1±2.9*	8.8±2.0*
$[CYS]_{tp}$	NAC	81.4±8.9	270.9±15.4*‡	268.4±16.2*‡	269.6±15.2*‡	288.7±17.5*‡	284.4±17.9*‡	208.7±15.1*‡
	CON	79.0±6.3	84.3±14.8*	87.3±13.4*	86.2±14.6*	87.0±12.1*	96.5±16.2*	67.9±10.8*
$[CYS]_{rp}$	NAC	9.7±3.5	71.8±7.8*‡	68.4±5.3*‡	66.5±5.0*‡	60.9±4.2*‡	65.3±4.1*‡	35.1±2.8*‡
	CON	8.8±2.1	15.2±4.8*	15.4±4.6*	16.4±5.5*	16.9±5.1*	15.4±5.9*	11.7±5.9*
$[CYS]_{trbc}$	NAC	15.5±2.1	35.2±3.1*‡	35.3 2.1*‡	41.0±2.0*‡	41.0±3.0*‡	37.7±2.4*‡	22.1±2.01*‡
	CON	14.1±2.6	13.5±2.6*	19.0±3.0*	12.8±2.9*	17.7±2.6*	17.8±2.6*	13.18±.2.6*
$[CYS]_{rrbc}$	NAC	5.1±1.2	9.6±1.1*†	8.6±1.0*†	12.1±0.9*†	13.1±.2.0*†	12.9±1.0*†	8.3±0.7*†
	CON	5.2±0.8	4.5± 0.9*	6.7±1.1*	5.6±1.1*	6.01±1.1*	6.8±1.4*	5.1±1.3*

EB, Exercise Bout; $[CYS]_{tb}$, Whole blood total cysteine; $[CYS]_{rb}$ whole blood reduced cysteine; $[CYS]_{tp}$, plasma total cysteine; $[CYS]_{rp}$, plasma reduced cysteine; $[CYS]_{trbc}$, calculated red blood cell cysteine, $[CYS]_{rrbc}$, calculated red blood cell reduced cysteine. significant main effect for treatment NAC > CON † $P<0.05$; ‡ $P<0.005$; * Significant main effect for time: different from rest ($P<0.005$). Mean ±SEM, n=8 except EB1 where n=7 for $[CYS]_{tb}$, $[CYS]_{rb}$ $[CYS]_{tp}$ and $[CYS]_{rp}$. For $[CYS]_{trbc}$ and $[CYS]_{rrbc}$ n=6 for Pre-Infusion, EB3 and 30min recovery and n=5 for EB1, EB2, and Fatigue.

Table 3.5. Calculated cystine concentrations ($\mu mol.l^{-1}$) in whole blood, plasma and red blood cells during high intensity, intermittent exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-EB1	EB1	EB2	EB3	Fatigue	30min Recovery
Plasma	NAC	71.7±9.9	199.1±16.9*‡	200.1±15.9*‡	203.1±15.9*‡	227.7±15.9*‡	219.0±18.9*‡	173.6±15.6*‡
	CON	70.2±8.9	68.9±10.2*	71.9±9.9*	69.7±8.9*	68.3±8.8*	45.5±6.6*	34.8±7.6*
Whole Blood	NAC	43.4±4.9	118.4±16.6*‡	117.8±17.6*‡	118.0±16.6*‡	128.1±15.9*‡	120.6±15.6*‡	100.2±13.9*‡
	CON	41.9±8.9	41.2±7.9*	44.2±6.9*	39.8±5.6*	40.9±5.6*	45.5±6.6*	34.8±5.6*
RBC	NAC	10.3±2.3	25.6±3.9*†	26.7±2.0*†	28.9±2.6*†	27.8±2.6*†	24.8±3.6*†	13.7±2.9*†
	CON	8.8±2.6	8.8±2.9*	12.3±2.3*	7.2±2.6*	11.7±3.8*	11.1±2.6*	8.0±2.1*

EB, Exercise Bout; Cystine calculated from total cysteine – reduced cysteine. significant main effect for treatment: NAC > CON † $P<0.05$; ‡ ($P<0.005$); * Significant main effect for time: different from rest ($P<0.005$). Mean ±SEM, n=6 for Pre-Infusion, EB3 and 30min recovery and n=5 for EB1, EB2 and Fatigue.

Table 3.6. Hematology and calculated fluid shifts during high intensity, intermittent exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-EB1	EB1	EB2	EB3	Fatigue	30min Recovery
[Hb] ($g.dl^{-1}$)	NAC	15.3±0.5	15.6±0.4	15.7±0.5	16.2±0.5 *	16.6±0.5 *	16.8±0.4 *	15.3±0.4
	CON	15.4±0.4	15.6±0.4	15.8±0.4	16.2±0.2 *	16.3±0.5 *	17.1±0.3 *	15.0±0.3
Hct (%)	NAC	46.1±1.1	46.5±1.1	47.4±1.4	48.8±1.0 *	49.8±0.9 *	50.7±1.2 *	46.3±0.9
	CON	46.1±1.0	46.2±0.9	46.4±1.1	47.8±1.1 *	48.3±1.0 *	50.8±1.5 *	44.4±1.3
ΔPV (%)	NAC	-	-	-7.6±1.1 *	-10.5±2.4 *	-14.4±1.1 *	-16.1±3.5 *	0.5±3.4
	CON	-	-	-6.4±0.6 *	-11.5±2.1 *	-12.7±2.6 *	-18.7±2.9 *	2.1±3.9

EB, Exercise Bout; * Significant main effect for time: different from rest $P<0.05$. Mean ±SEM, n=6 NAC, n=5 CON.

3.3.10. Plasma potassium and electrolyte concentrations

3.3.10.1. Potassium

Plasma [K^+] increased above pre-infusion levels in each EB, declined from fatigue at 1 and 2 min recovery and fell below pre-infusion levels at 5 and 10 min recovery ($P<0.05$, Figure 3.6A). No significant difference between NAC and CON was found for plasma [K^+].

The rise in plasma [K^+] ($\Delta[K^+]$) did not differ between EB1- EB3 but was increased in the final bout to fatigue ($P<0.001$). Plasma $\Delta[K^+]$ was higher in NAC than CON during EB2 and EB3 ($P<0.05$), with no differences found between treatments during EB1 and EB4. Similarly, the $\Delta[K^+]$-to-work ratio did not differ between EB1-3, but was greater in EB4 ($P<0.005$; Figure 3.6B). The $\Delta[K^+]$-to-work ratio was higher in NAC during EB2 and EB3 ($P<0.05$; Figure 3.6B).

3.3.10.2. Sodium, Chloride and Calcium

Plasma [Na^+] increased above pre-infusion levels at EB2, EB3 and at fatigue ($P<0.05$, Table 3.7), remained elevated above pre-infusion levels at 1 and 2 min recovery, but did not differ thereafter. Plasma [Cl^-] did not differ with time (Table 3.7). Plasma [Ca^{2+}] was increased above pre-infusion levels at fatigue, at 1 and 2 min recovery ($P<0.05$, Table 3.7), but did not differ thereafter. No differences between NAC and CON were found for any of plasma [Na^+], [Cl^-] or [Ca^{2+}].

3.3.10.3. Acid-base status

Plasma [H^+] was increased, whereas plasma PCO_2 and [HCO_3^-] fell during EB2, and remained lower during exercise and recovery, compared to pre-infusion levels ($P<0.05$, Table 3.7). A slightly lower [H^+] was found in NAC compared to CON ($P<0.05$), whereas no differences were found for plasma [HCO_3^-] or PCO_2.

Figure 3.6. Effect of NAC (▲) and CON (△) infusion on plasma [K^+], prior to, during and after high intensity, intermittent exercise (A); and the Δ[K^+]-to-work ratio (B). Shaded bars represent exercise bout at 130% $\dot{V}O_{2peak}$. EB 1-3 were 45s in duration, whilst EB4 was continued to fatigue (F). *Significant time main effect ($P<0.005$); † Significant treatment main effect ($P<0.05$). Mean ± SEM: n=8 except EB1 where n=7.

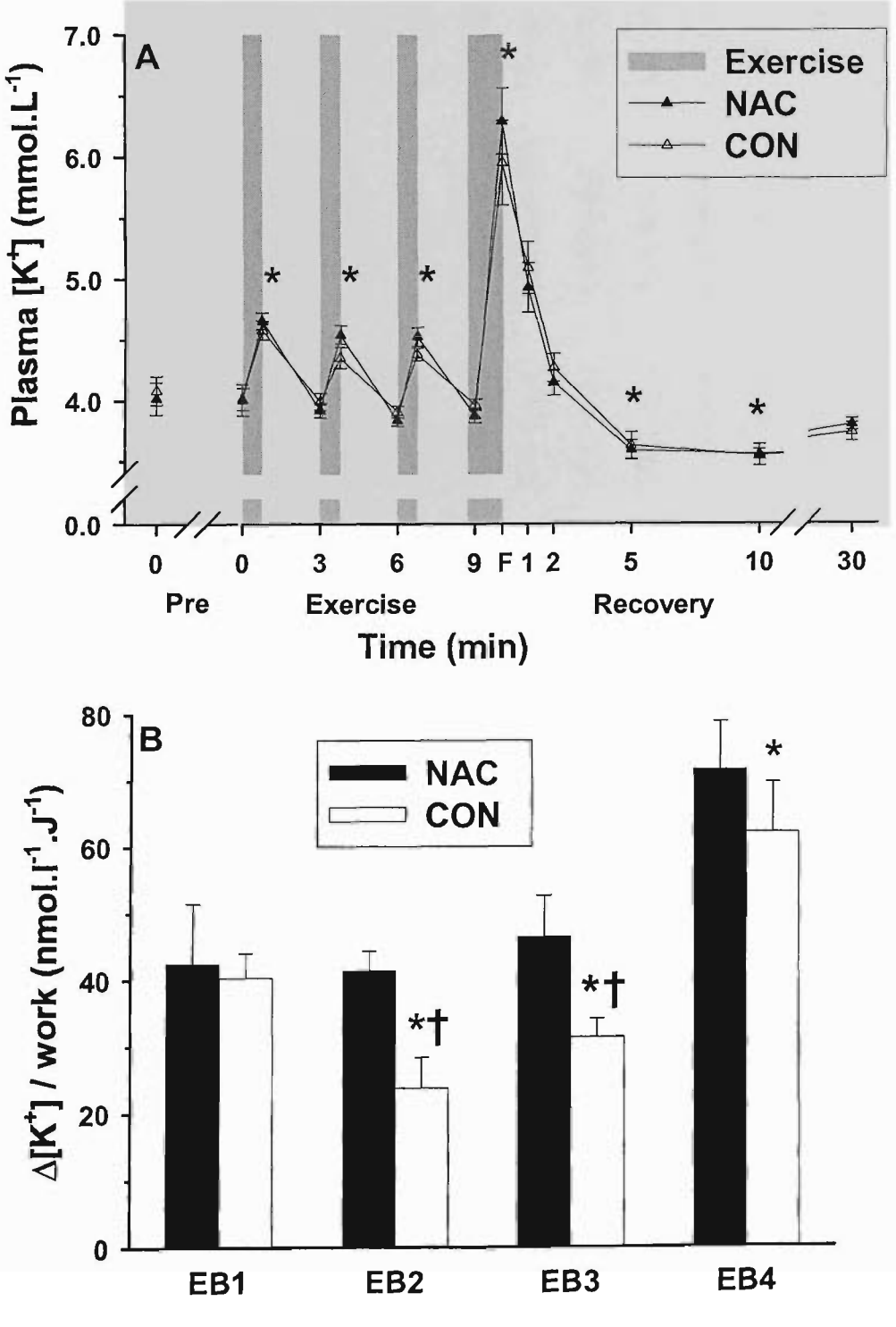


Table 3.7. Plasma acid-base variables and electrolyte concentrations during high intensity, intermittent exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-EB1	EB1	EB2	EB3	Fatigue	30 min Recovery
$[H^+]$(nmol.l^{-1})	NAC †	38.9±0.3 †	37.4±0.6 †	39.0±2.0 *†	46.2±1.4 *†	48.5±0.7 *†	54.7±1.7 *†	44.6±2.8 *†
	CON	39.1±0.4	39.1±0.6	38.6±0.5 *	48.5±0.7 *	48.5±0.7 *	55.7±2.3 *	48.4±2.6 *
$[HCO_3^-]$ (mmol.l^{-1})	NAC	26.7±0.5	25.5±0.7	25.1±1.2	21.6±2.0 *	17.6±0.5 *	14.7±0.5 *	16.5±1.3 *
	CON	25.5±0.4	24.3±0.5	25.2±0.5	19.6±0.5 *	17.4±0.5 *	14.9±0.7 *	17.9±1.6 *
PCO_2 (mmHg)	NAC	43.2±0.7	39.5±0.8	40.3±0.4	38.4±1.2 *	38.8±1.4 *	34.0±1.4 *	30.1±2.5 *
	CON	41.3±0.6	39.7±0.5	40.5±0.4	39.1±0.5 *	35.9±0.7 *	34.7±1.4 *	34.8±1.0 *
$[Na^+]$ (mmol.l^{-1})	NAC	141.0±0.7	141.9±0.7	141.8±0.6	144.7±0.4 *	144.6±1.0 *	151.0±0.8 *	141.4±0.7
	CON	138.5±1.4	142.6±0.6	139.1±1.7	144.9±1.7 *	143.8±1.0 *	147.1±1.4 *	141.2±0.7
$[Cl^-]$ (mmol.l^{-1})	NAC	104.0±0.5	103.9±0.9	103.5±0.7	102.1±0.6	103.1±0.7	105.1±0.9	101.1±0.8
	CON	104.0±0.3	103.2±0.9	104.8±1.4	103.0±0.8	102.0±1.3	107.7±1.6	105.7±2.7
$[Ca^{2+}]$ (mmol^{-1})	NAC	1.23±0.01	1.22±0.01	1.24±0.01	1.25±0.01	1.25±0.01	1.31±0.01 *	1.20±0.01
	CON	1.21±0.01	1.23±0.01	1.23±0.01	1.28±0.01	1.27±0.01	1.30±0.02 *	1.20±0.02
$\Delta[K^+]$ (mmol.l^{-1})	NAC	-	0.07±0.11	0.62±0.15	0.61±0.04 †	0.67±0.04 †	2.23±0.21 *	-2.30±0.20
	CON	-	0.01±0.09	0.58±0.07	0.35±0.07	0.45±0.04	2.09±0.33 *	-2.63±0.45

EB, Exercise Bout; $\Delta[K^+]$, rise in plasma $[K^+]$ during each EB; †significant main effect for treatment. * Significant main effect for time: different from pre-infusion $P<0.05$. Mean ±SEM, n=8 except EB1 where n=7

3.4. Discussion

This study investigated the effects of an antioxidant (*N*-acetylcysteine, NAC) drug infusion on blood redox state and intense intermittent exercise performance in healthy humans. The first important observation was that the modified NAC infusion protocol was free of serious adverse reactions and well tolerated under resting and vigorous exercise conditions. Whilst NAC altered blood redox status, plasma K^+ regulation was also impaired. Finally, NAC infusion did not enhance performance during voluntary, high intensity intermittent cycling exercise in humans.

3.4.1. No severe adverse reactions with NAC infusion

This study utilized a modified NAC infusion protocol, with major improvements on previous studies (Prescott et al. 1989; Reid et al. 1994; Travaline et al. 1997). Specifically, the modified infusion protocol attained relatively stable concentrations during exercise and the experiments were completed without anti-histamine pre-treatment. The peak plasma [NAC] was ~240 mg/l (~1.5 $mmol.l^{-1}$) at the end of the NAC loading phase and ~191 mg/l prior to exercise, in contrast to the estimated peak [NAC] of ~500 $mg.l^{-1}$ (~3 $mmol.l^{-1}$) in Reid and colleagues (1994). The unchanged [NAC] during the exercise period allowed the effects of a relatively stable [NAC] during exercise, in contrast to the expected rapidly declining NAC during the experimental period in a previous study (Reid et al. 1994).

An important finding is that the NAC infusion protocol was free of any major adverse reactions in these eight healthy volunteers. The most common side effect resulting from the NAC infusion was erythema at the site of infusion, but this was also evident during some CON trials. Of the side effects unique to the NAC infusion trial (flushing, localized swelling and altered moods), none were serious enough to result in termination of the protocol or require treatment. Furthermore, the protocol avoided other reported adverse reactions to NAC

infusion including loss of co-ordination, nausea, bronchospasm, conjunctival irritation, diarrhoea, angiodema, tachycardia and dyspepsia (Mant et al. 1984; Reid et al. 1994; Travaline et al. 1997). Therefore, this modified NAC infusion protocol may be utilized in future studies investigating NAC effects in healthy humans.

3.4.2. N-acetylcysteine modulates exercise effects on blood redox state

The results of this study demonstrate two important findings with respect to intense exercise and NAC effects on blood redox state. Firstly, intense intermittent exercise decreased whole blood [GSH], with a concomitant increase in whole blood [cGSSG]. Together with unchanged whole blood [TGSH], this demonstrates a shift in whole blood redox status during exercise. The assay used was unable to detect glutathione in plasma, consistent with others (Gohil et al. 1988).

The reported effects of high intensity exercise on [GSSG] and [GSH] in humans are inconsistent. Blood [GSSG] following graded exercise to exhaustion was increased in two studies (Sastre et al. 1992; Sen et al. 1994), but unchanged in another (Gohil et al. 1988); whilst blood [GSH] was decreased (Sastre et al. 1992) or unchanged (Gohil et al. 1988; Sen et al. 1994). These discrepancies may in part reflect the subjects' differing training status, which influences the response of the glutathione system (Michelet et al. 1995). However, it would be expected that both a decline in the GSH, together with a rise in GSSG would occur, yet only one of the three above studies reported this (Sastre et al. 1992). The results of this study demonstrate a clear effect of intense exercise on blood [GSH] and [cGSSG], indicating that exercise directly modulates blood redox state. Whole blood glutathione oxidation reflects defence against ROS formation (Sastre et al. 1992) and is often used as a sensitive marker of oxidative stress (Ji and Fu 1992). Therefore, the rise in [cGSSG] indicates increased extramuscular accumulation of ROS during intermittent, high intensity exercise, consistent

with ROS efflux from contracting leg muscles during exercise (Bailey et al. 2003). Since plasma glutathione was undetectable, these results indicate increased oxidative stress within red blood cells.

The second major finding was that NAC attenuated both the decline in blood [GSH] and rise in blood [cGSSG] during intense, intermittent exercise. A previous study found that oral NAC attenuated the rise in [GSSG] during high intensity exercise, although surprisingly, they reported no effect of exercise or NAC on GSH, and blood [NAC] was also not reported (Sen et al. 1994).

Cysteine is a precursor to glutathione synthesis (Prescott et al. 1989; Holdiness 1991; Cotgreave 1997) and meets the intracellular needs for synthesis of GSH (Cotgreave 1997). NAC infusion increased reduced [CYS] and increased the ratio of reduced [CYS] to oxidized [CYS] and may therefore have spared glutathione oxidation. This likely explains the attenuated decline in [GSH] and increased [cGSSG]. However, NAC is rapidly deacetylated to cysteine, which is itself a ROS scavenger (Cotgreave 1997) and this could also be a factor for the attenuated decrease and increase in [GSH] and [cGSSG], respectively.

3.4.3. Lack of ergogenic effect

This is the first study to investigate possible ergogenic effects of NAC infusion in healthy humans undertaking voluntary whole body exercise. The intra-subject variability during the intermittent exercise protocol was 2.4%, well within the typical variation for high intensity exercise (~5%; McKenna et al. 1997). NAC did not increase time to fatigue during high intensity, intermittent exercise, in contrast to the attenuation of fatigue in stimulated muscle contractions with NAC (Reid et al. 1994), or in diaphragm fatigue induced by inspiratory loading (Travaline et al. 1997).

The lesser rise in [cGSSG] during exercise with NAC indicates enhanced glutathione synthesis and greater protection against ROS formation. Thus, it is unlikely that the lower [NAC] in this study compared to Reid and colleagues (1994) can account for our lack of performance enhancement. This finding is consistent with the lack of effect of NAC on muscle fatigability during high frequency electrical stimulation in human tibialis anterior muscle (Reid et al. 1994). Together these suggest that NAC does not affect contractile performance during heavy muscular contractions. The attenuated fatigue with NAC in human tibialis anterior muscle during low frequency muscle stimulation (Reid et al. 1994) and in the diaphragm during inspiratory loading (Travaline et al. 1997) suggests that NAC may be more effective in modulating performance during low frequency fatigue protocols (Reid 1999). Therefore, research into NAC effects on prolonged exercise performance appears warranted.

3.4.4. NAC, intracellular actions and muscle performance

The capacity of NAC to act as an effective antioxidant during exercise may depend upon whether it influences intracellular processes in skeletal muscle. An important clinical application of NAC is the treatment of paracetamol overdose, which relies on NAC's reducing capacity within liver cells (Cotgreave 1997). Tissues such as bladder, bone marrow, erythrocytes and liver all take up NAC and/or its reduced cysteine derivatives (McLellan et al. 1995; Cuzzocrea et al. 2001), with the exact mechanism unknown. NAC infusion increased $[NAC]_{rbc}$ in healthy humans, indicating that NAC penetrates healthy cell membranes and suggesting that NAC may also permeate the sarcolemma. However, to knowledge, no studies have investigated this possibility. Nonetheless, several studies demonstrate that NAC directly affects skeletal muscle contractile function (Khawli and Reid 1994; Heunks, Machiels et al. 2001). Together these studies suggest that NAC exerts an important intracellular role in tissues

including skeletal muscle. However, further work investigating NAC and consequent effects on redox state in skeletal muscle is required.

An important question is whether altered blood redox state may alter muscle intracellular redox state and function. Extracellular administration of hydrogen peroxide increased dichlorofluorescin oxidation within muscle (Murrant et al. 1999), suggesting that extracellular redox perturbations can influence the intracellular milieu. Since NAC attenuated the increase in red blood cell [cGSSG], this suggests that it is likely that the intracellular concentrations of ROS and of glutathione may also be affected, however this remains to be determined.

NAC counteracts oxidative stress (Benrahmoune et al. 2000), and acts as an antioxidant by supporting glutathione synthesis (Cotgreave 1997). Supplementation with the cysteine donor Immunocol, which increased lymphocyte [GSH] by 36%, induced a 13% increase in work and peak power during 30 s cycle sprint exercise (Lands et al. 1999). Intraperitoneal injection of L-buthioninie SR-sulfoximine, which decreased total glutathione in the liver, lung, blood, plasma, skeletal muscle and heart by 50-90%, induced a 50% decrease in running endurance time in rats (Sen et al. 1994). Other studies demonstrated that exogenous GSH supplementation increased endurance swimming by up to 141% in rats (Cazzulani et al. 1991; Novelli et al. 1991). Therefore, enhancing glutathione synthesis can augment performance. However, our results indicate that attenuating the rise in [cGSSG] and fall in [GSH], reflecting enhanced glutathione synthesis, did not improve performance during high intensity intermittent exercise. A further fatigue sparing effect may be due to ROS scavenging, since NAC is a potent ROS scavenger (Aruoma et al. 1989; Travaline et al. 1997). The amount of NAC infused was likely to decrease both intracellular and extracellular ROS concentration, as indicated by lesser perturbations in blood redox status. Whilst an increased NAC dose would

increase ROS scavenging, this is probably not viable during exercise, due to the severe adverse reactions (Mant et al. 1984; Reid et al. 1994).

3.4.5. NAC impairs plasma K^+ regulation, but had minimal effects on other electrolytes

Whilst NAC had minimal effects on most plasma electrolytes during exercise, NAC impaired plasma K^+ regulation and lowered plasma $[H^+]$. Our laboratory has recently demonstrated that fatiguing muscle contractions inhibited the maximal Na^+,K^+-ATPase activity in human skeletal muscle (Fraser et al. 2002), with increased ROS production postulated as one possible candidate, since ROS can inhibit Na^+,K^+-ATPase activity (Kourie 1998). Hence, it was hypothesized that NAC would reduce the $\Delta[K^+]$-to-work ratio, but this was increased during the second and third exercise bouts with NAC. The $\Delta[K^+]$-to-work ratio has been used previously as a marker of plasma K^+ regulation during exercise (McKenna et al. 1993; McKenna et al. 1997; Harmer et al. 2000; Fraser et al. 2002). Therefore the higher $\Delta[K^+]$-to-work ratio indicates that NAC impaired K^+ regulation during intense, intermittent exercise. The mechanism is unknown, but might include increased K^+ release from contracting muscle and/or reduced K^+ clearance by contracting and inactive muscle, or other tissues. These changes might also be mediated by alterations in muscle blood flow, as NAC is a potent vasodilator (Andrews et al. 2001). Since muscle K^+ regulation is linked with muscle performance (Nielsen and Clausen 2000), the impaired K^+ regulation with NAC might also help explain why performance was unaltered, despite a marked effect of NAC on blood redox state.

Finally, NAC attenuated plasma $[H^+]$ during exercise. This effect in plasma was small and likely to be of minor physiological significance. However, if NAC also lowers $[H^+]$ in skeletal muscle, this may have important implications, since a semi-quinone radical in the presence of

hydrogen peroxide and a high [H^+] may form the potent hydroxyl radical (Jenkins 1988), which denatures the Ca^{2+}ATPase enzyme (Xu et al. 1997).

3.4.6. Conclusions

N-acetylcysteine did not induce any serious adverse reactions in healthy volunteers, at rest, during or after vigorous exercise. NAC blunted the decline in [GSH] as well as the concomitant rise in [cGSSG] in whole blood during exercise, indicating its efficacy as a modulator of blood redox status during exercise. NAC failed to enhance performance during high intensity, intermittent exercise, suggesting that ROS may not exert an important role in muscle fatigue under these exercise conditions. However, it is possible that the adverse effects of impaired K^+ regulation with NAC counterbalanced any positive effects due to redox modulation. Further research is required to investigate the cellular actions of NAC and in particular the effects on prolonged exercise performance in humans.

CHAPTER 4: STUDY 2

EFFECTS OF INTRAVENOUS *N*-ACETYLCYSTEINE INFUSION ON TIME TO FATIGUE AND POTASSIUM REGULATION DURING PROLONGED CYCLING EXERCISE.

4.1. Introduction

Skeletal muscle produces reactive oxygen species (ROS) at low rates under resting conditions (Jackson et al. 1985; Reid et al. 1993; McArdle et al. 2001), but this rate is greatly enhanced during muscular contraction (Reid et al. 1992; Diaz et al. 1993; Bailey et al. 2003). Endogenous antioxidants, including the enzymes superoxide dismutase, catalase and glutathione peroxidase, and thiol compounds such as glutathione and cysteine, are present in skeletal muscle and protect against the harmful effects of ROS (Reid 1999). However, during exercise the endogenous antioxidant system is overwhelmed and an increased ROS concentration occurs (Davies et al. 1982; Bailey et al. 2003).

In animal models, ROS are known to accelerate muscular fatigue (Barclay and Hansel 1991; Reid, et al. 1992; Diaz et al. 1993; Nashawati et al. 1993), which is attenuated by antioxidants (Shindoh et al. 1990; Barclay and Hansel 1991; Reid et al. 1992; Supinski et al. 1997). Importantly, studies implicating ROS in muscular fatigue have generally involved low frequency muscle stimulation protocols. In rat diaphragm bundles stimulated at 30 Hz, force was 20% greater after treatment with superoxide dismutase, catalase and dimethyl sulfoxide (Reid et al. 1992). Similarly, superoxide dismutase increased tension by ~20% in canine diaphragm stimulated at 15 Hz (Nashawati et al. 1993). *N*-acetylcysteine (NAC), a thiol containing compound, also attenuated skeletal and diaphragm muscle fatigue (Shindoh et al. 1990; Diaz et al. 1994; Khawli and Reid 1994). In rat diaphragm, NAC attenuated fatigue when stimulated at 20 Hz for 4 min (Diaz et al. 1994) and in rabbit diaphragm stimulated at 20

Hz for 20 min (Shindoh et al. 1990). In humans, NAC attenuated diaphragm muscle fatigue induced by loaded breathing (Travaline et al. 1997) and reduced by 15% the force loss with fatigue induced by electrical stimulation of tibialis anterior muscle at 10 Hz (Reid et al. 1994). However, the adverse reactions reported in the latter study, including loss of co-ordination, vomiting, diarrhoea and nausea, would preclude voluntary exercise in humans (Reid et al. 1994).

Study 1 demonstrated that a modified NAC infusion protocol, without antihistamine pretreatment, was free of severe adverse reactions and was well tolerated by healthy humans during voluntary high intensity, intermittent exercise. Although no fatigue sparing effects of NAC during intense voluntary cycling exercise in humans (Study 1), this was consistent with a lack of an effect of NAC during high frequency (40 Hz) stimulation in human muscle (Reid et al. 1994). Given the apparent involvement of ROS in fatigue induced by low frequency muscle stimulation in animal and human models, it is likely that a similar involvement may be evident in prolonged submaximal exercise, with scavenging of ROS (Aruoma et al. 1989) and supporting glutathione synthesis a possible mechanism (Cotgreave 1997). No studies have investigated the effects of intravenous NAC infusion on performance in healthy humans during prolonged exercise. The hypothesis tested in this study was that intravenous NAC infusion would prolong the time to fatigue during exhaustive, submaximal exercise.

A further possible mechanism may include effects on skeletal muscle Na^+,K^+-ATPase. Maximal Na^+,K^+-ATPase activity in human skeletal muscle is depressed with exhaustive dynamic (Fraser et al. 2002) and isometric contractions (Fowles et al. 2002), and in rat muscle after prolonged running and recovery (Fowles et al. 2002), with one proposed causal mechanism being ROS accumulation (Sen et al. 1995; Kourie 1998). Furthermore, several studies have demonstrated a decline in muscle intracellular K^+ with prolonged exercise and

suggested a link with fatigue (see refs in McKenna 1992). Hence, the ROS scavenging effects of NAC might alleviate Na^+,K^+-ATPase inactivation, enhance muscle K^+ regulation and, thereby improve prolonged exercise performance. However, Study 1 demonstrated converse effects, that NAC infusion in untrained humans impaired plasma K^+ regulation during intense exercise. The mechanism is unknown, but this possible conflict is of potential importance and requires resolution. During prolonged submaximal exercise, K^+ fluxes are expected to be markedly less compared to the intense exercise protocol completed in Study 1. Therefore, this study investigated whether NAC would improve plasma K^+ regulation during prolonged submaximal exercise. The hypothesis tested was that NAC would enhance K^+ regulation, as evidenced by a decreased rise in plasma $[K^+]$ and a lesser $\Delta[K^+]$-to-work^{-1} ratio. Finally, since NAC infusion attenuated plasma $[H^+]$ during intense intermittent exercise (Study 1), this study also explored the effects of NAC on plasma $[H^+]$ and other electrolytes during prolonged, submaximal exercise.

4.2. Methods

4.2.1. Subjects

Eight male subjects (age, 21.3±2.3 yr; body mass, 77.81±10.50 kg; height, 179.4±4.4 cm; mean±SD) volunteered for the study after being informed of all risks and giving written informed consent (Appendix 5). The subjects comprised four recreationally active team sports participants who trained or competed 1-2 times per week and four endurance trained cyclists who trained 4-5 times per week. Subjects refrained from vigorous activity and avoided ingesting caffeine, alcohol, or other drugs in the 24 h prior to exercise trials. Ethical approval was obtained from the Victoria University of Technology Human Research Ethics Committee (Appendix 3).

4.2.2. Exercise trials

4.2.2.1. Overview

Subjects attended the laboratory on six separate occasions, separated by a 7 d period. All exercise trials were completed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands). *Peak oxygen consumption:* Subjects first completed an incremental exercise test to determine their peak oxygen consumption ($\dot{V}O_{2peak}$), with all equipment, calibration and procedures as previously detailed (Li et al. 2002; Study 1).

4.2.2.2. Prolonged, Submaximal Exercise Protocol

Subjects completed a total of five prolonged, submaximal exercise trials. The first trial was for familiarization purposes, the second and third trials were to determine the within-subject variability of their time to fatigue, whilst the final two trials were the *N*-acetylcysteine (Parvolex™, Faulding Pharmaceuticals) or saline (CON) infusion trials. The prolonged, submaximal cycling exercise test comprised an initial 45 min at a workrate corresponding to 70% $\dot{V}O_{2peak}$, followed by exercise at 90% $\dot{V}O_{2peak}$, with subjects cycling at 100rpm until fatigue. Fatigue was defined as an inability to maintain pedal cadence above 60 rpm. The time to fatigue at 90% $\dot{V}O_{2peak}$ was used as an index of performance. Prolonged exercise was chosen since NAC attenuates fatigue during repetitive, low frequency electrical stimulation of limb muscle and diaphragm in humans (Reid et al. 1994; Travaline et al. 1997).

4.2.2.3. Experimental trials

The two experimental trials investigated the effects of intravenous NAC during voluntary, prolonged, submaximal exercise. These trials were conducted in a double-blind, randomized, counterbalanced design to determine the effects of NAC or saline (CON) infusion on exercise performance and K^+ regulation. For ethical reasons, the attending medical practitioner was

non-blinded. Arterialised venous blood was sampled from a dorsal hand vein (Fraser et al. 2002) at rest, during exercise at 15, 30, 45 min and fatigue and at 1, 2, 5, 10 and 30 min recovery. Expired gases were measured over 5 min periods at 10 min intervals during exercise. Subjects also consumed standard food packages for 24h prior to their two experimental trials (Appendix 6).

4.2.3. N-acetylcysteine Infusion

The NAC intravenous infusion protocol was as previously described in healthy subjects (Study 1). Briefly, an initial loading dose of 125 $mg.kg^{-1}.hr^{-1}$ was used for 15 min to increase plasma [NAC], followed by a constant infusion of 25 $mg.kg^{-1}.hr^{-1}$ to achieve a plateau in [NAC]. This was continued for 20 min prior to exercise and continued throughout exercise until fatigue. Study 1 demonstrated that this protocol considerably reduced the initial high NAC concentrations and avoided the associated adverse reactions compared to previously reported bolus NAC infusion protocols. Furthermore, this protocol preserved blood redox status during exercise, by attenuating the decline of reduced glutathione and the rise in oxidized glutathione in blood during exercise (Study 1).

4.2.4. Assessing reaction severity

Adverse reactions to NAC were continually assessed throughout the experimental trials. Reactions were graded as either none - no adverse effects were observed; mild - adverse events observed but not causing discomfort to subject and/or interruption of exercise protocol; moderate - adverse effects causing discomfort to subject and interruption of exercise protocol, but no active treatment after stopping infusion; or severe -adverse effects causing discomfort to subject, interruption of the exercise protocol and active treatment after stopping infusion. Subjects were also monitored for more serious adverse reactions including angiodema,

tachycardia, bronchospasm, dyspepsia and conjunctival irritation, as reported in previous studies (see references in Study 1).

4.2.5. Blood processing and analyses

Two blood samples were drawn in rapid succession at each sample point. The first 1 ml sample was taken using a syringe containing lithium heparin (RapidLyte, Chiron Diagnostics, MA, USA), for immediate plasma pH, gas and electrolyte analyses, including sodium ($[Na^+]$), chloride ($[Cl^-]$) and calcium concentrations ($[Ca^{2+}]$), using an automated analyzer (Ciba Corning 865, Bayer, MA, USA). A second 5 ml sample was used for measurement of blood haemoglobin concentration ([Hb]) and haematocrit (Hct) using an automated analyzer (Sysmex, K-800, Kobe, Japan), plasma K^+ concentration ($[K^+]$) and reduced and total thiols in blood and plasma. All blood and plasma were processed as previously reported (Study 1). NAC concentration was determined by high pressure liquid chromatography analysis (HPLC; Waters Associates, MA, USA), with fluorescence detection (Hitachi, Tokyo, Japan; Study 1). Glutathione and cysteine unfortunately could not be analyzed due to a laboratory freezer failure.

4.2.6. Calculations

The decline in plasma volume (ΔPV) from rest with exercise was calculated from changes in [Hb] and Hct (Harrison, 1985). The rise in plasma $[K^+]$ above rest ($\Delta[K^+]$) was calculated for each exercise value. The ratio of $\Delta[K^+]$ divided by cumulative work output during exercise ($\Delta[K^+]$-to-work ratio, $nmol.l^{-1}.J^{-1}$) was calculated as an index of plasma K^+ regulation (Harrison 1985; McKenna et al. 1993). Red blood cell NAC concentration ($[NAC]_{rbc}$) was calculated as described earlier (Study 1).

4.2.7. Statistical analyses

All data are presented as mean ± SEM, except anthropometric data. Single comparisons (eg. time to fatigue) were analyzed using a paired Student t-test. A one-way ANOVA with repeated measures was used for [NAC]. All other blood analyses were analyzed using a two-way (treatment, time) ANOVA with repeated measures on both factors. Post-hoc analyses used the Newman-Kuels test. Correlation analyses used least square linear regression. Significance was accepted at $P<0.05$. Individual coefficients of variation (CV) for time to fatigue were calculated for all subjects within the exercise protocol and averaged to obtain an overall CV (Jeukendrup et al. 1996).

4.3. Results

4.3.1. N-acetylcysteine and adverse reactions

Total NAC content infused was 5.09±0.23 g. One subject suffered nausea during NAC infusion and did not complete the exercise trial. With cessation of the NAC infusion, no treatment was required. Importantly, no severe adverse reactions were observed (Table 4.1).

4.3.2. Exercise performance variability and effects of NAC

The subjects' $\dot{V}O_{2peak}$ was 52.3±2.8 $ml.kg^{-1}.min^{-1}$ and their work rates corresponding to 70% and 90% $\dot{V}O_{2peak}$ were 156±12 W and 230±18 W, respectively. Good reproducibility was seen in the time to fatigue at 90% $\dot{V}O_{2peak}$ during the two variability trials, with a CV of 7.4±1.2% (Table 4.2). No significant differences were found between trials for time to fatigue at 90% $\dot{V}O_{2peak}$ (NAC 14.68±1.72 vs CON 12.5±2.06 min), or total work (NAC 627.7±69.8 kJ vs CON 588.2±30.9 kJ). However, individual time to fatigue data showed tremendous variability in responsiveness to NAC, with a CV of 31.8±16.1% (Figure 4.1A). This was clearly at odds

with their performance stability in the trials used to determine variability (Table 4.2). To attempt to explain this result, time to fatigue with NAC for each individual was expressed as a percentage change relative to CON trials and then plotted against aerobic power (Figure 4.1B). A strong positive correlation was found between percentage change in time to fatigue during NAC trials and $\dot{V}O_{2peak}$ ($r=0.78$; $P<0.05$; Figure 4.1B), indicating a different responsiveness to NAC in subjects with divergent $\dot{V}O_{2peak}$.

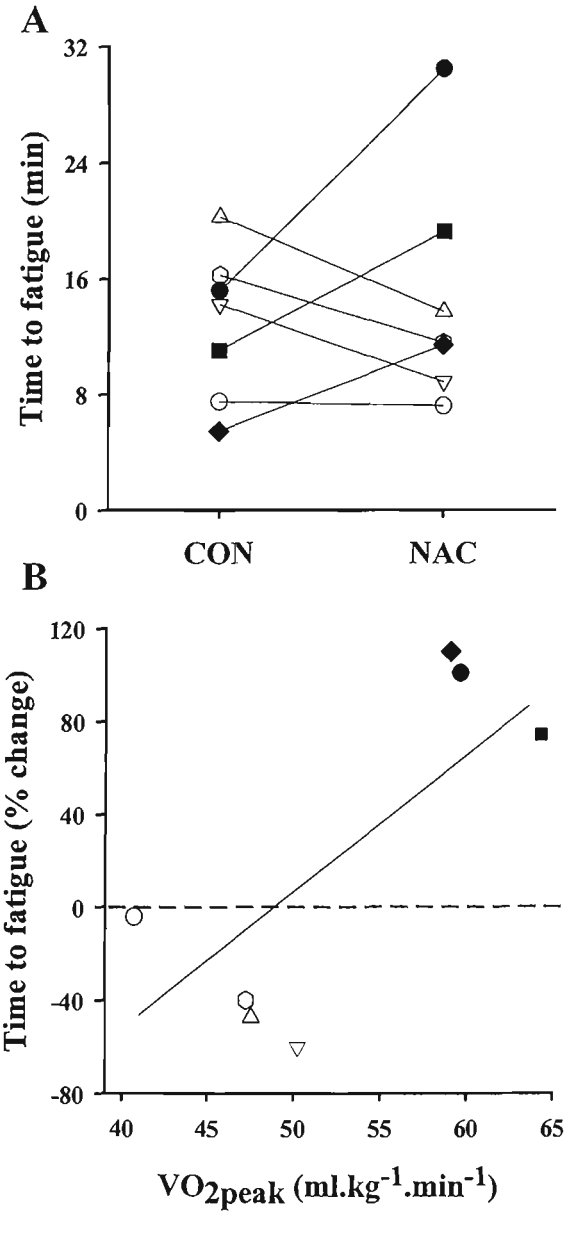
Table 4.1. Lack of severe adverse reactions with *N*-acetylcysteine (NAC) or saline (CON) infusion, before and during prolonged, submaximal exercise in eight healthy subjects.

	Reaction Frequency and Severity							
	None		**Mild**		**Moderate**		**Severe**	
Reaction	CON	NAC	CON	NAC	CON	NAC	CON	NAC
Vomiting	8	8	0	0	0	0	0	0
Erythema	4	2	4	6	0	0	0	0
Swelling	8	2	0	6	0	0	0	0
Flushing	8	7	0	1	0	0	0	0
Rash	7	8	1	0	0	0	0	0
Coughing	8	8	0	0	0	0	0	0
Nausea	8	7	0	0	0	1	0	0

Table 4.2. Individual time to fatigue during pre-experimental familiarization and variability prolonged, submaximal cycling exercise trials. Each exercise trial comprised 45 min at 70% $\dot{V}O_{2peak}$, then 90% $\dot{V}O_{2peak}$ continued to fatigue. Time to fatigue at 90% $\dot{V}O_{2peak}$ was used as an index of performance. Coefficient of variation (CV) was calculated from variability trial #1 and #2.

	Fatigue time (min)			CV (%)
Subject	Familiarization Trial	Variability Trial #1	Variability Trial #2	
1	9.6	11.6	12.6	5.8
2	11.2	15.2	14.1	4.9
3	19.3	24.6	25.6	2.8
4	9.8	13.7	12.4	6.8
5	23.2	21.9	18.5	11.8
6	8.6	13.0	14.2	6.2
7	12.3	17.4	15.1	9.9
8	3.6	5.30	6.2	11.1
Mean±SEM	12.2±2.1	15.3±2.1	14.8±1.9	7.4±1.1

Figure 4.1. Effect of *N*-acetylcysteine (NAC) and saline (CON) infusion on time to fatigue during prolonged exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, then to fatigue at 90% $\dot{V}O_{2peak}$ in healthy humans. (A) Individual time to fatigue at 90% $\dot{V}O_{2peak}$. (B) Scatter plot showing percentage change in time to fatigue at 90% $\dot{V}O_{2peak}$ with NAC relative to CON. A positive correlation was found between percentage change with NAC and $\dot{V}O_{2peak}$ ($y = 6.3x + 307.3$; $r = 0.78$; $P<0.05$). n=7. mean±SEM.



4.3. Plasma potassium

Plasma [K^+] was increased above pre-infusion levels throughout exercise, increased further at fatigue and then declined during recovery ($P<0.05$), returning to pre-infusion levels at 10 min (Figure 4.2). No significant differences were found between NAC and CON. The rise in plasma [K^+] during exercise ($\Delta[K^+]$) did not differ between 15-45 min, but was increased at fatigue ($P<0.005$). The $\Delta[K^+]$ during exercise at 70% $\dot{V}O_{2peak}$ tended to be lower with NAC ($P<0.07$) compared to CON, and at fatigue was lower in NAC compared to CON ($P<0.05$, Figure 4.3).

The $\Delta[K^+]$-to-work ratio decreased during exercise, being higher at 15 min than subsequent exercise times ($P<0.05$), with no differences between NAC and CON (Figure 4.4A). The $\Delta[K^+]$-to-work ratio and percentage change in time to fatigue tended to be inversely related ($r=-0.71$; $P<0.07$; Figure 4.4B).

Figure 4.2. Effect of NAC (▲) and CON (△) infusion on plasma [K^+] during prolonged exercise. Shaded bar denotes exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, then to fatigue (F) at 90% $\dot{V}O_{2peak}$. *Significant time main effect; greater than pre-infusion (-35 min, $P<0.005$). ** Significantly different to 45 min ($P<0.05$). n=7, mean±SEM.

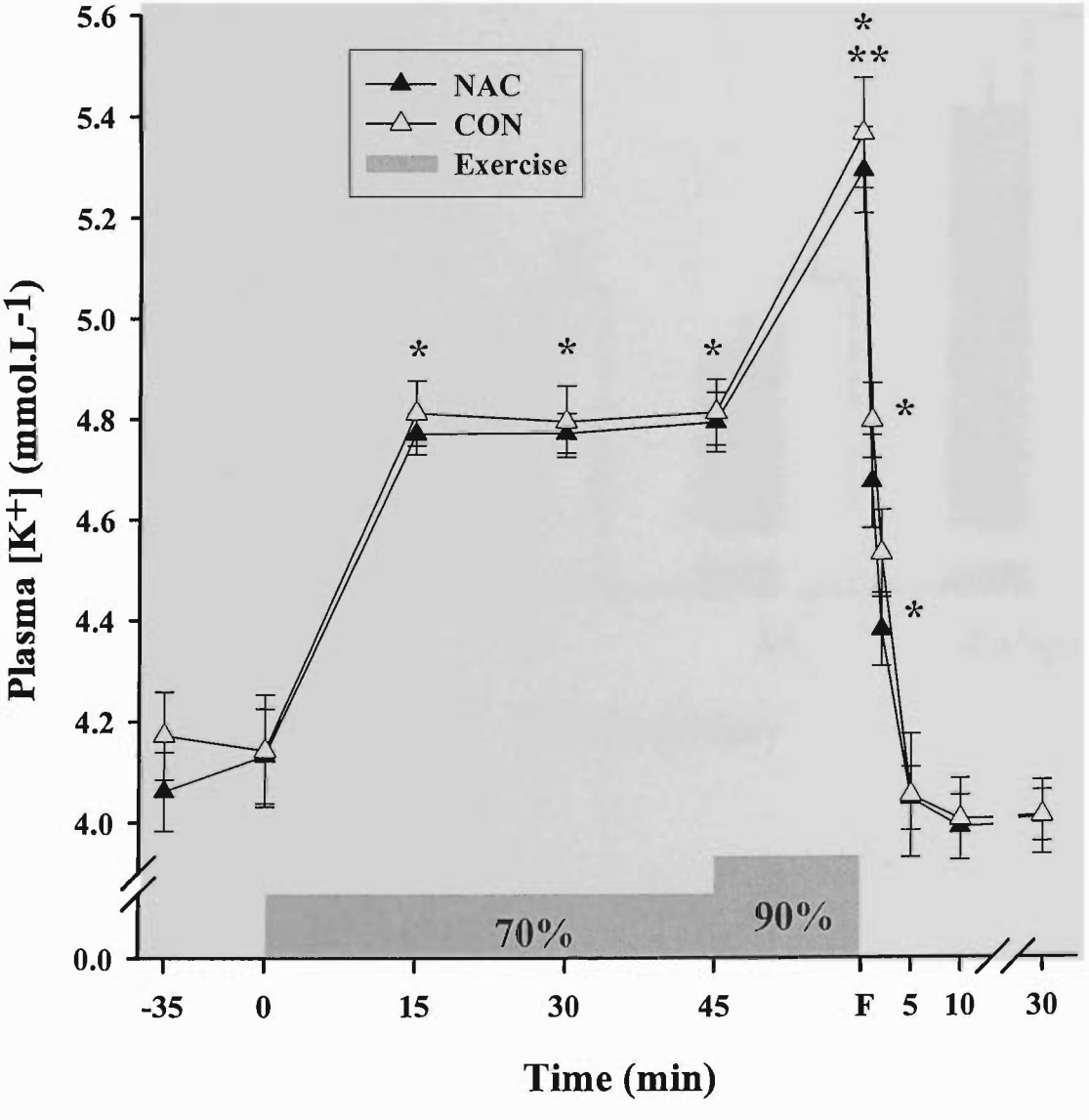


Figure 4.3. Effect of NAC and CON infusion on the rise in plasma potassium ($\Delta[K^+]$) during prolonged submaximal exercise. Open bars, CON; shaded bars, NAC. * Significant time main effect; greater than 15 min ($P<0.05$); † NAC < CON ($P<0.05$), n = 7, mean±SEM.

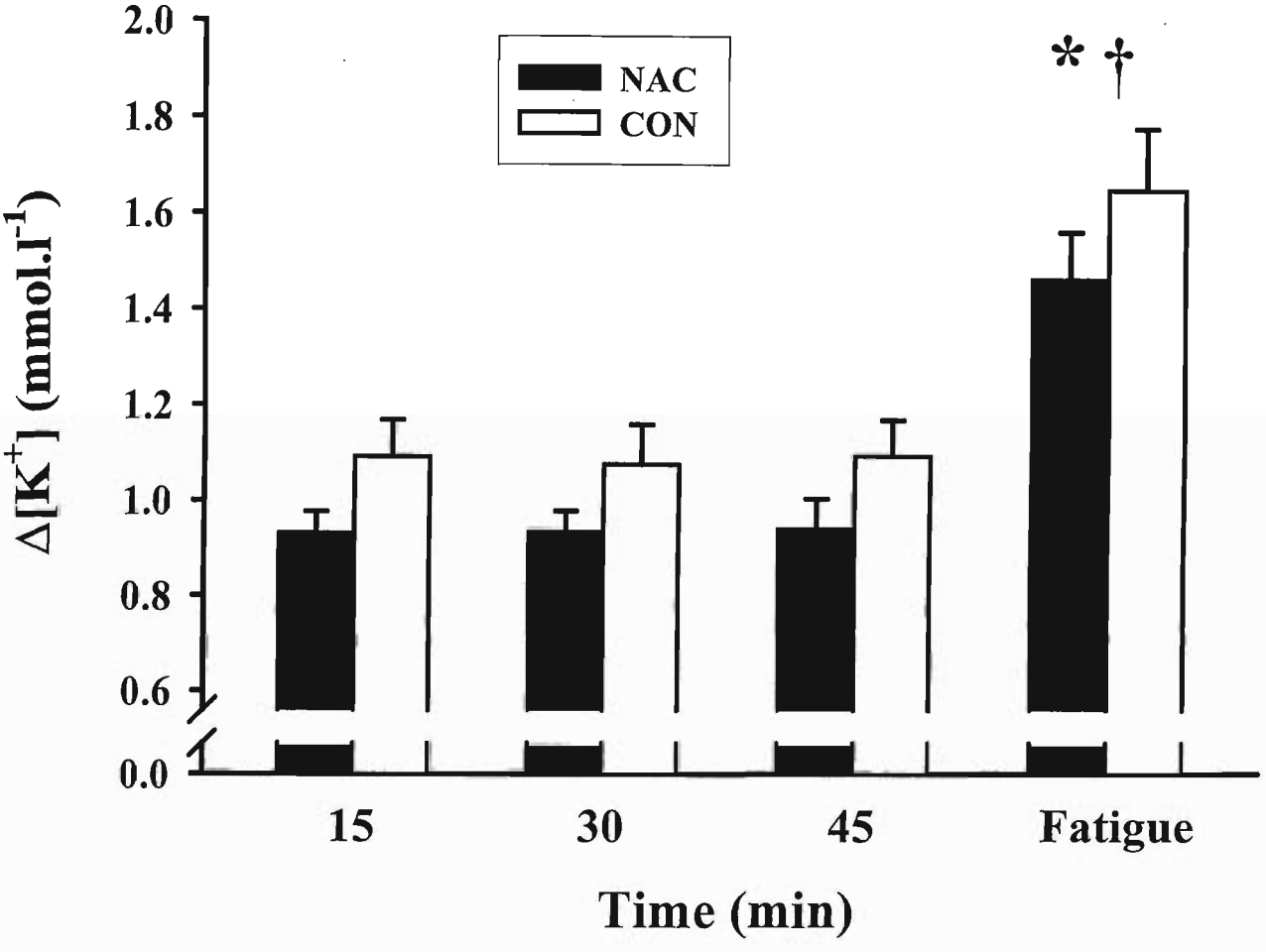
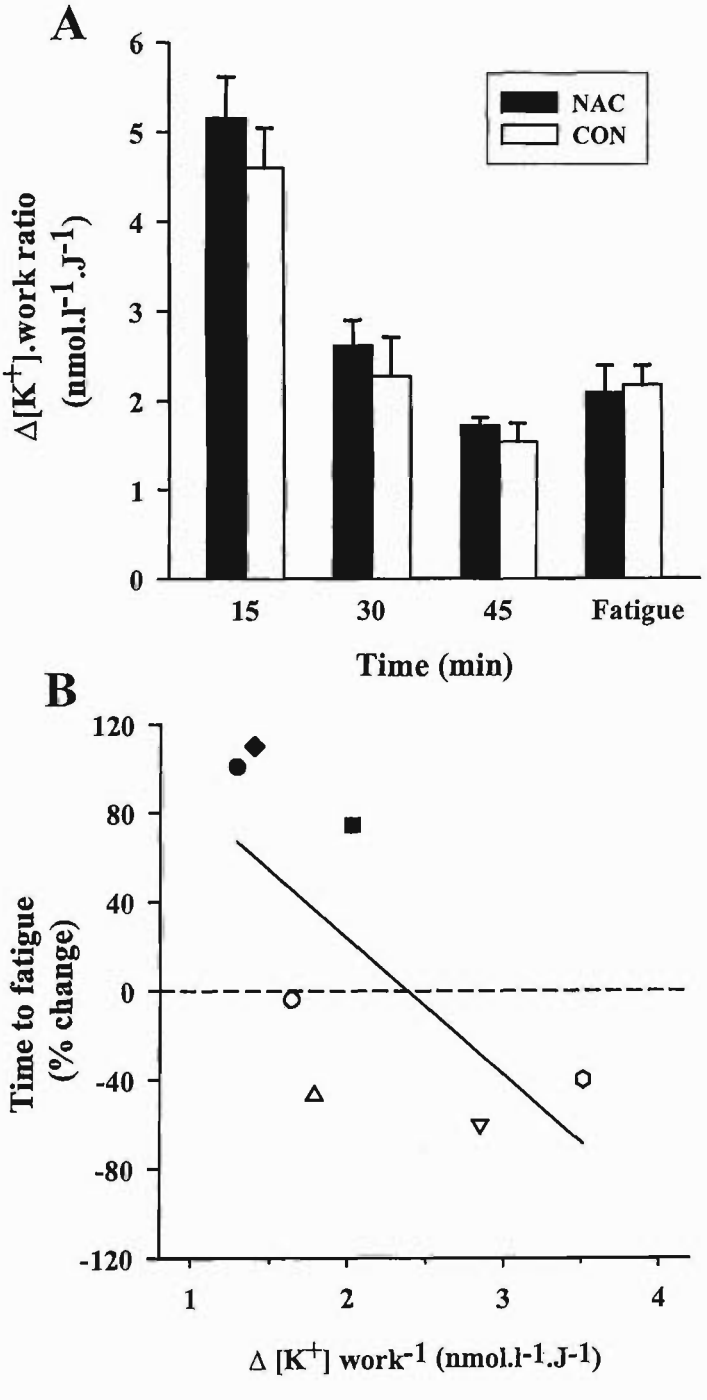


Figure 4.4. (A) Effect of NAC and CON infusion on $\Delta[K^+]$-to-work ratio during prolonged exercise. Open bars, CON; shaded bars, NAC. (B) Scatter plot showing percentage change in time to fatigue at 90% $\dot{V}O_{2peak}$ with NAC versus $\Delta[K^+]$-to-work ratio (y = -61.3x + 146.1; r = -0.71; P=0.07). * Significant time main effect; less than 15 min (P<0.005); n = 7, mean±SEM.



4.3.4. N-acetylcysteine

4.3.4.1. Plasma, blood and RBC NAC

During the 15 min loading infusion phase $[NAC]_{tp}$ increased progressively until a peak of 253.51±34.94 $mg.l^{-1}$ at 15 min ($P<0.05$, Figure 4.5A). During the maintenance infusion phase, $[NAC]_{tp}$ decreased to 182.02±29.70 $mg.l^{-1}$ immediately prior to exercise and then plateaued with no further changes during exercise. In recovery, $[NAC]_{tp}$ decreased rapidly from fatigue levels, but remained higher than pre-infusion at 30 min after stopping the infusion ($P<0.05$). A similar pattern of change was found for $[NAC]_{rp}$ (Figure 5B), except that the decline in $[NAC]_{rp}$ at 30 min recovery was not significant when compared to fatigue levels. An identical pattern of change was found for both total and reduced forms of NAC in whole blood and red blood cells during the loading, maintenance, exercise and recovery periods (Figure 4.5).

4.3.5. Fluid shifts, plasma electrolyte concentrations and acid-base status

Both [Hb] and Hct were higher than pre-infusion levels, and thus plasma volume declined, during exercise and until 30 min recovery ($P<0.05$, Table 4.3). No differences between NAC and CON were found for [Hb], Hct or ΔPV. Plasma $[Na^+]$ increased above pre-infusion levels throughout the exercise period until 2 min recovery ($P<0.05$, Table 4.3). Plasma $[Cl^-]$ did not differ during exercise or recovery, whilst plasma $[Ca^{2+}]$ was increased above pre-infusion levels at fatigue until 2 min recovery ($P<0.05$, Table 3). Compared to pre-infusion levels, plasma $[H^+]$ was increased, whereas plasma PCO_2 and $[HCO_3^-]$ fell, throughout exercise and recovery ($P<0.05$, Table 4.3). No differences between NAC and CON were found for these plasma electrolyte or acid-base variables.

Figure 4.5. Total (A) and reduced (B) *N*-acetylcysteine concentration ([NAC]) in plasma, blood and red blood cell prior to, during and after prolonged, submaximal exercise. Shaded bar denotes exercise comprising 45 min at 70% $\dot{V}O_{2peak}$, then to fatigue (F) at 90% $\dot{V}O_{2peak}$. n=7; mean±SEM. Pre-exercise infusion lasted 35 minutes. Significant time main effect with all times greater than pre-infusion ($P<0.05$, asterisks not shown for clarity).

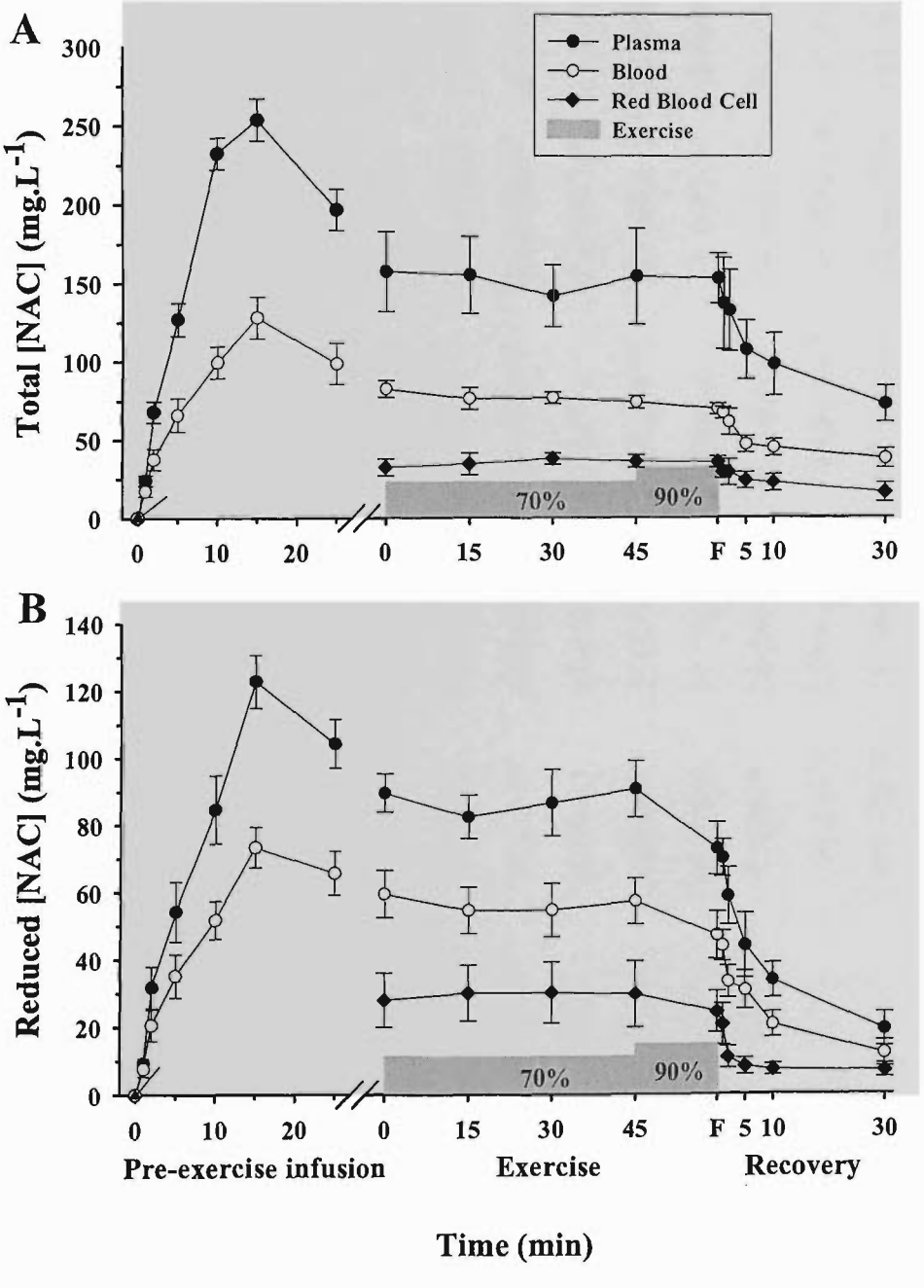


Table 4.3. Haematology, calculated fluid shifts, plasma acid-base variables and electrolyte concentrations during prolonged, submaximal exercise prior to, during and following *N*-acetylcysteine (NAC) and saline (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-Exercise	Exercise Time				Recovery
				15	30	45	Fatigue	30 min
[Hb] ($g.dl^{-1}$)	CON	14.6±0.8	14.8±0.2	15.5±0.1 *	15.6±0.2 *	15.5±0.2 *	15.8±0.1 *	14.4±0.2
	NAC	14.7±0.3	14.7±0.3	15.6±0.3 *	15.5±0.2 *	15.5±0.2 *	15.8±0.2 *	14.4±0.2
Hct (%)	CON	44.5±0.6	45.0±0.6	47.2±0.7 *	47.1±0.5 *	47.0±0.6 *	47.9±0.7 *	43.8±0.4
	NAC	44.5±0.8	44.8±0.8	47.4±0.7 *	47.0±0.7 *	46.7±0.5 *	48.2±0.6 *	43.2±0.2
ΔPV (%)	CON	-	-1.9±0.4	-10.4±0.8 *	-10.6±0.5 *	-9.7±0.7 *	-13.7±0.9 *	3.3±2.1 *
	NAC	-	-1.0±0.7	-10.9±0.9 *	-9.4±1.2 *	-9.0±1.6 *	-13.3±1.4 *	4.7±2.1 *
$[H^+]$($nmol.l^{-1}$)	CON	39.1±0.4	38.4±0.3	42.0±0.7 *	40.8±0.8 *	39.8±0.9 *	45.2±1.4 *	42.6±0.3 *
	NAC	39.1±1.0	37.8±1.1	42.4±0.5 *	41.5±0.5 *	40.7±0.5 *	46.0±1.3 *	42.0±0.6 *
$[HCO_3^-]$ ($mmol.l^{-1}$)	CON	26.4±0.5	25.4±0.6	23.1±0.4 *	23.0±0.3 *	23.9±0.3 *	18.0±0.9 *	23.1±1.4 *
	NAC	26.0±0.4	25.0±0.2	22.4±0.4 *	22.4±0.6 *	23.2±0.6 *	18.0±0.9 *	24.1±0.5 *
PCO_2 (mmHg)	CON	41.3±0.6	39.7±0.5	40.5±0.4	39.1±0.5 *	35.9±0.7 *	34.7±1.4 *	34.8±1.0 *
	NAC	43.2±0.7	39.5±0.8	40.3±0.4	38.4±1.2 *	38.8±1.4 *	34.0±1.4 *	30.1±2.5 *
$[Na^+]$ ($mmol.l^{-1}$)	CON	139.2±1.3	138.8±1.0	140.5±1.1 *	141.5±0.7 *	140.4±0.8 *	143.0±0.8 *	138.5±0.7
	NAC	139.1±0.8	139.9±0.6	141.8±0.8 *	141.8±0.8 *	141.6±0.5 *	143.4±0.7 *	138.9±0.6
$[Ca^{2+}]$ ($mmol^{-1}$)	CON	1.21±0.01	1.23±0.01	1.23±0.01	1.28±0.01	1.27±0.01	1.30±0.02 *	1.20±0.02
	NAC	1.23±0.01	1.22±0.01	1.24±0.01	1.25±0.01	1.25±0.01	1.31±0.01 *	1.20±0.01

* Significant main effect for time: different from pre-infusion $P<0.05$. Mean ±SEM, n=7 NAC, n=8 CON.

4.4. Discussion

4.4.1. Ergogenic effects of NAC are dependent upon $\dot{V}O_{2peak}$

This study hypothesized a performance enhancement with NAC, based on the findings that NAC infusion attenuated fatigue during low frequency electrical stimulation of human tibialis anterior muscle (Reid et al. 1994). Although there was no effect on time to fatigue in the whole group, the markedly different responsiveness of individuals to NAC necessitated more careful evaluation. For the first time, this study demonstrates that the effects of NAC on prolonged exercise are dependent upon $\dot{V}O_{2peak}$, with a tendency for time to fatigue to be increased in the fitter subjects. Further research in a larger group of subjects with homogenous $\dot{V}O_{2peak}$ is required to validate these intriguing preliminary findings.

4.4.2. NAC improves K^+ regulation during exercise

An interesting finding was the reduced $\Delta[K^+]$ at fatigue, which is consistent with the hypothesis that NAC would enhance K^+ regulation during exercise. A possible underlying mechanism for such an effect might reflect ROS inhibition of Na^+,K^+-ATPase activity, as shown in cardiac (Vinnikova et al. 1992), sarcolemmal vesicles (Kukreja et al. 1990) and skeletal muscle-derived L6 cells (Sen et al. 1995). Importantly, acute exercise also depresses maximal Na^+,K^+-ATPase activity in skeletal muscle (Fowles et al. 2002; Fowles et al. 2002; Fraser et al. 2002), which may also be consequent to increased ROS. Thus, one possible explanation for the lesser $\Delta[K^+]$ during exercise with NAC is an attenuation of the ROS effect on skeletal muscle Na^+,K^+-ATPase activity. However, no studies have investigated the effects of ROS and/or antioxidants on human skeletal muscle Na^+,K^+-ATPase activity. The non-significant negative correlation between time to fatigue and $\Delta[K^+]$-to-work ratio ($r = -0.71$, $n=7$) does suggest a possible link between exercise performance and K^+ regulation with NAC.

The lack of significance may reflect a Type II error due to the small sample size. This possible relationship should be explored in further studies.

4.4.3. Other potential mechanisms

It is possible that several factors may underlie the observed relationship between percentage change in time to fatigue with NAC and $\dot{V}O_{2peak}$. Study 1 showed that this intravenous NAC infusion protocol had marked effects on blood redox status during intense, intermittent exercise, such that NAC attenuated the decline in reduced glutathione (GSH) and rise in oxidized glutathione (GSSG) with exercise. This study demonstrates almost identical [NAC] changes in plasma, whole blood and red blood cell occurred during prolonged, as in intense, exercise. Therefore, it is likely that NAC could have exerted similar effects on blood redox status in prolonged exercise. Exogenous glutathione supplementation enhanced glutathione biosynthesis and increased swimming endurance by up to 141% in rats (Cazzulani et al. 1991; Novelli et al. 1991). Thus, enhanced glutathione synthesis (Study 1) is consistent with an apparent increased time to fatigue in fitter individuals with NAC infusion.

Fiber type specific effects may also underlie the observed positive relationship between percentage change in time to fatigue with NAC and $\dot{V}O_{2peak}$. Individuals with high VO_{2peak} possess a higher proportion of slow twitch fibers (Li et al. 2002), which show a greater increase in isometric force with the reducing agent dithiothreitol, than fast muscle (Plant et al. 2001). Slow twitch fibers also have increased mitochondrial content (Gollnick et al., 1986), which is considered a major source of ROS (Cadenas and Davies 2000). It is likely that individuals with a high $\dot{V}O_{2peak}$ produce a greater amount of ROS than subjects with a lower $\dot{V}O_{2peak}$, which would be exacerbated when cycling at a higher absolute exercise intensity (Lovlin et al. 1987; Alessio and Goldfarb 1988). This may be counterbalanced by increased

skeletal muscle endogenous antioxidant enzyme activities with training (Alessio and Goldfarb 1988). However, there is reduced protection of the mitochondria against ROS after endurance training (Tonkonogi et al. 2000). Thus, NAC protection of susceptible oxidative fibers from ROS could underlie the relationship between percentage change in time to fatigue with NAC and $\dot{V}O_{2peak}$. Further studies should address possible different fiber type effects.

Sarcoplasmic reticulum Ca^{2+} regulation is sensitive to redox modulation, with ROS enhancing ryanodine receptor opening, which may also diminish with prolonged exposure (Reid 2001). Hydrogen peroxide also decreases myofibrillar Ca^{2+} sensitivity and force, which is reversed by the reducing agent dithiothreitol (Andrade et al. 1998). This effect is more prominent in slow twitch fibers and may be glutathione-dependent (Posterino et al. 2003). In addition, Ca^{2+} uptake is decreased with increased ROS concentration (Posterino and Lamb 1996; Xu et al. 1997). Whether these effects occur in human muscle remain to be elucidated and are worthy of further investigation. Finally, NAC had no effects on fluid shifts, acid base status or plasma electrolyte concentrations during exercise.

4.4.4. Conclusions

NAC infusion effects on fatigue during prolonged cycling exercise may be dependent upon $\dot{V}O_{2peak}$. Furthermore, NAC improved K^+ regulation as evidenced by a decreased rise in plasma $[K^+]$. These intriguing findings demand further studies for verification and to determine possible mechanisms.

CHAPTER 5: STUDY 3 – PART I

N-ACETYLCYSTEINE ENHANCES MUSCLE CYSTEINE AND GLUTATHIONE AVAILABILITY AND ATTENUATES FATIGUE DURING PROLONGED EXERCISE IN ENDURANCE-TRAINED INDIVIDUALS.

5.1. Introduction

In skeletal muscle, an integrated system of endogenous antioxidants and protein and non-protein thiol compounds, including superoxide dismutase, glutathione peroxidase, catalase and glutathione minimizes the accumulation of reactive oxygen species (ROS; Sen 1995). However, this antioxidant capacity is small and is overwhelmed during exercise, resulting in increased ROS (Davies et al. 1982; Jackson et al. 1985; Bailey et al. 2003). Increased ROS production accelerates muscle fatigue in rat (Reid et al. 1992) and canine diaphragm (Nashawati et al. 1993; Supinski et al. 1997) and mouse limb skeletal muscle (Barclay and Hansel 1991). Therefore, enhancing the skeletal muscle antioxidant capacity may be beneficial for muscle performance.

N-acetylcysteine (NAC), a thiol-containing compound, attenuated fatigue in rabbit and rat diaphragm (Shindoh et al. 1990; Diaz et al. 1994; Supinski et al. 1995). Furthermore, NAC infusion attenuated fatigue of human muscle, during both low frequency electrical stimulation of the tibialis anterior muscle (Reid et al. 1994) and inspiratory resistive loading of the diaphragm (Travaline et al. 1997). This thesis has developed an NAC infusion model for use during voluntary whole body exercise in humans, finding no effect on intense, intermittent exercise performance (Study 1). However, during prolonged, submaximal exercise, time to fatigue was greater in three well-trained individuals during NAC infusion, with performance change induced by NAC correlated to $\dot{V}O_{2peak}$ (Study 2). This suggested a performance

enhancing effect of NAC, but the limited sample size precluded definitive conclusions. Therefore, the first hypothesis tested in this study was that NAC infusion would increase time to fatigue during prolonged submaximal exercise performance in a homogenous group of well-trained individuals.

The mechanism(s) underlying this potential ergogenic effect during prolonged exercise were not investigated (Study 2), but also deserve investigation. The first problem is that it is not known whether NAC crosses the sarcolemma and thereby directly affects muscle ROS or redox status during exercise. Study 1 and Study 2 found that NAC infusion increased erythrocyte NAC concentration ([NAC]) during exercise, suggesting that NAC might also enter myocytes, but there are no reports of muscle NAC. Thus, the second hypothesis tested was that NAC infusion would increase NAC content in both skeletal muscle and erythrocytes during prolonged exercise.

NAC potentially reduces the deleterious effects of ROS by direct scavenging of ROS (Aruoma et al. 1989) and/or supplying cysteine (CYS) for enhanced glutathione synthesis (Cotgreave 1997). Study 1 demonstrated that NAC maintained blood redox status during high intensity, intermittent exercise, indicated by an attenuated decline in reduced glutathione (GSH) and rise in oxidized glutathione (GSSG). It is therefore possible that muscle ROS and glutathione status might also be protected by NAC during prolonged exercise, but these effects of NAC in human muscle are unknown.

A complicating factor is that the reported effects of exercise on human skeletal muscle glutathione are scarce and their findings conflicting, with reports of GSH being unchanged (Cooper et al. 1986) or decreased (Svensson et al. 2002), and of GSSG being increased (Cooper et al. 1986) or unchanged (Sahlin et al. 1992). A critical role is suggested for endogenous GSH in alleviating exercise-induced oxidative stress and affecting exercise

performance (Sen et al. 1994). In rats, exogenous glutathione administration enhanced glutathione synthesis and increased swim performance by up to 141% (Cazzulani et al. 1991; Novelli et al. 1991), whilst glutathione deficiency reduced endurance time by ~50% (Sen et al. 1994).

Cysteine is a precursor to glutathione synthesis and increased intracellular CYS availability enhanced intracellular glutathione (Holdiness 1991; Cotgreave 1997; Sen and Packer 2000). Supplementation with a CYS donor increased performance during sprint cycling (Lands et al. 1999), suggesting that enhanced glutathione synthesis can augment performance in humans. No studies have comprehensively investigated the effects of prolonged exercise or NAC infusion on muscle total glutathione (TGSH), GSH, CYS and cystine. Therefore, this study also tested the hypotheses that fatiguing exercise would decrease muscle GSH and increase GSSG, and that each of TGSH, GSH and CYS would be augmented by NAC infusion.

5.2. Methods

5.2.1. Subjects

Eight healthy males (age, 27.1±5.6 yr; body mass, 76.7±10.9 kg; height, 180.3±5.4 cm; mean±SD) volunteered for the study after being informed of all risks and giving written informed consent (Appendix 7). The subjects were endurance trained, completing either running or cycling activity, 4-5 times per week for 1-2 hours, for a minimum of 2 years. Subjects refrained from vigorous activity and avoided ingesting caffeine, alcohol, or other drugs and also consumed standard food packages (Appendix 6) for 24 h prior to their two experimental trials. Ethical approval was obtained from the Victoria University of Technology Human Research Ethics Committee (Appendix 3).

5.2.2. Exercise trials

5.2.2.1. Overview

Subjects attended the laboratory on six separate occasions, separated by a 7 d period. All exercise trials were completed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands). *Peak oxygen consumption:* Subjects first completed an incremental exercise test to determine their peak oxygen consumption ($\dot{V}O_{2peak}$), with all equipment, calibration and procedures as previously detailed (Li et al. 2002; Study 1).

5.2.2.2. Prolonged, Submaximal Exercise

An identical exercise protocol was used as previously described (Study 2), with subjects cycling at 70% $\dot{V}O_{2peak}$ for 45 min and then to volitional fatigue at 90% $\dot{V}O_{2peak}$ (Study 2).

5.2.2.3. Experimental trials

The two experimental prolonged, submaximal exercise trials were conducted in a double-blind, randomized, cross-over design, to determine the effects of NAC (Parvolex™, Faulding Pharmaceuticals) or saline (CON) infusion on exercise performance and on muscle and blood thiols (Study 1 and 2). For ethical reasons, the attending medical practitioner was non-blinded. To prevent possible un-blinding of experimenters due to the NAC odour, all ampoules and syringes containing NAC and saline were handled and sealed in a room separate to the laboratory. The medical practitioner also removed the cannulae post experiment.

5.2.2.4. N-acetylcysteine Infusion

The NAC intravenous infusion protocol comprised an initial loading dose of 125 $mg.kg^{-1}.hr^{-1}$ for 15 min to increase plasma [NAC], followed by a constant infusion of 25 $mg.kg^{-1}.hr^{-1}$ to achieve a plateau in [NAC], with exercise commencing after 20 min of constant infusion

(Study 1 and 2). NAC infusion was continued throughout exercise until fatigue and any adverse reactions were assessed according to the scale previously detailed (Study 2).

5.2.3. Blood processing and analyses

A 20G catheter was inserted into a dorsal hand vein for arterialised venous blood sampling and a 22G catheter inserted into a superficial median forearm vein for infusion of either NAC or saline. Arterialized venous blood was sampled from a dorsal hand vein (Fraser et al. 2002) at 0, 1, 2, 5, 10, 15, 25 and 35 min during the pre-exercise infusion period. Further samples were taken at 15, 30, 45 min and at fatigue during exercise and during recovery at 1, 2, 5, 10 and 30 min. A 5 ml sample was used for measurement of reduced and total thiols in blood and plasma with all processing and analyses as previously detailed (Study 1). Blood and plasma thiol concentrations, including NAC, total and reduced glutathione and cysteine, were analyzed by high-pressure liquid chromatography (HPLC; Waters Associates, MA, USA; Study 1), with fluorescence detection (Hitachi, Tokyo, Japan). Due to laboratory freezer failure, for 3 subjects whole blood total and reduced cysteine could not be measured, and consequently cystine and RBC concentrations could not be calculated.

5.2.4. Muscle biopsy sampling and analyses

5.2.4.1. Muscle biopsy sampling

After injection of a local anaesthetic (1% Xylocaine) into the skin and fascia, three small incisions were made in the mid-portion of the vastus lateralis muscle. Muscle samples were taken pre-infusion, after 45 min of exercise and at fatigue and analyzed for muscle thiols, including NAC, glutathione and cysteine. The subject's contralateral leg was biopsied on their second experimental trial. The time taken for the subject to stop pedalling at 45 min, undergo a

muscle biopsy and recommence cycling did not differ between trials (CON 55±7s vs. NAC 53±7 s).

5.2.4.2. Muscle thiol analyses

The muscle sample was immediately blotted on filter paper, frozen in liquid nitrogen and stored at -80°C for later analysis of muscle thiols. Approximately 20 mg frozen muscle was homogenized for 20 s in 200 ml 20mM monobromobimane (mBrB) using a hand-held homogeniser (Omni 1000; Omni International, Gainesville, VA). For analysis of total thiols 100 ml of homogenate was added to 200 ml 4mM DTT in an eppendorf tube. The sample was immediately vortexed and left at room temperature for 10 min to reduce oxidized thiols. Free thiols were derivatized with 50 ml of 20 mM mBrB, vortexed and incubated at room temperature in darkness for 10 min. Proteins were precipitated with 25 ml sulphosalicylic acid (50% wt, vol) and immediately vortexed and centrifuged at 1000 g for 5 min. The supernatant (50 ml) was injected into the HPLC for analysis. For reduced thiols 100 ml of muscle homogenate was mixed with 250 ml H_2O and 25 ml sulphosalicylic acid (50% wt, vol) in an eppendorf tube, immediately vortexed and centrifuged at 1000 g for 5 min, before injection of 50 ml of the supernatant into the HPLC for analysis. The HPLC method and conditions were identical to those used for plasma and blood thiol analyses (Study 1).

The HPLC mobile phase was methanol (18:82 vol/vol) and 20 mM KH_2PO_4 at pH 2.9 and 5 mM octanesulphonic acid running through a 150 by 3.9 mm NovapakC_{18} column (Waters Associates, MA, USA) at 1 ml.min^{-1}, with fluorescence detection at 400 nm excitation and 475 nm emission. This gives baseline separation of thiol compounds from each other and the reagent peaks, with a quantitation limit of approximately 100 nM and coefficient of determination of <5% for each. Total concentrations of the thiols were determined in a similar

manner except that the oxidized thiols were reduced with DTT before a second derivitisation with mBrB was performed. The supernatant was then extracted and injected for HPLC analysis.

5.2.5. Calculations

Thiol concentrations in red blood cells were calculated as previously detailed (Study 1 and 2). Hct could not be measured in one subject due to technical difficulties and consequently RBC NAC data are reported for seven subjects. Individual coefficients of variation (CV) for time to fatigue were calculated for all subjects within the exercise protocol and averaged to obtain an overall CV (Jeukendrup et al. 1996). This allowed clearer delineation of NAC effects from typical test variation.

5.2.6. Statistical analyses

All data are presented as mean ± SEM, except anthropometric data. Single comparisons (eg. time to fatigue) were analyzed using a paired Student t-test. A one-way ANOVA with repeated measures was used for blood and plasma [NAC]. All other blood and muscle analyses were analyzed using a two-way (treatment, time) ANOVA with repeated measures on both factors. Post hoc analyses were conducted using Student Newman-Kuels test. Significance was accepted at $P<0.05$.

5.3. Results

5.3.1. Exercise performance variability and effects of NAC

The subjects' $\dot{V}O_{2peak}$ was 65.6±2.2 $ml.kg^{-1}.min^{-1}$ and their submaximal workrates were 239±20 W and 336±25 W, corresponding to 71±1.3% and 92±1.9% $\dot{V}O_{2peak}$, respectively. Time to fatigue at 92% $\dot{V}O_{2peak}$ was reproducible during the two variability trials (CV 5.6±0.6%, Table 5.1) and no trial order effect was observed (data not shown). NAC increased,

by 26.3±9.1% ($P<0.05$), time to fatigue at 92% $\dot{V}O_{2peak}$ (CON 5.3±0.7 vs NAC 6.4±0.6 min) and thus also work done (CON 104.9±15.3 vs. NAC 126.5±11.6 kJ).

Table 5.1. Individual time to fatigue during pre-experimental prolonged sub-maximal exercise trials. Each exercise trial comprised 45 min at 71% of peak oxygen consumption ($\dot{V}O_{2peak}$) and then 92% $\dot{V}O_{2peak}$ continued to fatigue. Time to fatigue at 92% $\dot{V}O_{2peak}$ was used as an index of performance.

	Fatigue time (min)		**CV (%)**
Subject	**Variability trial#1**	**Variability trial#2**	
1	5.32	5.91	7.4
2	4.94	5.40	6.3
3	6.06	5.62	5.3
4	5.05	4.67	5.5
5	3.25	3.56	6.4
6	4.15	4.58	7.0
7	5.06	5.21	2.1
8	4.79	5.13	4.9
Mean±SEM	4.8±0.3	5.0±0.3	5.6±0.6

5.3.2. N-acetylcysteine and adverse reactions

No moderate or severe adverse reactions to NAC were observed during the pre-infusion, exercise or recovery periods (Table 5.2).

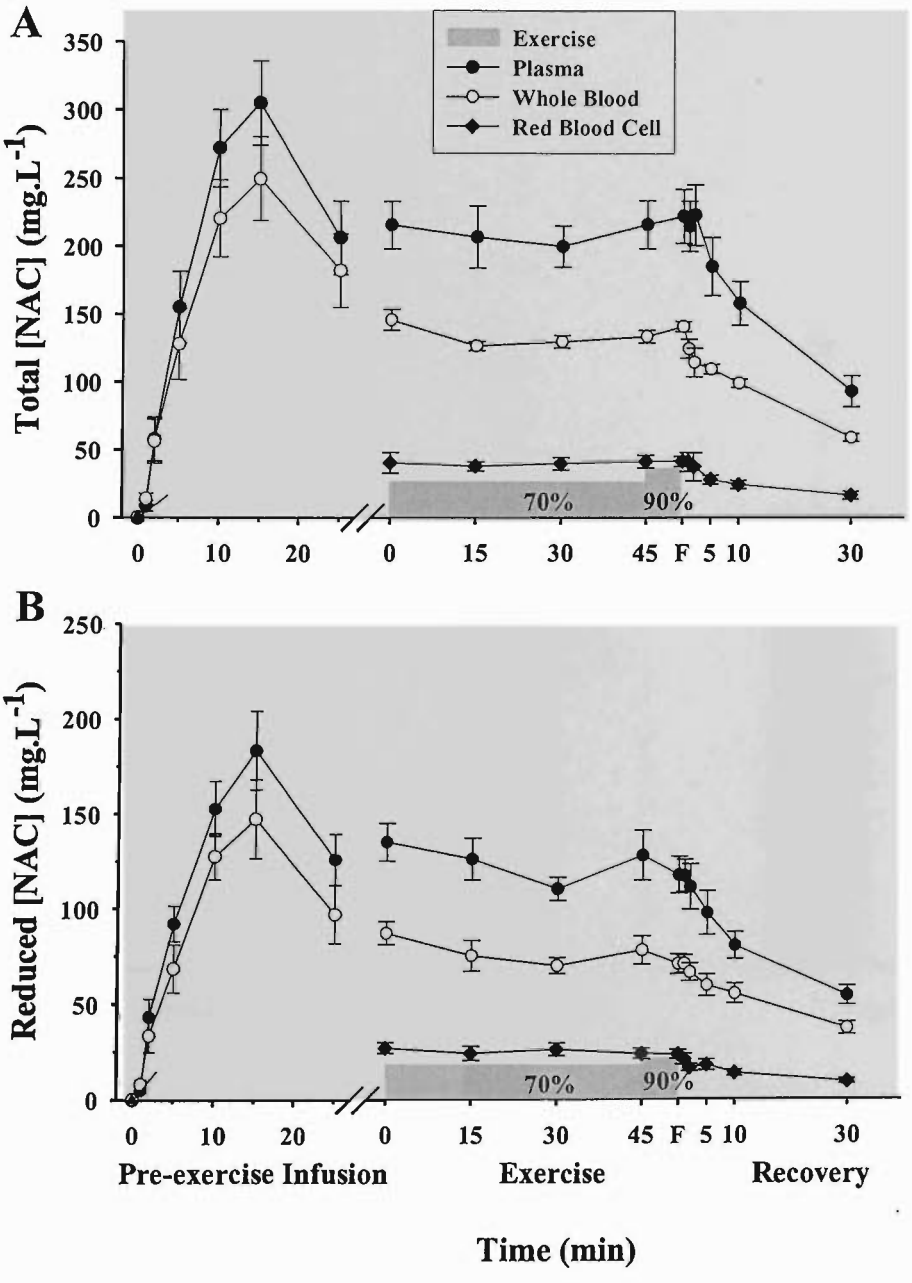
Table 5.2. Lack of severe or moderate adverse reactions with *N*-acetylcysteine (NAC) or saline (CON) infusion, before and during prolonged submaximal exercise. n=8.

	Reaction frequency and severity							
Reaction	**None**		**Mild**		**Moderate**		**Severe**	
	NAC	CON	NAC	CON	NAC	CON	NAC	CON
Erythema	6	8	2	0	0	0	0	0
Swelling	7	8	1	0	0	0	0	0
Flushing	6	7	2	1	0	0	0	0
Coughing	7	8	1	0	0	0	0	0
Sweating	5	4	3	4	0	0	0	0
Itchy skin	7	7	1	1	0	0	0	0

5.3.3. Plasma, blood and RBC NAC

The [NAC] measured in whole blood and plasma, and calculated for RBC is shown in Figure 5.1. During the 15 min loading infusion phase total plasma [NAC] increased progressively until a peak of 305.2±38. 6 $mg.l^{-1}$ at 15 min ($P<0.005$), decreased during the maintenance infusion phase ($P<0.005$), to 214.7±17.5 $mg.l^{-1}$ immediately prior to exercise and then plateaued, with no further changes during exercise. In recovery, total plasma [NAC] decreased rapidly from fatigue levels, but remained higher than pre-infusion at 30 min post-infusion ($P<0.05$). A similar pattern of change was found for reduced plasma [NAC] (Figure 5.1). An identical pattern of change was found for both total and reduced forms of NAC in whole blood during the loading, maintenance, exercise and recovery periods. Red blood cell NAC also followed a similar pattern during exercise and recovery periods being 38.9 $mg.l^{-1}$ and 23.8 $mg.l^{-1}$ during exercise for total and reduced forms, respectively (Figure 5.1).

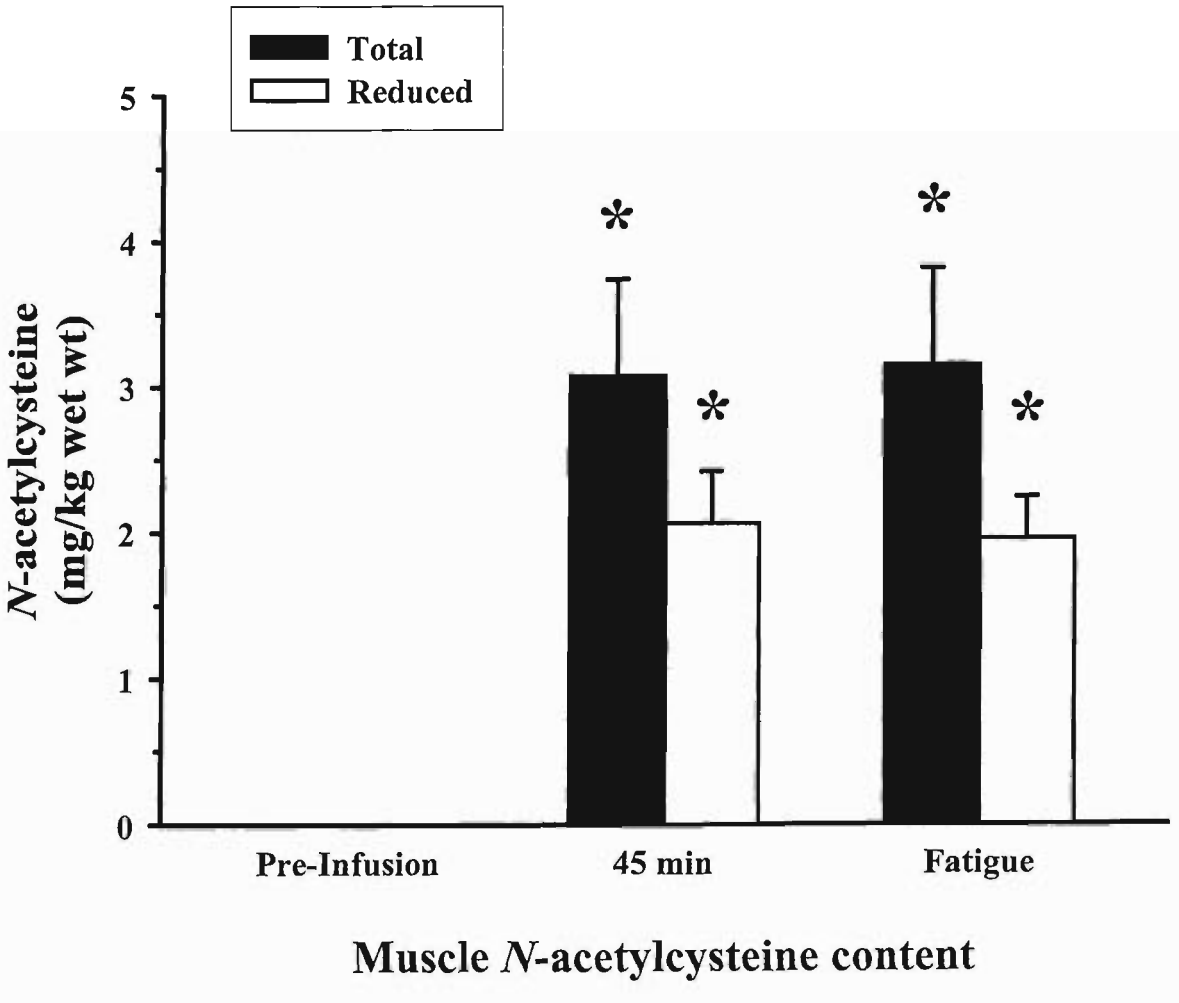
Figure 5.1. Total (A) and reduced (B) N-acetylcysteine concentration ([NAC]) in plasma, whole blood and red blood cells (RBC) prior to, during and after prolonged, submaximal exercise. Shaded bar denotes exercise comprising 45 min at 71% $\dot{V}O_{2peak}$, then to fatigue (F) at 92% $\dot{V}O_{2peak}$. n=8 except for total and reduced RBC where n=7; mean±SEM. Pre-exercise infusion lasted 35 minutes. Significant time main effect, with all times greater than pre-infusion ($P<0.05$, asterisks not shown for clarity).



5.3.4. Muscle NAC

NAC was not detected in muscle at pre-infusion or during CON trials. During NAC infusion, muscle total and reduced NAC were elevated at 45 min and at fatigue, with 72.8±7.3 % and 68.7±6.3 % present in the reduced form, respectively (Figure 5.2).

Figure 5.2. Increased muscle NAC content with NAC infusion. Open bars, Reduced NAC; shaded bars, Total NAC. NAC was not detected in muscle samples during CON trials or pre-infusion. * Significant time main effect; greater than pre-infusion ($P<0.005$); n = 8, mean±SEM.



5.3.5. Muscle thiols

5.3.5.1. Cysteine and Cystine

Muscle total and reduced CYS, as well as cystine were unchanged from pre-infusion levels during CON and were not different between NAC and CON prior to infusion (Figure 5.3). However, NAC markedly increased both total and reduced CYS and cystine in muscle compared to pre-infusion levels ($P<0.001$). Muscle total and reduced CYS and cystine were greater at 45 min and fatigue with NAC than in CON ($P<0.005$, Figure 5.3).

5.3.5.2. Glutathione

Muscle TGSH was decreased by 37.7±17.9% at 45 min of exercise and remained depressed at fatigue (Figure 5.4; $P<0.05$). Exercise tended to decrease muscle GSH ($P=0.06$), whilst muscle cGSSG was unaltered with exercise (Figure 5.4). Both muscle TGSH and GSH were higher during NAC compared to CON ($P<0.05$; Figure 5.4). No change in muscle GSH:TGSH, GSSG:TGSH or GSSG:GSH ratios were found with either exercise or NAC (data not shown).

Figure 5.3. Increased muscle (A) total cysteine; (B) reduced cysteine and (C) calculated cystine with NAC infusion during prolonged, submaximal exercise. Open bars, NAC; shaded bars, CON * Significant time main effect; greater than pre-infusion ($P<0.05$); # Significant interaction effect: no difference at pre-infusion but NAC > CON at 45 min and Fatigue ($P<0.05$), n = 8, mean±SEM.

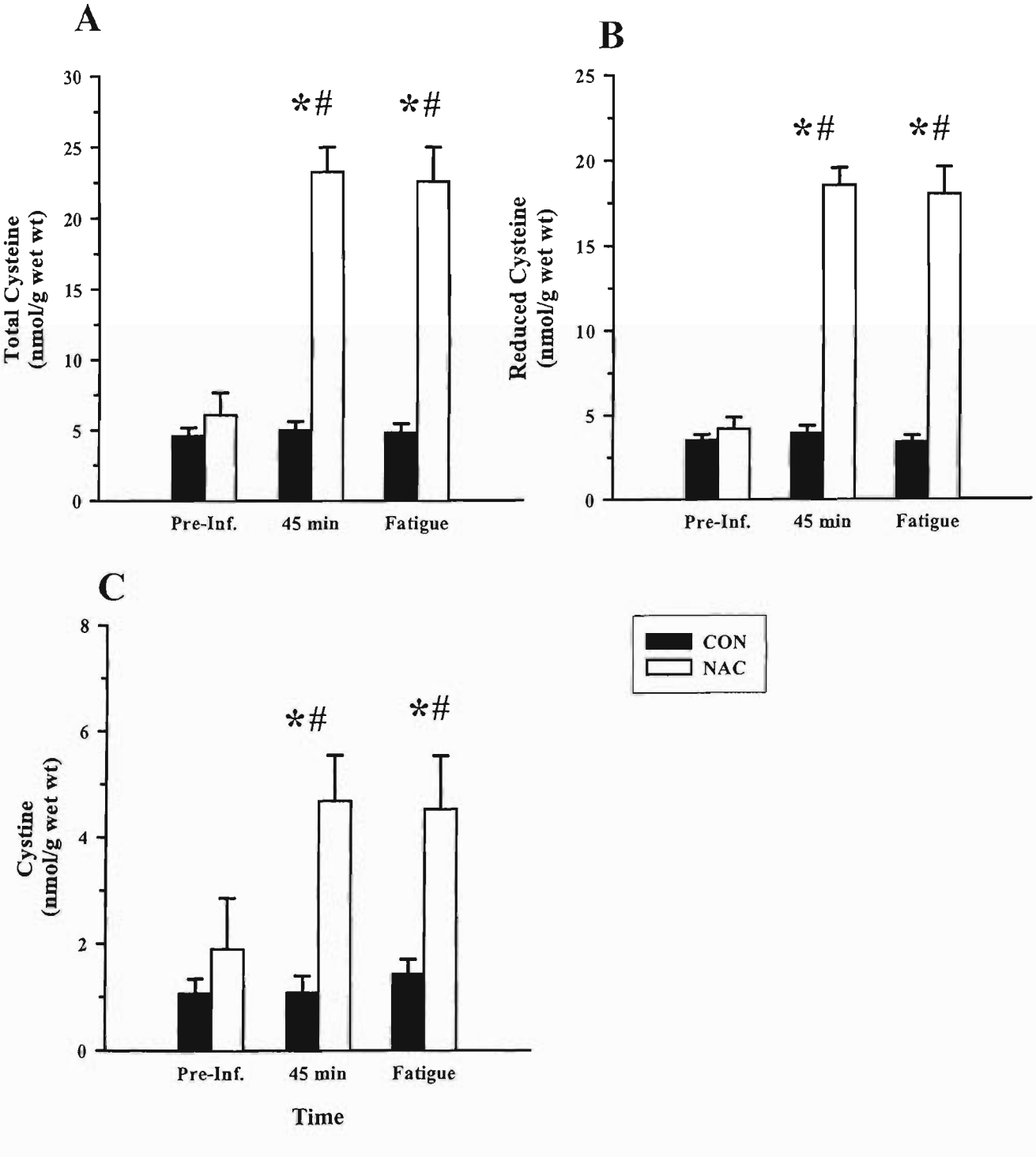
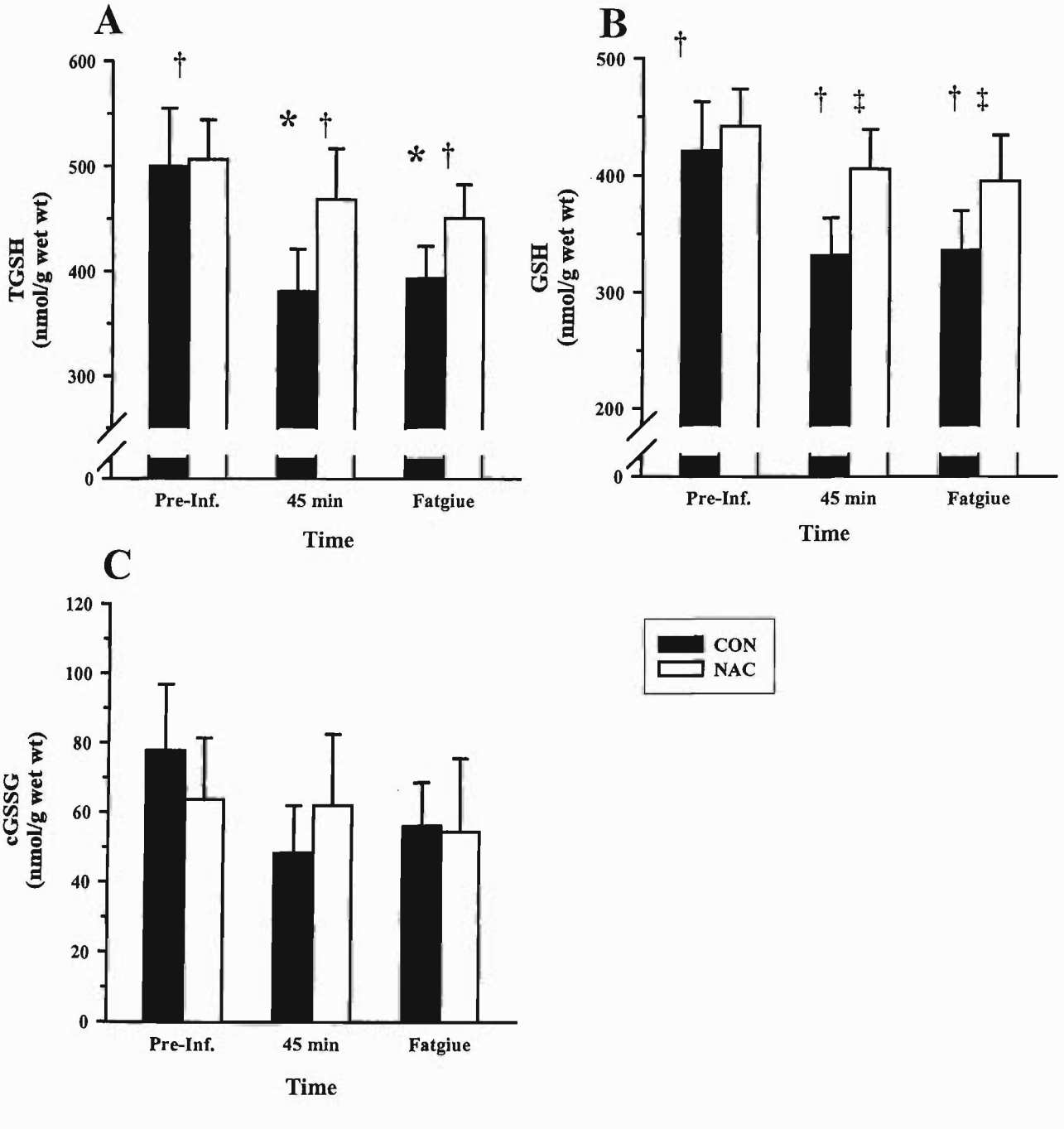


Figure 5.4. Effects of NAC infusion on muscle (A) total glutathione (TGSH); (B) reduced glutathione (GSH) and (C) calculated oxidised glutathione (cGSSG) contents prior to, during and after prolonged, submaximal exercise. Open bars, NAC; shaded bars, CON. * Significant time main effect; less than pre-infusion ($P<0.05$); † Significant treatment main effect, NAC > CON, ($P<0.05$), ‡ Time main effect; less than pre-infusion ($P=0.06$), n = 8, mean±SEM.



5.3.6. Blood Thiols

5.3.6.1. Cysteine and cystine

No change in total and reduced [CYS] or cystine from pre-infusion levels occurred in CON at any time (Tables 5.3 and 5.4). Prior to infusion, no differences were found between NAC and CON in whole blood, plasma or RBC total and reduced cysteine ([CYS], Table 5.3) or cystine (Table 5.4). However, NAC increased total and reduced [CYS] and cystine in whole blood, plasma and RBC by up to 3-fold above pre-infusion levels ($P<0.005$). Consequently, NAC increased total and reduced [CYS] and cystine in whole blood, plasma and RBC immediately prior to and throughout the exercise and recovery periods ($P<0.005$).

5.3.6.2. Glutathione

In contrast to muscle, whole blood [TGSH] was unchanged from pre-exercise infusion, at any time during exercise and recovery (Figure 5.5). Whole blood [GSH] was unchanged during the pre-exercise infusion, but [GSH] had declined at 15 min during exercise ($P<0.05$) and remained lower during the subsequent exercise and recovery periods ($P<0.05$). Whole blood [cGSSG] was unchanged during pre-infusion, increased during exercise and remained elevated above pre-infusion levels at 30 min recovery ($P<0.05$, Figure 5.5). No differences between treatments were found for blood [TGSH], [GSH] or [cGSSG] (Figure 5.5).

The whole blood GSH:TGSH ratio (data not shown) did not change during the pre-infusion period but decreased at 15 min exercise and remained lower throughout the remainder of exercise and throughout the recovery period, compared to pre-infusion ($P<0.05$). No differences in the GSH:TGSH ratio were observed at any time between CON or NAC.

5.3.7. Plasma Glutathione

Plasma glutathione levels were too low to be reliably detected, consistent with other studies (Gohil et al. 1988).

Table 5.3. Total and reduced cysteine concentrations ([CYS]; $\mu mol.l^{-1}$) in whole blood, plasma and erythrocytes (RBC) during prolonged submaximal exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-Exercise	Exercise				Recovery
				15 min	30 min	45 min	Fatigue	30 min
Blood total [CYS]	CON	37.85±2.25	36.61±3.72	41.38±5.02	38.56±4.88	37.71±3.48	41.75±2.30	35.89±3.41
	NAC	29.80±3.58	138.08±19.41*§	139.24±17.31*§	134.93±17.66*§	138.55±17.98*§	148.01±25.75*§	74.08±4.62*§
Blood reduced [CYS]	CON	8.37±1.79	9.26±1.46	9.21±1.28	10.74±1.21	10.38±1.81	10.10±0.95	9.40±2.05
	NAC	7.12±0.57	51.34±6.15*§	45.50±3.83*§	44.64±3.89*§	49.32±5.43*§	41.57±2.60*§	31.49±6.77*§
Plasma total [CYS]	CON	51.33±2.65	49.41±4.52	55.14±4.66	53.25±3.32	49.54±3.66	59.31±5.09	53.38±5.16
	NAC	44.97±8.00	204.93±24.52*§	195.13±39.04*§	181.98±26.83*§	199.26±27.96*§	175.00±24.99*§	116.02±12.54*§
Plasma reduced [CYS]	CON	6.59±0.80	8.98±0.62	10.93±0.97	12.69±1.49	10.54±1.47	11.79±1.11	9.61±1.15
	NAC	7.73±1.22	75.07±7.54*§	61.49±5.90 *§	61.69±5.54*§	74.81±6.16*§	63.16±3.11*§	51.24±9.10*§
RBC total [CYS]	CON	22.78±3.18	21.93±4.20	25.36±6.13	21.21±4.44	25.97±4.73	26.76±0.84	16.80±1.31
	NAC	18.38±7.42	20.67±4.48	30.44±8.05*§	30.99±5.43*§	34.08±2.53*§	29.67±5.83*§	19.12±7.67*§
RBC reduced [CYS]	CON	11.17±3.39	9.83±3.51	6.56±2.99	7.34±2.59	9.09±3.76	7.49±2.00	9.58±4.65
	NAC	8.28±1.56	7.24±2.74§	16.13±9.73*§	19.30±7.90*§	14.59±4.90*§	13.90±5.04*§	5.18±0.90*§

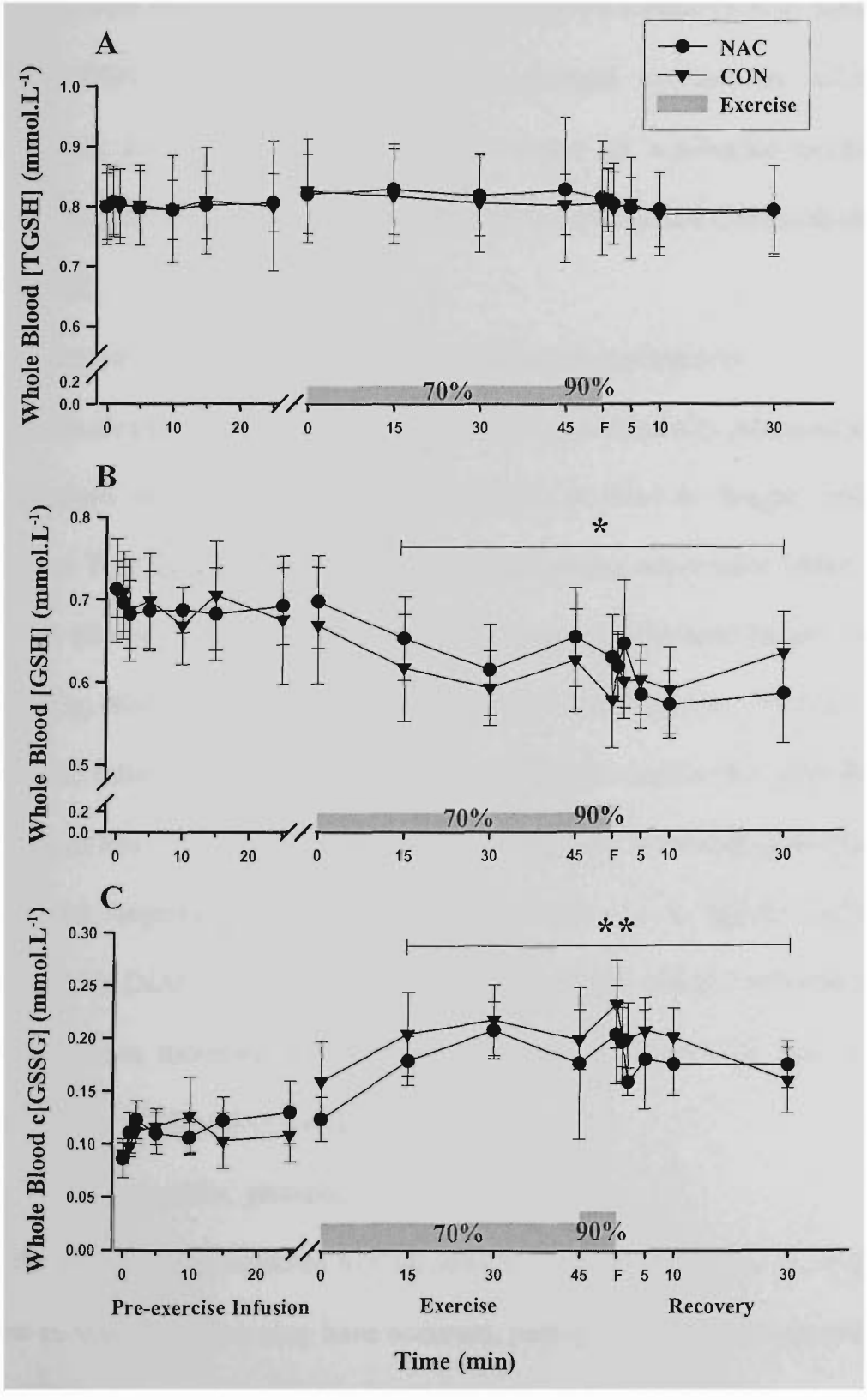
Values are means ± SEM, n=8 for total and reduced plasma and n=5 for total and reduced whole blood and erythrocytes (RBC). *significant interaction effect greater than pre-infusion ($P<0.005$) § significant interaction effect NAC>CON, ($P<0.005$).

Table 5.4. Calculated cystine concentrations ($\mu mol.l^{-1}$) in whole blood, plasma and erythrocytes (RBC) during prolonged submaximal exercise prior to, during and following *N*-acetylcysteine (NAC) and placebo (CON) infusion.

Variable	Treatment	Pre-Infusion	Pre-Exercise	Exercise Time				Recovery
				15 min	30 min	45 min	Fatigue	30 min
Whole blood	CON	29.07±2.90	27.32±2.56	32.17±4.38	28.36±3.67	27.32±1.96	31.65±2.11	26.49±3.87
	NAC	22.68±3.05	86.74±15.48*§	94.41±21.00*§	90.28±16.22*§	89.24±15.23*§	106.44±25.89*§	42.60±9.51*§
Plasma	CON	44.73±2.52	40.50±4.33	44.21±4.24	39.99±2.82	38.50±2.91	47.54±4.35	44.97±4.84
	NAC	36.70±6.84	129.86±19.99*§	131.52±31.69*§	170.09±24.47*§	124.04±23.72*§	112.79±23.84*§	64.60±15.99*§

Values are means ± SEM, n=8 for total and reduced plasma and n=5 for total and reduced whole blood and erythrocytes (RBC). * Significant interaction effect: different from pre-infusion; § significant interaction effect NAC>CON ($P<0.005$).

Figure 5.5. No effect of NAC infusion on whole blood [TGSH], [GSH] and [cGSSG] prior to, during and after prolonged, submaximal exercise. NAC (•) and CON (▼); Shaded bar denotes exercise comprising 45 min at 71% $\dot{V}O_{2peak}$, then to fatigue (F) at 92% $\dot{V}O_{2peak}$. n = 8, mean±SEM. * Significant time effect; less than pre-infusion ($P<0.05$); ** Significant time effect; greater than pre-infusion ($P<0.05$).



5.4. Discussion

This study provides several novel findings in regard to muscle glutathione and performance in humans. First, these findings demonstrated a dramatic enhancement of submaximal exercise performance with NAC infusion, with time to fatigue increased by 26%. Second, an increase in NAC was detected in human skeletal muscle with NAC infusion. This study also reports for the first time the effects of NAC infusion on whole blood TGSH, GSH and CYS during prolonged exercise in endurance trained individuals. Finally, this study provides evidence for a potential mechanism for this ergogenic effect, with an increased muscle CYS, TGSH and GSH availability observed with NAC.

5.4.1. Increased muscle NAC and increased muscle performance

This data clearly demonstrate that NAC infusion substantially enhanced performance in well-trained individuals, with a 26% increase in time to fatigue during prolonged exercise. This finding confirms the earlier preliminary observation based on only a few athletes (Study 2) and provides the first evidence of attenuated fatigue by NAC during voluntary, whole body exercise in humans. These findings also show, for the first time, that NAC infusion increased NAC content in skeletal muscle. It is probable that a small portion of this NAC detected in muscle was due to contamination by blood. However, given the magnitude of increase in muscle NAC, it is highly likely that muscle intracellular [NAC] was elevated. Furthermore Study 1 and 2 also demonstrated that NAC infusion increased RBC [NAC]. Together these indicate that an elevation in muscle intracellular [NAC] is highly likely.

5.4.2. Muscle cysteine, glutathione and exercise.

As the proportion of oxidized to total muscle glutathione was ~15%, it is possible that some ex-vivo oxidation may have occurred, perhaps due to the small delay in freezing

muscle samples. However, this is an inevitable limitation when obtaining muscle samples via needle biopsy and our proportion of oxidized to total muscle glutathione is consistent with others (Svensson et al. 2002). These findings show that muscle TGSH content decreased during submaximal cycling exercise, which differs to other studies (Sahlin et al. 1992; Svensson et al. 2002). The reason for this discrepancy is unclear, but may be related to the fact that the subjects utilised in this study were more highly trained than subjects used in previous studies (Sahlin et al. 1992; Svensson et al. 2002). However, the findings of a tendency for a decrease in muscle GSH ($P=0.06$) and no change in muscle cGSSG during submaximal exercise are in agreement with others (Sahlin et al. 1992; Svensson et al. 2002). More importantly, this study demonstrates for the first time that NAC was able to increase both muscle TGSH and GSH during whole-body exercise. The maintenance of GSH is dependent on regeneration from GSSG by glutathione reductase (Sen et al. 1992). However, this seems unlikely as a mechanism responsible for the elevation in GSH, as there was no change in GSSG with exercise or NAC, therefore implicating another mechanism. This was probably due to increased availability of the glutathione pre-cursor, CYS, as evidenced by increases in each of muscle, RBC and plasma.

Cysteine can be actively transported into cells (Bannai and Tateishi 1986) and increased intracellular CYS availability enhances intracellular GSH (Sen et al. 1992). Therefore, the increased extracellular CYS consequent to NAC infusion increases the intracellular availability of this amino acid to regenerate GSH (Sen et al. 1992; Sen and Packer 2000). In addition, this study also reports that NAC infusion increased muscle NAC. NAC is rapidly deacylated to produce free CYS (Deneke 2000) and this may have also contributed to the 3-fold increase in muscle cysteine. Although the increase in muscle CYS and cystine with NAC could also be due to contamination with blood, this is

unlikely as there was no increase in CON trials. Thus, these results strongly suggest that the greater muscle GSH with NAC may be consequent to increased intra- and extracellular CYS availability. Furthermore, NAC attenuated the decline in muscle TGSH, which being predominantly in the reduced form (Deneke 2000), would have also increased muscle GSH availability.

These results are consistent with conclusions from other studies that increasing glutathione availability improved exercise performance in rats (Cazzulani et al. 1991; Novelli et al. 1991; Sen et al. 1994) and in humans (Lands et al. 1999). This study further demonstrates that the greater muscle GSH and TGSH during exercise with NAC was associated with increased time to fatigue. However, this effect was already evident by 45 minutes of exercise and suggests that factors additional to increased CYS and GSH availability may have also contributed to muscle fatigue.

5.4.3. Muscle ROS scavenging

Numerous studies implicate ROS production as an important factor in muscle fatigue (Barclay and Hansel 1991; Reid et al. 1992; Diaz et al. 1994; Lawler et al. 1997; Supinski et al. 1997). Although this study did not measure ROS concentration, NAC is known to scavenge a number of ROS including hypochlorus acid, hydroxyl radical and hydrogen peroxide (Aruoma et al. 1989). Furthermore, NAC is rapidly deacetylated to cysteine, which itself is a known free radical scavenger (Cotgreave 1997). Thus, in addition to supporting glutathione synthesis, NAC can scavenge ROS within skeletal muscle, which may be another possible mechanism explaining enhanced performance with NAC. Further research is required to determine whether NAC scavenges ROS *in vivo* and whether this is sufficient to improve exercise performance.

Increased GSH availability with NAC could have indirect effects on antioxidant enzyme activity. GSH acts as a substrate for glutathione peroxidase activity (GPX), which

decreases hydrogen peroxide (H_2O_2) and also leads to the formation of the deleterious hydroxyl radical. It is possible that the increased GSH availability with NAC may also have supported the function of GPX, thus facilitating the removal of H_2O_2. However, this study did not measure GPX activity and the concentration of H_2O_2 in human muscle is unknown.

5.4.4. Other potential mechanisms of NAC on exercise performance

Although enhanced glutathione status and NAC scavenging of ROS may have contributed to increased exercise performance, other potential mechanisms may also be responsible. NAC blunts unpleasant sensations produced during exhaustive exercise (Travaline et al. 1997). Whether this occurred in this study cannot be determined. However, this seems unlikely due to the large effect of NAC on muscle TGSH, GSH and cysteine, which were already evident at 45 min, where work was matched to CON trials.

It is possible that elevated muscle antioxidant capacity may have exerted a protective effect on key ion transporting or ion channel proteins in muscle, including the Na^+,K^+ATPase enzyme, the sarcoplasmic reticulum Ca^{2+} release channel (ryanodine receptor) and Ca^{2+}-ATPase enzyme, each of which are deleteriously affected by increased ROS production (Kourie 1998). Recent reports have found that the activity of Na^+,K^+ATPase, Ca^{2+}-ATPase and also the rate of Ca^{2+} release are depressed at fatigue in human muscles (Booth et al., 1997; Fraser et al. 2002; Li et al. 2002) with increased ROS a proposed mechanism. Animal models demonstrate that ROS deleteriously affects Na^+,K^+ATPase activity (Kukreja et al. 1990; Sen et al. 1995), ryanodine receptor function (Oba et al. 1996; Posterino and Lamb 1996) and Ca^{2+} ATPase activity (Xu et al. 1997; Andrade et al. 2001). Whether NAC blunts these effects in human skeletal muscle remains to be elucidated.

5.4.5. Blood and plasma thiols

The decreased whole blood [GSH] and increased [cGSSG] with prolonged exercise is consistent with other human studies (Gohil et al. 1988; Sen et al. 1994; Groussard et al. 2003). However, the lack of modification with NAC contrasts the previous findings during high intensity, intermittent exercise (Study 1), where a clear effect of NAC on blood thiol status during exercise was found. This may reflect differences in the training status of subjects and the exercise intensity and duration. Study 1 utilised untrained individuals, whereas this study utilised endurance trained individuals. Endurance trained individuals have increased blood antioxidant activity compared to sedentary subjects (Marzatico et al. 1997) and training increases blood antioxidant enzyme capacity (Miyazaki et al. 2001) which may reduce the overall oxidative stress in erythrocytes (Ji et al. 1993). Increasing exercise intensity results in increased blood glutathione oxidation (Sastre et al. 1992) and ROS production (Bailey et al. 2003), which might also explain an effect of NAC on blood thiol status during intense (Study 1) but not in submaximal exercise. However, more profound changes in blood glutathione may have occurred in blood draining the exercising muscles and hence NAC effects on GSH and cGSSG may be greater at these sites.

5.4.6. Conclusions

This study demonstrates for the first time that NAC infusion during prolonged submaximal exercise increased muscle NAC, skeletal muscle cyst(e)ine and glutathione availability during exercise and substantially enhanced performance in well trained individuals.

CHAPTER 6: STUDY 3 – PART II

N-ACETYLCYSTEINE INFUSION ENHANCES SKELETAL MUSCLE NA^+,K^+-ATPASE ACTIVITY AND PLASMA K^+ REGULATION DURING PROLONGED SUBMAXIMAL EXERCISE IN WELL-TRAINED INDIVIDUALS.

6.1 Introduction

The Na^+,K^+-ATPase enzyme in skeletal muscle is primarily located in the sarcolemmal and t-tubular membranes and is critical in maintaining trans-membrane sodium (Na^+) and potassium (K^+) concentration gradients and, consequently, preserving membrane excitability (Overgaard et al., 1997, Clausen, 2003; Sejersted and Sjøgaard, 2000). Both exercise and electrical stimulation may increase Na^+,K^+-ATPase activity in skeletal muscle by up to 18-22-fold above rest (Everts and Clausen, 1994, McKenna, 2003, Clausen, 2003). This increase is important since impaired muscle force development is evident at high interstitial potassium concentrations ([K^+]; Bouclin et al., 1995; Juel, 1988; Clausen et al., 1993; Cairns et al., 1995; Lannergren and Westerblad, 1986). Furthermore studies in humans have linked muscle K^+ loss with fatigue (Verburg et al., 1999; Sjøgaard et al., 1985).

The importance of the Na^+,K^+-ATPase enzyme in muscle fatigue is demonstrated in studies that have utilised the Na^+,K^+-ATPase specific inhibitor, ouabain. In rat skeletal muscle, ouabain-induced inhibition of muscle Na^+,K^+-ATPase accelerated muscle fatiguability and slowed recovery, whereas hormonal or excitation-induced stimulation of Na^+,K^+-ATPase activity delayed muscle fatiguability and enhanced recovery (Clausen and Everts, 1991). Several recent reports have demonstrated that exercise depressed maximal Na^+,K^+-ATPase activity in human skeletal muscle, as measured by the 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity assay, including intense

(Fraser et al, 2002 Aughey et al., 2004; Fowles et al., 2002) and prolonged exercise (Leppik et al, 2004; Sandiford et al., 2004). These persistent findings of depressed maximal Na^+,K^+-ATPase activity with exercise suggest a role in loss of muscle K^+ and Na^+ homeostasis and thus an important link to muscle fatigue.

The precise mechanism(s) underlying this depressed maximal Na^+,K^+-ATPase activity in skeletal muscle during exercise remain to be elucidated. However, a role for exercise-induced increase in ROS production in depressed Na^+,K^+-ATPase enzyme mediated muscle function seems plausible. Several studies report that ROS depressed Na^+,K^+-ATPase activity (Kukreja et al., 1990; Vinnikova et al., 1992; Sen et al., 1995; Kim and Akera, 1987). In canine myocardium sarcolemmal vesicles, Na^+,K^+-ATPase activity was inhibited by hydrogen peroxide (H_2O_2), which was dependent on both the concentration and time of incubation (Kukreja et al., 1990). In guinea pig cardiac sarcolemma, ischaemia-reperfusion induced a reduction in Na^+,K^+-ATPase activity, which was attenuated by the endogenous antioxidants superoxide dismutase (SOD) and catalase (CAT; Kim and Akera, 1987). In skeletal muscle derived L6 cells, the Na^+,K^+-ATPase enzyme was also suggested to be under redox modulation (Sen et al., 1995). Whilst tert-butyl hydroperoxide activated Na^+,K^+-ATPase activity at 75 mM, there was a significant inhibition of Na^+,K^+-ATPase activity at 200 mM (Sen et al., 1995). These studies suggest that increased ROS production may be involved in the depression of maximal Na^+,K^+-ATPase activity in skeletal muscle during exercise and thereby contribute to disturbed muscle ionic homeostasis and muscle fatigue.

No studies have investigated the possible interaction between ROS and Na^+,K^+-ATPase activity in skeletal muscle during exercise. Study 1 demonstrated that NAC infusion increased the rise in plasma $[K^+]$ ($\Delta[K^+]$) and rise in plasma K^+ concentration to work ratio ($\Delta[K^+]$-to-work^{-1} ratio) during intense, intermittent exercise (Chapter 3). This

indicates that NAC impaired K^+ regulation during high intensity, intermittent exercise in untrained individuals. In contrast, Study 2 demonstrated that NAC enhanced plasma K^+ regulation during prolonged submaximal exercise, as evidenced by a decreased $\Delta[K^+]$. Furthermore, the $\Delta[K^+]$-to-work^{-1} ratio tended to be inversely related to time to fatigue (Study 2, Chapter 4). Therefore, this study tested two hypotheses. First, that NAC would attenuate the decline in skeletal muscle maximal Na^+,K^+-ATPase activity observed during prolonged, submaximal exercise in well-trained individuals. Second, that NAC would enhance plasma K^+ regulation during prolonged exercise, as evidenced by a decreased rise in plasma $[K^+]$ ($\Delta[K^+]$) and $\Delta[K^+]$-to-work^{-1} ratio.

6.2 Methods

6.2.1. Subjects and exercise trials

The subject characteristics, exercise undertaken and NAC infusion protocol are identical to Study 3 – Part I and are detailed in sections 5.2.1 and 5.2.2. Briefly, 8 subjects cycled for 45 min at 71% $\dot{V}O_{2peak}$ and then to volitional fatigue at 92% $\dot{V}O_{2peak}$. NAC or saline (CON) was intravenously infused at an initial loading dose of 125 mg.kg^{-1}.hr^{-1} for 15 min, followed by a constant infusion of 25 mg.kg^{-1}.hr^{-1} until fatigue (Studies 1-3). The two experimental trials were conducted in a double-blind, randomised, crossover design to contrast the effects of NAC or CON infusion.

6.2.2. Blood processing and analyses

Blood was sampled at pre-infusion, immediately prior to exercise, during exercise at 15, 30, 45 min and at fatigue and during recovery at 1, 2, 5, 10 and 30 min. Blood was analysed for plasma PCO_2, pH and electrolyte concentrations including K^+ ($[K^+]$), as well as blood haemoglobin concentration ([Hb]) and haematocrit (Hct), as detailed in sections 4.2.5 and 5.2.3.

6.2.3. Muscle biopsy sampling and analyses

6.2.3.1 Muscle biopsy sampling

A vastus lateralis muscle biopsy was taken at pre-infusion, 45 min of exercise and at fatigue in both CON and NAC trials, as detailed in section 5.2.4.1.

6.2.3.2 Maximal 3-O-MFPase activity analyses

Muscle samples (~20 mg) were rapidly blotted on filter paper, weighed then homogenized (5% wt/vol) on ice for 2 x 20 s at 20,000 rpm (Omni 1000, Omni International, Warrenton, USA) in a homogenate buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris at pH 7.40 (Fraser and McKenna, 1998). Muscle homogenates were immediately frozen and stored in liquid N_2 for later analyses of maximal Na^+,K^+-ATPase activity.

Maximal muscle Na^+,K^+-ATPase activity was determined using the K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity assay, as previously used in human skeletal muscle in our laboratory (Fraser and McKenna, 1998; Fraser et al., 2002; Leppik et al., 2004; Aughey et al., 2004). Before analysis, muscle homogenates were freeze-thawed 4 times and then diluted 1/5 in cold homogenate buffer. The 3-*O*-MFPase activity was measured in an assay medium containing 5mM $MgCl_2$, 1.25 mM EDTA, 100 mM Tris and an 80 nM 3-*O*-methyl fluorescein standard at pH 7.40. A 30 µl homogenate was incubated in 2.5 ml of assay medium at 37°C for 5 min before addition of 40 µl of 10 mM 3-*O*-MFP to initiate the reaction. After 60 s, 10 µl of 2.58 M KCl was added to stimulate K^+-dependent phosphatase activity and the reaction was measured for a further 60 s. All assays were performed at 37°C, using continuous stirring, with data sampled at 1 Hz on a spectrofluorimeter (Aminico Bowman AB2 SLM, Thermospectronic, Madison, USA). Excitation wavelength was 475 nm and emission wavelength 515 nm, with 4 nm slit widths. The 3-*O*-MFPase activity was

calculated from the slope after addition of 10 μM KCl minus the slope prior to KCl addition.

6.2.4 Calculations

The decline in plasma volume (ΔPV), $\Delta[K^+]$ and $\Delta[K^+]$-to-work^{-1} ratio (nmol.l^{-1}.J^{-1}) were calculated as described in section 4.2.6.

6.2.5 Statistical analyses

All data are presented as mean ± SEM, except anthropometric data. All blood and muscle measures were analysed using a two-way (treatment, time) ANOVA with repeated measures on both factors. Post-hoc analyses used the Newman-Kuels test. The percentage change in the *in vitro* maximal 3-*O*-MFPase activity from rest was contrasted between conditions using a paired Student's t-test. Correlations between maximal 3-*O*-MFPase activity and exercise performance variables were determined by least squares regression. Significance was accepted at $P<0.05$.

6.3 Results

6.3.1 Exercise performance

NAC increased time to fatigue at 92% $\dot{V}O_{2peak}$ by 26.3±9.1% (Section 5.3.1).

6.3.2 Muscle 3-O-MFPase activity

No difference in pre-infusion maximal *in vitro* 3-*O*-MFPase activity was observed between trials (Figure 6.1A). Maximal *in vitro* 3-*O*-MFPase activity declined at 45 min of exercise by 21.6±2.8% and at fatigue by 23.9±2.2%, when compared to pre-infusion ($P<0.001$; time main effect). These changes did not reflect fluid shifts into muscle as similar reductions were observed in maximal *in vitro* 3-*O*-MFPase when expressed per gram of protein (Figure 6.1B).

No significant difference was found in 3-*O*-MFPase activity between trials when expressed per gram protein. To minimise inter-subject variation in results, the maximal

in vitro 3-*O*-MFPase activity was expressed as the percentage change from pre-infusion (Figure 6.2A). The percentage change in maximal 3-*O*-MFPase at 45 min was significantly attenuated by NAC ($P<0.05$). In contrast, at fatigue there was no difference between trials in the percentage change from pre-infusion in maximal *in vitro* 3-*O*-MFPase activity (Figure 6.2A).

To account for the 26% longer time to fatigue with NAC the percentage change in maximal 3-*O*-MFPase activity was expressed relative to work done. The percentage change from pre-infusion to work ratio was lower at both 45 min and at fatigue during NAC trials ($P<0.05$; Figure 6.2B).

6.3.3 Plasma Potassium

Plasma $[K^+]$ was increased above pre-infusion levels throughout exercise at 72% $\dot{V}O_{2peak}$, increased further during exercise at 92% $\dot{V}O_{2peak}$ at fatigue and then declined during recovery ($P<0.05$), returning to pre-infusion levels at 30 min (Figure 6.3). No significant differences in plasma $[K^+]$ were found between NAC and CON.

The $\Delta[K^+]$ did not differ from 15 to 45 min during exercise at 71% $\dot{V}O_{2peak}$, but was increased at fatigue at 92% $\dot{V}O_{2peak}$ ($P<0.01$; Figure 6.4A). NAC attenuated plasma $\Delta[K^+]$ during exercise ($P<0.05$; treatment main effect; Figure 6.4A).

The $\Delta[K^+]$-to-work ratio decreased from 15 min throughout exercise at 72% $\dot{V}O_{2peak}$ ($P<0.05$), but was higher at fatigue than at 45 min ($P<0.05$; Figure 6.4B). NAC attenuated the plasma $\Delta[K^+]$-to-work ratio ($P<0.05$; treatment main effect).

Figure 6.1 Skeletal muscle maximal *in vitro* K^+ stimulated 3-*O*-methylfluorescein phosphatase activity (3-*O*-MFPase, Na^+,K^+-ATPase activity) pre-infusion and during prolonged submaximal exercise to fatigue in well trained individuals. Activity is expressed as (A) nmol.min^{-1}.(g wt weight)$^{-1}$ or (B) nmol.min^{-1}.(g protein)$^{-1}$. * time main effect; less than pre-infusion ($P<0.001$). Values are mean±SEM; n=8.

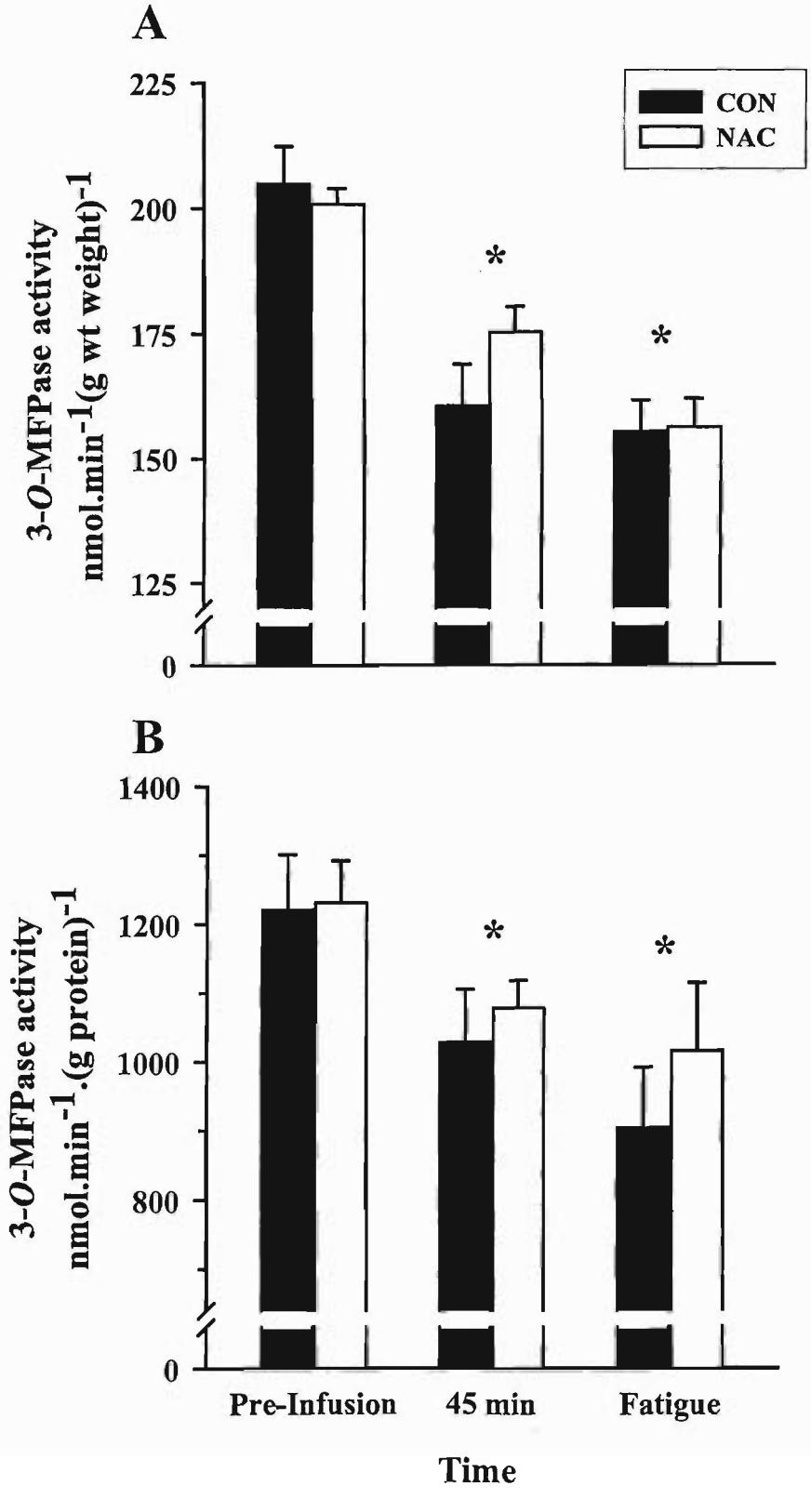


Figure 6.2 Percentage (%) change from pre-infusion in maximal *in vitro* K^+ stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (Na^+,K^+-ATPase activity) during prolonged submaximal exercise in well trained individuals. (A) Percentage change in maximal *in vitro* 3-*O*-MFPase activity; (B) % change in maximal *in vitro* 3-*O*-MFPase activity-to-work^{-1} ratio, at 45 min and at fatigue. † NAC < CON ($P<0.05$). Values are mean±SEM; n=8.

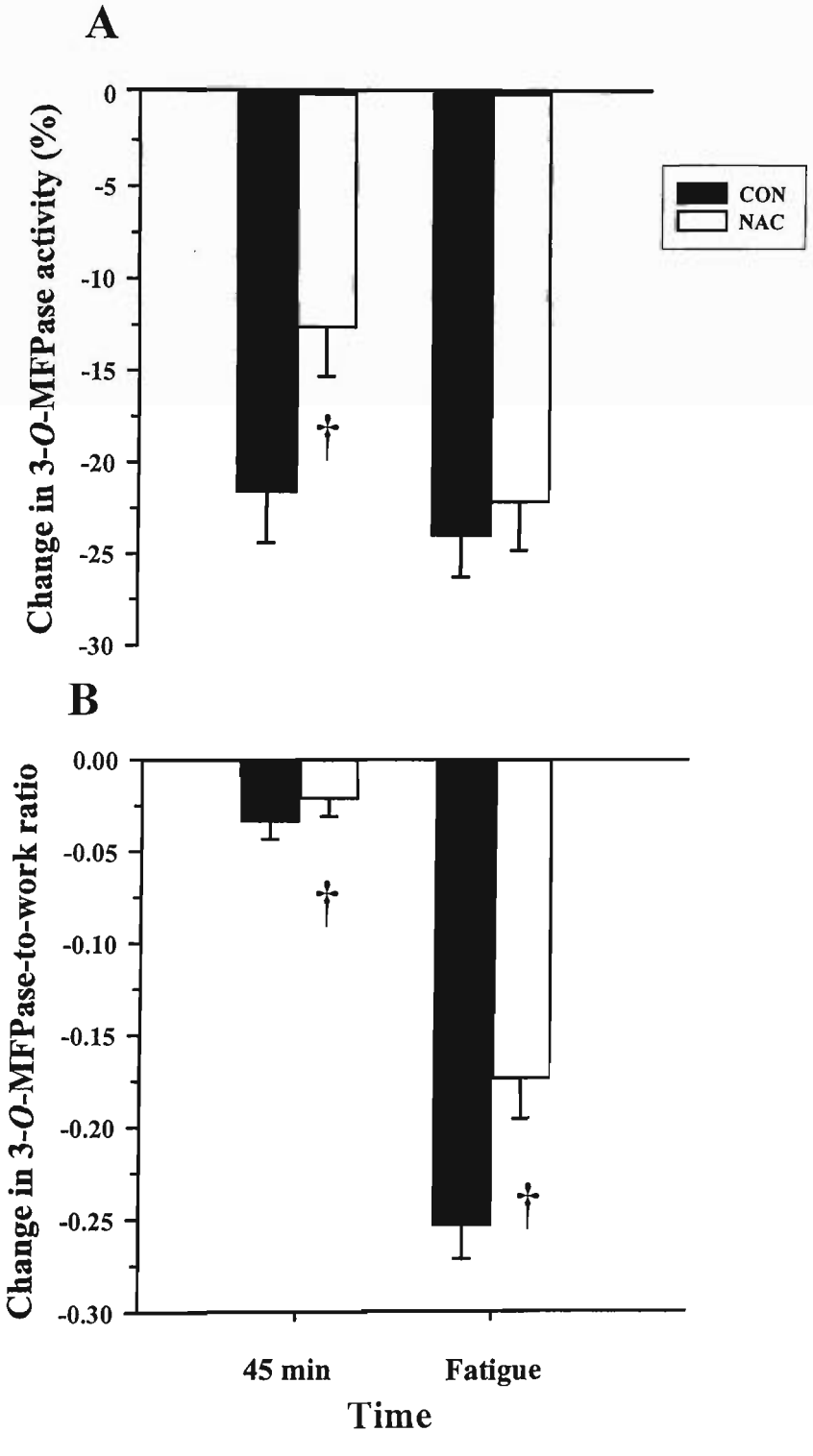


Figure 6.3 Effect of NAC (Δ) and CON (•) infusion on plasma potassium concentration ($[K^+]$) during and after prolonged exercise. Shaded bar denotes exercise comprising 45 min at 71% $\dot{V}O_{2peak}$, then to fatigue (F) at 92% $\dot{V}O_{2peak}$. * time main effect; different to pre-infusion (-35 min, $P<0.005$), ** greater than 45 min ($P<0.05$). n=7, mean±SEM.

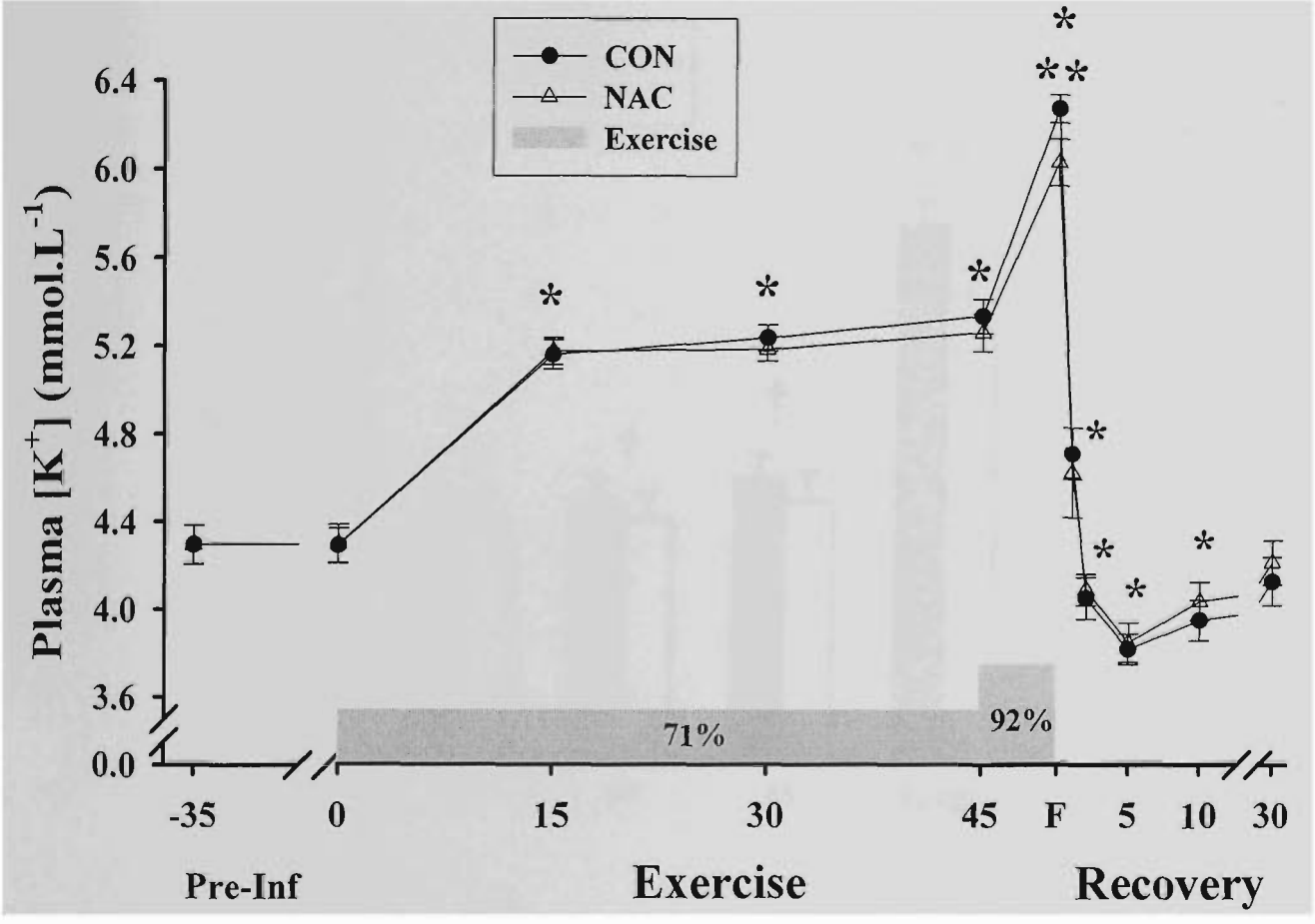
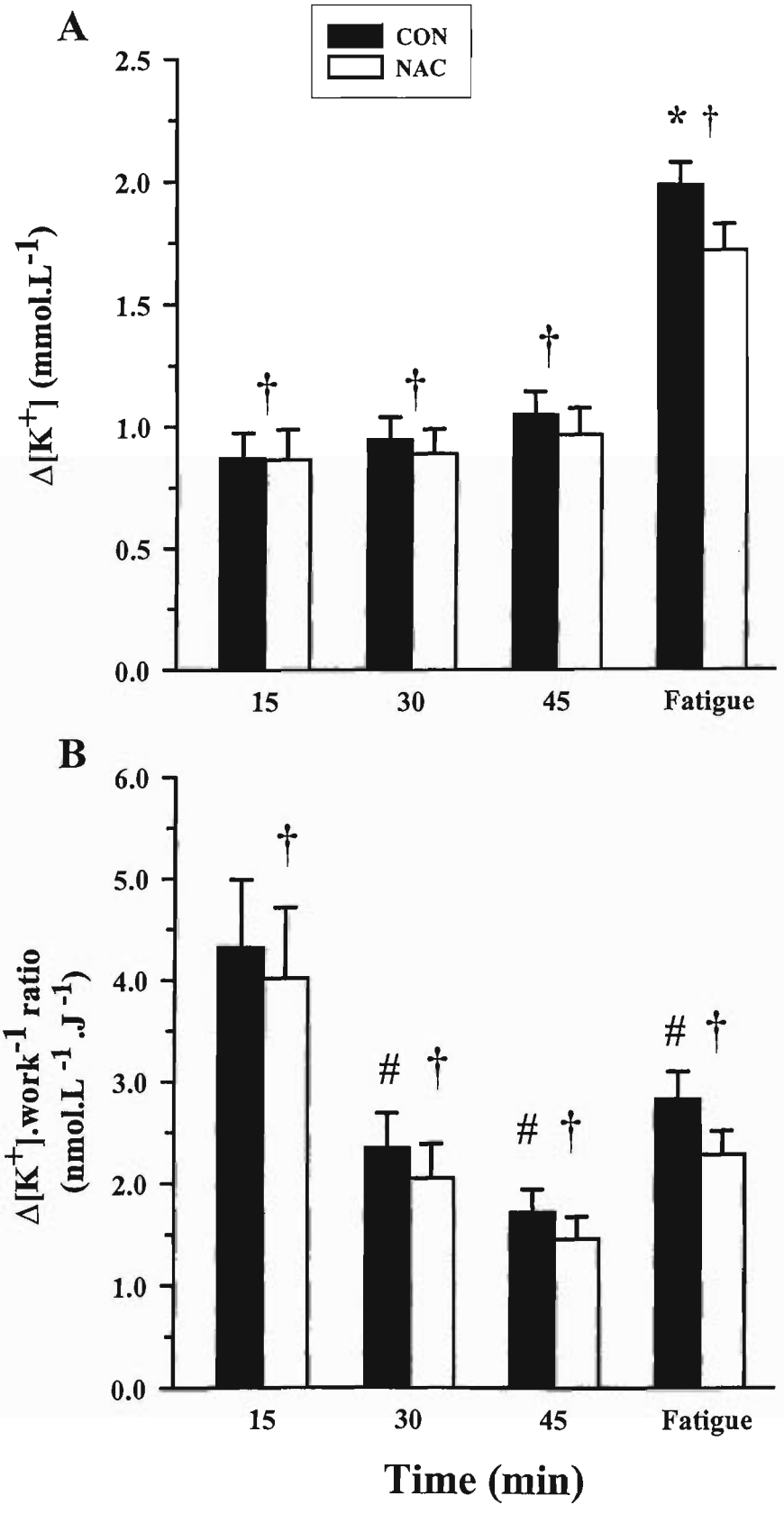


Figure 6.4 Effect of NAC and CON infusion on (A) the rise in plasma K^+ above pre-infusion ($\Delta[K^+]$) and (B) the $\Delta[K^+]$-to-work ratio, during prolonged submaximal exercise. Shaded bars, CON; open bars, NAC. * time main effect; greater than 15 min ($P<0.05$); # time main effect; less than 15 min ($P<0.05$); † NAC < CON ($P<0.05$; treatment main effect); n=7, mean±SEM.



6.3.4 Relationships between Na^+,K^+-ATPase and plasma K^+ and exercise performance

No significant correlations were observed between time to fatigue and each of maximal 3-*O*-MFPase activity, percentage change from pre-infusion in maximal 3-*O*-MFPase activity, $\Delta[K^+]$, plasma $\Delta[K^+]$-to-work^{-1} ratio or $\dot{V}O_{2peak}$. Pre-infusion maximal 3-*O*-MFPase activity was also not significantly correlated to $\dot{V}O_{2peak}$.

6.3.5 Fluid shifts, plasma electrolyte concentrations and acid-base status

Both [Hb] and Hct were higher than pre-infusion levels, and thus plasma volume declined, during exercise and until 30 min recovery ($P<0.05$, Table 6.1). However, no differences between NAC and CON were found for [Hb], Hct or ΔPV (Table 6.1).

Plasma $[Na^+]$ increased above pre-infusion levels throughout the exercise period until fatigue ($P<0.05$, Table 6.1). Plasma $[Cl^-]$ did not differ during exercise or recovery, whilst plasma $[Ca^{2+}]$ was increased above pre-infusion levels only at fatigue ($P<0.05$, Table 6.1). Compared to pre-infusion levels, plasma $[H^+]$ was increased, whereas plasma PCO_2 and $[HCO_3^-]$ fell, throughout exercise and recovery ($P<0.05$, Table 6.1). No differences between NAC and CON were found for any of these plasma electrolyte or acid-base variables (Table 6.1).

Table 6.1 Hematology, calculated fluid shifts, plasma acid-base variables and electrolyte concentrations during prolonged, submaximal exercise prior to, during and following saline (CON) and *N*-acetylcysteine (NAC) infusion.

Variable	Treatment	Pre-Infusion	Pre-Exercise	Exercise Time (min)				Recovery
				15	30	45	Fatigue	30 min
[Hb] ($g.dl^{-1}$)	CON	15.4±0.3	15.7±0.3	16.4±0.3 *	16.4±0.2 *	16.3±0.2 *	16.7±0.2 *	15.2±0.2
	NAC	15.5±0.3	15.7±0.3	16.5±0.3 *	16.4±0.2 *	16.3±0.2 *	16.9±0.2 *	15.3±0.2
Hct (%)	CON	44.1±1.3	44.4±1.3	46.6±1.2 *	46.8±1.2 *	46.7±1.1 *	47.9±1.2 *	43.1±1.2
	NAC	43.9±1.2	44.3±1.0	47.0±1.0 *	47.4±1.0 *	47.0±0.8 *	48.8±1.2 *	43.8±1.0
ΔPV (%)	CON	-	-2.0±1.0	-10.0±0.7 *	-10.4±1.4 *	-9.5±1.3 *	-12.7±2.4 *	5.8±1.9 *
	NAC	-	-1.0±0.5	-11.6±1.3 *	-11.7±1.3 *	-10.5±1.3 *	-16.0±1.3 *	1.9±2.3 *
$[H^+]$($nmol.l^{-1}$)	CON	39.1±0.6	39.0±0.6	43.5±0.9 *	42.6±1.0 *	42.0±1.0 *	51.0±2.4 *	41.0±1.1 *
	NAC	39.2±0.6	38.7±0.7	43.7±0.8 *	42.3±0.9 *	41.9±1.2 *	53.1±1.6 *	41.1±1.3 *
$[HCO_3^-]$ ($mmol.l^{-1}$)	CON	27.3±0.8	26.3±0.9	20.8±1.0 *	20.0±1.0 *	20.6±0.8 *	14.0±0.7 *	22.7±0.7 *
	NAC	28.0±0.9	26.2±0.8	19.3±0.5 *	19.2±0.6 *	19.6±0.6 *	12.8±0.6 *	21.0±0.8 *
PCO_2 (Torr)	CON	43.4±1.3	42.2±1.2	36.5±1.3	36.0±1.2 *	36.1±0.9 *	30.7±1.6 *	38.3±1.0 *
	NAC	45.5±0.7	42.4±1.5	37.3±1.4	34.4±0.4 *	34.9±1.1 *	28.7±0.7 *	36.7±1.0 *
$[Na^+]$ ($mmol.l^{-1}$)	CON	140.3±0.8	139.4±0.8	142.0±0.7 *	142.2±0.6 *	141.2±0.6 *	145.0±0.6 *	137.9±0.5
	NAC	139.3±0.7	139.7±0.7	141.3±0.6 *	141.2±0.7 *	141.8±0.7 *	146.2±1.0 *	139.0±0.6
$[Cl^-]$ ($mmol.l^{-1}$)	CON	104.8±1.0	103.5±0.9	105.3±1.0	102.3±1.1	104.1±0.9	104.8±1.6	104.9±1.5
	NAC	105.3±0.6	105.6±1.0	105.1±0.7	104.1±0.4	103.5±0.8	104.4±0.9	103.3±0.6
$[Ca^{2+}]$ ($mmol.l^{-1}$)	CON	1.24±0.02	1.27±0.02	1.24±0.02	1.28±0.02	1.27±0.02	1.28±0.01 *	1.23±0.02
	NAC	1.24±0.02	1.25±0.02	1.25±0.01	1.24±0.02	1.25±0.01	1.30±0.02 *	1.21±0.01

* Significant main effect for time: different from pre-infusion $P<0.05$. Mean ±SEM, n=7.

6.4 Discussion

This study demonstrated for the first time that NAC infusion affects changes in maximal Na^+,K^+-ATPase activity in skeletal muscle and on plasma K^+ regulation, during prolonged submaximal exercise to fatigue in well-trained individuals. The first important finding was that NAC attenuated the decline in maximal Na^+,K^+-ATPase activity that occurred after 45 min exercise at 72% $\dot{V}O_{2peak}$. Whilst NAC did not attenuate the decrease in Na^+,K^+-ATPase activity at fatigue, exercise time was prolonged by 26% with NAC. When this increased work was taken into account an effect of NAC was evident, with a lower percentage change in 3-*O*-MFPase to work ratio during NAC compared to CON trials. NAC also enhanced plasma K^+ regulation, as evidenced by a smaller rise in plasma $[K^+]$ during exercise and a lower $\Delta[K^+]$-to-work^{-1} ratio throughout exercise including at fatigue. Thus, NAC modulated both maximal Na^+,K^+-ATPase activity and plasma K^+ regulation during exercise. This data provides evidence in support of both hypotheses and suggest that ROS play an important role in depressed maximal Na^+,K^+-ATPase activity during prolonged exercise in well-trained subjects. Although these changes were not directly correlated with increased time to fatigue it seems plausible that ROS-mediated decreased Na^+,K^+-ATPase activity may contribute to fatigue.

6.4.1 Depression of Na^+,K^+-ATPase activity with exercise

A clear finding was that prolonged submaximal exercise decreased maximal Na^+,K^+-ATPase activity by ~22% at 45 min and ~ 24% at fatigue in well-trained individuals. This finding of a decreased Na^+,K^+-ATPase activity with exercise is consistent with findings from our laboratory (Fraser et al., 2002; Leppik et al., 2004; Aughey et al., 2004) and others (Fowles et al., 2002; Sandiford et al., 2004). The values for maximal *in vitro* 3-*O*-MFPase activity in this study are slightly lower than those previously reported for endurance trained individuals in our laboratory (Fraser et al., 2002; Aughey et al.,

2004). The exact reason for this difference is unclear. Whilst the 22-24% decrease in maximal Na^+,K^+-ATPase activity with exercise is slightly larger than previously reported by our laboratory (Fraser et al., 2002; Aughey et al., 2004; Leppik et al., 2004), this is consistent with findings by others (Fowles et al., 2002).

Although this study did not quantify Na^+,K^+-ATPase content, decreased maximal Na^+,K^+-ATPase activity with prolonged exercise is unlikely to have occurred due to a loss of Na^+,K^+-ATPase. Recent reports demonstrate no reduction in the [^{3}H]-ouabain binding site content in muscle after either prolonged or brief exercise in humans (Leppik et al., 2004; Aughey et al., 2004; Fowles et al., 2002). Similarly, the [^{3}H]-ouabain binding site content was not decreased following brief, high frequency or prolonged low frequency electrical stimulation of rat soleus and EDL muscle (McKenna et al., 2003). Although a recent report demonstrated a 10% increase in vastus lateralis muscle [^{3}H]ouabain binding in humans with endurance running (Overgaard et al., 2002), this occurred after 10-11 h of exercise, during which time Na^+,K^+-ATPase synthesis is probable (Wolitzky and Fambrough, 1986). Thus, it is unlikely that the decreased Na^+,K^+-ATPase activity observed in this study was due to a reduction in [^{3}H]-ouabain binding site content.

It is not possible to determine the exact underlying mechanism(s) that cause the depression in maximal *in vitro* Na^+,K^+-ATPase activity. However, given the identical and controlled 3-*O*-MFPase assay conditions for the rest and exercising muscle samples, depressed Na^+,K^+-ATPase activity most likely reflects a structural alteration in the Na^+,K^+-ATPase enzyme and/or altered characteristics of the membrane in which it is embedded (Fraser et al., 2002). In previous work conducted by our laboratory it was speculated that one possible factor mediating decreased Na^+,K^+-ATPase activity was increased ROS (Fraser et al., 2002; Leppik et al., 2004; Aughey et al., 2004).

6.4.2 NAC attenuated the decline in skeletal muscle Na^+,K^+-ATPase activity during exercise

The maximal *in vitro* 3-*O*-MFPase activity after 45 min exercise and at fatigue was not attenuated by NAC when activity was expressed in absolute units. This may reflect considerable inter-subject variability, the small sample size and consequently a Type II error. To account for this variability, the decline in maximal Na^+,K^+-ATPase activity from pre-infusion was then expressed as a percentage change from pre-infusion in each individual. This allowed us to detect that the reduction in percentage change in maximal Na^+,K^+-ATPase activity from pre-infusion at 45 min was almost halved with NAC, from ~22% during CON trials, to ~12% during NAC trials. At this time, power output and thus work performed was matched between the two trials. Interestingly, the percentage change in Na^+,K^+-ATPase activity was almost identical during both NAC and CON trials at fatigue, but ~26% more work was performed during NAC trials (Study 3 – Part I). When the normalized for work done, the ratio of percentage change in maximal Na^+,K^+-ATPase activity from pre-infusion to work was 32% lower with NAC infusion. This lower ratio with NAC suggests that ROS may play an important role in the change in maximal Na^+,K^+-ATPase activity in skeletal muscle evidenced during fatiguing exercise in well-trained individuals. It is speculated that the depressed 3-*O*-MFPase activity during exercise may be due to structural and conformational changes caused by the accumulation of ROS as shown for mouse cerebral Na^+,K^+-ATPase activity (Jamme et al., 1995). However, the effects in skeletal muscle are unknown.

NAC is a non-specific antioxidant that scavenges ROS (Arouma et al., 1989) and it seems likely that the attenuated decrease in Na^+,K^+-ATPase activity with NAC may, in part, be due to ROS scavenging. Vinnikova et al (1992) demonstrated in canine cardiac sarcolemmal muscle that the $^1O_2$-induced inhibitory effect on Na^+,K^+-ATPase activity

was prevented by histidine, a $^1O_2$ scavenger, whereas superoxide dismutase (SOD), catalase (CAT) and mannitol had no effect on Na^+,K^+-ATPase activity. Since SOD, CAT and mannitol remove $O_2^{-\bullet}$, H_2O_2 and $^\bullet OH$, respectively, this might suggest that $O_2^{-\bullet}$, H_2O_2 and $^\bullet OH$ do not inhibit the Na^+,K^+-ATPase enzyme. In contrast, others have demonstrated that H_2O_2 inhibited Na^+,K^+-ATPase activity in canine myocardium sarcolemmal vesicles (Kukreja et al., 1990) and in guinea pig cardiac sarcolemma (Kim and Akera, 1987). Further work is required to determine whether specific ROS inhibit Na^+,K^+-ATPase activity in skeletal muscle.

Whether glutathione exerts a stabilising on skeletal muscle Na^+,K^+-ATPase activity is not known. However, reduced glutathione (GSH) acts as a substrate for glutathione peroxide, which scavenges H_2O_2. In the previous chapter an increased GSH availability was demonstrated with NAC infusion in these subjects (Study 3 – Part I). Increased GSH availability may have facilitated the intramuscular removal of H_2O_2 during exercise and, thus, help explain the attenuated percentage decline in Na^+,K^+-ATPase activity during fatiguing exercise. Furthermore, NAC infusion resulted in a threefold increase in muscle cysteine (Study 3 – Part I), which is also a ROS scavenger (Cotgreave, 1997). Increased muscle glutathione and cysteine contents, together with increased muscle NAC, would facilitate the removal of intramuscular ROS and might therefore protect the Na^+,K^+-ATPase enzyme from their deleterious effects.

6.4.3 Functional implications of enhanced Na^+,K^+-ATPase activity with NAC

Both the $\Delta[K^+]$ and the $\Delta[K^+]$-to-work ratio during exercise were attenuated by NAC, thus indicating enhanced K^+ regulation with NAC during exercise. This is consistent with the findings of Study 2 using an identical exercise protocol, but differs with the effects of NAC on K^+ regulation during intense exercise in Study 1. The effects of NAC on K^+

channels in skeletal muscle need to be explored in future studies to explain the different effects of NAC on K^+ regulation with the different exercise intensities studied.

Although arterialized-venous plasma [K^+] only peaked at ~6.3 mM in this study, a much greater increase in muscle interstitial [K^+] is likely to have occurred (Nielsen et al., 2004). An increase in interstitial [K^+] and decrease in muscle intracellular [K^+] would reduce the intracellular-to-extracellular [K^+] ratio. Together with the observed depression in maximal Na^+,K^+-ATPase activity, this may reduce membrane potential and impair excitability in skeletal muscle (Nielsen et al., 2004). Numerous studies have reported impaired muscle force development with interstitial [K^+] increased to 10-14 mM (Cairns et al., 1995; Clausen et al., 1993; Juel, 1988; Lannergren and Westerblad, 1986; Bouclin et al., 1995). During human isometric exercise, compromised Na^+,K^+-ATPase activity was linked with impaired sarcolemmal excitability and muscle fatigue (Fowles et al., 2002). Consequently, the enhanced plasma K^+ regulation and muscle Na^+,K^+-ATPase activity with NAC may also reflect improved muscle K^+ regulation, delay fatigue and thereby contributed to the increased time to fatigue that was observed in this study.

Against this possibility, no significant correlation was found between any 3-*O*-MFPase variable and time to fatigue. However, the use of correlational analysis with such a small sample size, coupled with the typical variability in the 3-*O*-MFPase assay, is problematic (Leppik et al., 2004). Moreover, it is well accepted that fatigue is a multifactorial process and the lack of a significant correlation between Na^+,K^+-ATPase and time to fatigue may not necessarily reflect the contribution of Na^+,K^+-ATPase and K^+ regulation to fatigue.

6.4.4. Conclusions

In conclusion, NAC significantly attenuated the percentage decline in maximal Na^+,K^+-ATPase activity during submaximal fatiguing exercise, which suggests that ROS play an

important role in Na^+,K^+-ATPase inactivation. The enhanced plasma K^+ regulation observed during exercise with NAC is consistent with enhanced Na^+,K^+-ATPase function with NAC. This study, and results from the previous chapter, suggest enhanced muscle K^+ regulation and glutathione availability contribute to the 26% increase in time to fatigue during prolonged submaximal exercise in well-trained individuals.

CHAPTER 7: GENERAL DISCUSSION

This thesis provides several novel findings regarding NAC and the role of ROS in muscle fatigue during intense, intermittent and prolonged, submaximal exercise. Firstly, NAC can be infused during voluntary exercise without severe adverse reactions (Studies 1-3). Although there was no performance enhancement during high intensity exercise (Study 1), NAC effects during prolonged submaximal exercise were dependent upon the training status of individuals (Study 2), and a 26% increase in time to fatigue was observed in well-trained individuals (Study 3). During prolonged submaximal exercise increased muscle cysteine and glutathione availability, as well as enhanced a Na^+,K^+-ATPase activity and K^+ regulation, were likely mechanisms.

7.1 Implementation of a modified infusion protocol: lack of serious adverse reactions

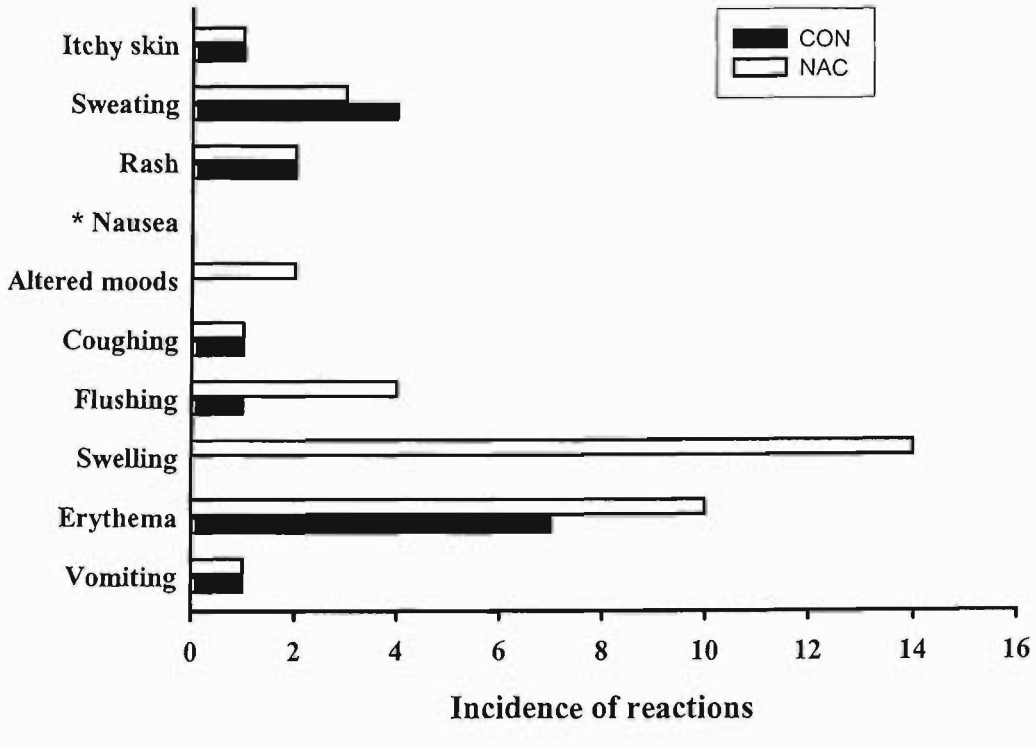
This thesis utilised an NAC infusion protocol modified from previous studies (Reid et al., 1994; Prescott et al., 1989). Although Reid et al., (1994) and Travaline et al., (1997) respectively demonstrated attenuated skeletal muscle and diaphragm fatigue, with NAC infusion in healthy individuals, the nature and incidence of adverse reactions to NAC in these studies would preclude voluntary exercise. Although no adverse reactions were reported with oral NAC (Sen et al., 1994), this approach is limited due to the low bioavailability of NAC (Holdiness, 1991).

The most common adverse reaction was local swelling at the infusion site and erythema, although erythema also occurred during some CON trials (Figure 7.1). In all 24 subjects tested in this thesis, no severe adverse reactions were observed that required active treatment. Of these 24 subjects, only one suffered moderate nausea during the pre-

exercise infusion period, which upon cessation of the NAC infusion required no further treatment.

The modified infusion protocol utilised in this thesis avoided the high concentrations of plasma NAC and allowed healthy human volunteers to complete voluntary, whole body exercise (Studies 1-3). Plasma [NAC] peaked at 305 $mg.l^{-1}$ (Study 3), which is well below the estimated values of 500 $mg.l^{-1}$ predicted from Reid et al. (1994). Since adverse reactions to NAC appear to follow a time course suggesting a dose dependent effect (Reid et al., 1994; Figure 2.7), the likely reason for the absence of adverse reactions in this thesis compared to other studies is the lower plasma [NAC]. Therefore, the modified infusion protocol utilised in this study was free of severe adverse actions and did not preclude voluntary, whole body exercise in these healthy human volunteers.

Figure 7.1 Incidence of mild adverse reactions during all experimental trials with NAC and CON.



* Moderate adverse reaction was observed in this subject

7.2 *N*-acetylcysteine effects on performance

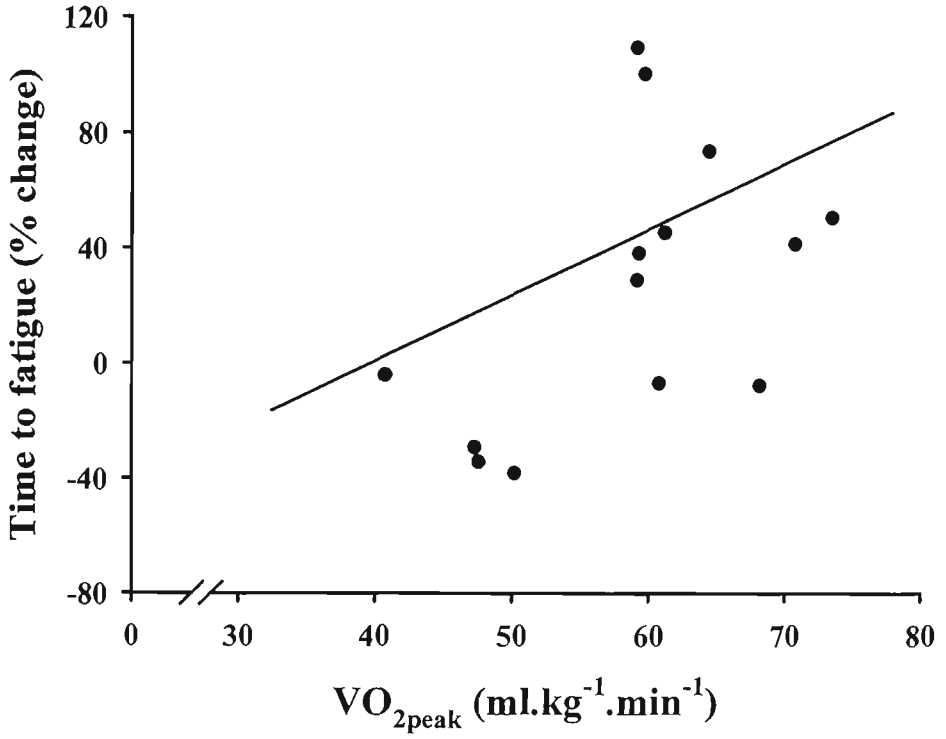
7.2.1 High intensity, intermittent exercise

NAC did not increase time to fatigue during high intensity, intermittent cycling exercise in healthy untrained individuals. This is consistent with the lack of effect of intravenous NAC on high frequency electrical stimulation of human tibialis anterior muscle (Reid et al., 1994) and suggests that NAC does not confer an ergogenic effect during intense exercise. There was no relationship between $\dot{V}O_{2peak}$ and time to fatigue during intermittent high intensity exercise.

7.2.2 Prolonged, submaximal exercise

The effects of NAC on prolonged, submaximal cycling exercise performance were training status dependent. In Study 2, time to fatigue at 90% $\dot{V}O_{2peak}$ was significantly correlated with $\dot{V}O_{2peak}$, such that NAC increased time to fatigue by 95% in trained individuals but decreased time to fatigue by 38% in untrained individuals. The improvement in well-trained individuals was confirmed in Study 3 where it was demonstrated that NAC increased time to fatigue by 26% in well-trained subjects. The percentage change in time to fatigue relative to CON tended to be correlated to $\dot{V}O_{2peak}$ in all subjects that completed prolonged submaximal cycling exercise (n= 14; P=0.06; Figure 7.2).

Figure 7.2 Relationship demonstrating percentage change in time to fatigue at 90% $\dot{V}O_{2peak}$ during prolonged submaximal exercise with NAC relative to CON during prolonged, submaximal exercise ($y = 2.6098x - 126.51$; $r = 0.504$; $P = 0.06$; $n = 14$).



7.3 *N*-acetylcysteine effects on muscle and blood thiols.

This thesis demonstrated an increased NAC content in skeletal muscle and [NAC] in RBC with NAC infusion during exercise in healthy individuals. This may allow NAC to exert intracellular effects. An increase in intracellular NAC would also provide increased antioxidant protection within the skeletal muscle, as NAC is a potent ROS scavenger (Arouma et al., 1989). Furthermore, NAC is rapidly deacetylated to cysteine, which also exhibits ROS scavenger properties (Cotgreave, 1997). Since NAC increased muscle CYS and cystine by up to 3 fold during NAC trials, with no change during CON trials (Study 3), also supports the rapid deaceteylation of NAC.

Cysteine can be actively transported into cells (Bannai, 1986) and increased intracellular CYS availability increases intracellular GSH (Sen et al., 1992). NAC increased cysteine

in each of muscle, plasma, blood and RBC and is therefore a likely mechanism explaining increased muscle TGSH and GSH content during prolonged, submaximal exercise. Several studies demonstrate a critical role for glutathione during exercise performance (Novelli et al., 1991; Cazzulani et al., 1991; Lands et al., 19999; Sen et al., 19994). The results from Studies 2 and 3 support these earlier findings.

Findings from these experiments also suggest that the effects of NAC on blood glutathione are also training status- and intensity-dependent. NAC attenuated the decline in blood GSH and increase in GSSG during high intensity, intermittent exercise (Study 1), but there was no effect during prolonged, submaximal exercise (Study 3). However, it is difficult to directly compare these results due to the different training status of subjects. Furthermore, since an increased exercise intensity also increased blood glutathione oxidation (Sastre et al., 1992) and ROS production (Bailey et al., 2003), this is also a likely explanation why NAC affected blood thiol status during high intensity, intermittent (Study 1) but not during prolonged, submaximal exercise (Study 3).

7.4 *N*-acetylcysteine effects on K^+ regulation.

In all studies, plasma [K^+] increased above pre-exercise values during exercise, but there was no effect of NAC. Surprisingly, this thesis found that NAC impaired plasma K^+ regulation during high, intensity exercise (Study 1), but improved it during prolonged, submaximal exercise (Study 3), as evidenced by a reduced $\Delta[K^+]$ and/or an increased $\Delta[K^+]$-to-work ratio. Enhanced K^+ regulation during prolonged submaximal exercise in part may be due to NAC attenuating the decrease in skeletal muscle Na^+,K^+-ATPase activity (Study 3 – Part II). Animal models demonstrate that ROS deleteriously affects Na^+,K^+ATPase activity (Kukreja et al. 1990; Sen et al. 1995). Hence, ROS scavenging by NAC (Arouma et al., 1989) may attenuate any ROS-induced inhibition of Na^+,K^+-ATPase activity in well-trained subjects, resulting in improved muscle K^+ regulation.

CHAPTER 8: CONCLUSIONS

A number of exciting conclusions can be drawn from the results of this thesis. Firstly, NAC can be infused without antihistamine pre-treatment, did not induce any serious adverse reactions and does not preclude voluntary whole body exercise in healthy humans (Studies 1-3).

There was no effect of NAC on time to fatigue during high intensity intermittent exercise in untrained subjects (Study 1). NAC effects on performance during prolonged, submaximal exercise are $\dot{V}O_{2peak}$ dependent (Study 2). The 26% increase in time to fatigue during prolonged, submaximal exercise (Study 3) demonstrated a marked effect on endurance performance in well-trained individuals.

The effects of exercise on blood glutathione were dependent upon exercise intensity. Intermittent high intensity exercise decreased GSH and increased GSSG, whereas there was no effect on blood glutathione status during prolonged submaximal exercise. Interestingly, NAC enhanced blood glutathione status during high intensity, intermittent (Study 1), but not prolonged submaximal (Study 3), exercise, with the exact mechanism unclear. NAC infusion increased NAC, cysteine and cystine concentrations in each of blood, plasma and RBC during both high intensity and prolonged cycling exercise.

NAC infusion significantly increased skeletal muscle NAC. NAC also increased muscle TGSH and GSH availability during prolonged submaximal exercise (Study 3) and this was most likely due to increased muscle cysteine availability. The increase in skeletal muscle NAC, cysteine, TGSH and GSH was a likely mechanism for the increased time to fatigue in well-trained individuals.

NAC impaired plasma K^+ regulation, as evidenced by an increased plasma $\Delta[K^+]$-to-work ratio during high intensity, exercise (Study 1). Conversely, NAC enhanced plasma K^+ regulation during prolonged submaximal exercise (Studies 2 and 3). Prolonged

submaximal exercise decreased in skeletal muscle in well-trained individuals. The percentage change in maximal Na^+,K^+-ATPase activity was attenuated by NAC. This suggests that ROS may play a role in decreased Na^+,K^+-ATPase activity during prolonged submaximal exercise in well trained individuals and may also contribute to fatigue (Study 3).

CHAPTER 9: DIRECTIONS FOR FUTURE RESEARCH

Although NAC increased time to fatigue in well-trained individuals during prolonged, submaximal exercise, these results should not encourage the use of NAC as an ergogenic aid exercise. Presumably, increasing the dose of NAC increases the antioxidant protection of this drug but this may also result in an increased incidence of adverse reactions and thus preclude its safe use in voluntary whole-body exercise. Rather, the modified NAC infusion protocol further extends the potential use of NAC as an experimental tool to investigate the effects of ROS and glutathione on muscle performance, with no requirement for subjects to be pre-treated with antihistamines.

There was no effect of NAC on performance during high, intensity exercise in untrained individuals (Study 1). As demonstrated in Study 2, the effects of NAC are training status dependent. Whether NAC improves high, intensity exercise in well-trained individuals should be investigated in future studies, as well as the effect of skeletal muscle fibre type.

Although the results of this thesis demonstrate that NAC augments time to fatigue in trained individuals during prolonged exercise, the precise mechanism(s) for this increase remain to be elucidated. As the mitochondria is a primary source of ROS, investigating the effects of NAC on mitochondrial function and isolated muscle fibres *in vitro* may further our understanding the potential mechanisms by which NAC improves performance.

NAC infusion also increased muscle NAC content, but the transport of NAC into the muscle cell was not investigated and remains to be elucidated. NAC increased muscle TGSH and GSH availability during prolonged submaximal exercise (Study 3), with increased NAC and CYS a likely mechanism. A paucity of data on muscle glutathione

during exercise remains and further studies are required to expand our knowledge on the role of muscle glutathione on muscle function during human exercise.

NAC scavenges a number of ROS *in vitro* (Arouma et al., 1989), but there are no such studies that have been completed *in vivo*. This needs to be elucidated in future studies as it will further our understanding of the mechanisms responsible for the benefits of NAC on increased time to fatigue during prolonged, submaximal exercise. Furthermore, whether NAC has any effect on endogenous antioxidant enzymes, including SOD, CAT, GPX and GR, should be investigated in future studies.

The effects of ROS on SR Ca^{2+}-ATPase and Ca^{2+} release are well documented (section 2.2.3.1 and 2.2.3.2), but the effects on human skeletal muscle are relatively unknown. Furthermore, SR Ca^{2+} regulation is sensitive to glutathione modulation (Lamb et al., 2003). Since NAC increased glutathione availability during exercise (Study 3), it may be possible that SR Ca^{2+} regulation may be enhanced with NAC infusion.

Further research is also required to elucidate the mechanism as to why NAC attenuated the decrease in percentage change from pre-infusion in skeletal muscle Na^+,K^+-ATPase activity. This may include investigating specific isoform expressions and mRNA levels. Since NAC enhanced K^+ regulation during exercise in well-trained individuals it would also be worthy to investigate whether NAC affects muscle K^+ content, resting membrane potential and K^+ channels, as well as muscle blood flow during exercise.

CHAPTER 10: REFERENCES

Adrian, R. H., and Peachey, L. D. Reconstruction of the action potential of frog sartorius muscle. *J Physiol* 235: 103-31., 1973.

Akova, B. Surmen-Gur, E. Gur, H. Dirican, M. Sarandol, E. and Kucukoglu, S. Exercise-induced oxidative stress and muscle performance in healthy women: role of vitamin E supplementation and endogenous oestradiol. *Eur J App Physiol Occup Physiol,* 84: 141-7, 2001.

Alessio, H. M., and Goldfarb, A. H. Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol,* 64: 1333-6, 1988.

Alessio, H. M. Goldfarb, A. H., and Cao, G. Exercise-induced oxidative stress before and after vitamin C supplementation. *Int J Sp Nutr,* 7: 1-9, 1997.

Alessio, H. M., Hagerman, A. E., Fulkerson, B. K., Ambrose, J., Rice, R. E., and Wiley, R. L. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Med Sci Sp Exer,* 32: 1576-81, 2000.

Allard, J. P., Royall, D., Kurian, R., Muggli, R., and Jeejeebhoy, K. N. Effects of beta-carotene supplementation on lipid peroxidation in humans. *Am J Clin Nutr,* 59: 884-90., 1994.

Allen, D. G., and Westerblad, H. Role of phosphate and calcium stores in muscle fatigue. *J Physiol* 536: 657-665, 2001.

Allen, D. G., Westerblad, H., Lee, J. A., and Lannergren, J. Role of excitation-contraction coupling in muscle fatigue. *Sports Medicine,* 13(2): 116-26, 1992.

Andrade, F. H., Reid, M. B., Allen, D. G., and Westerblad, H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. 509: 565-75, 1998.

Andrade, F. H., Reid, M. B., and Westerblad, H. Contractile response of skeletal muscle to low peroxide concentrations: myofibrillar calcium sensitivity as a likely target for redox-modulation. *Faseb J* 15: 309-11, 2001.

Andrews, N. P. Prasad, A. and Quyyumi, A. A. *N*-acetylcysteine improves coronary and peripheral vascular function. *J Am Coll Cardiol,* 37: 117-23., 2001.

Aruoma, O. I., Halliwell, B., Hoey, B. M., and Butler, J. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Rad Biol Med* 6: 593-7, 1989.

Ashton, T., Young, I. S., Peters, J. R., Jones, E., Jackson, S. K., Davies, B., and Rowlands, C. C. Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. *J Appl Physiol,* 87: 2032-6, 1999.

Atalay, M., Laaksonen, D. E., Niskanen, L., Uusitupa, M., Hanninen, O., and Sen, C. K. Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. *Acta Physiol Scand,* 161: 195-201, 1997.

Atalay, M., Seene, T., Hanninen, O., and Sen, C. K. Skeletal muscle and heart antioxidant defences in response to sprint training. *Acta Physiol Scand,* 158: 129-34, 1996.

Aughey, R. J., Gore, C. J., Hahn, A. G., Garnham, A. P., Clark, S. A., Petersen, A. C., Roberts, A. D., and McKenna, M. J. Chronic intermittent hypoxia and incremental cycling exercise independently depress muscle in-vitro maximal Na^+,K^+-ATPase activity in well-trained athletes. *J Appl Physiol In Press* 2004.

Bailey, D. M., Davies, B., Young, I. S., Jackson, M. J., Davison, G. W., Isaacson, R., and Richardson, R. S. EPR spectroscopic detection of free radical outflow from

an isolated muscle bed in exercising humans. *J Appl Physiol,* 94: 1714-1718, 2003.

Balog, E. M., and Fitts, R. H. Effects of fatiguing stimulation on intracellular Na^+ and K^+ in frog skeletal muscle. *J Appl Physiol,* 81: 679-85, 1996.

Balog, E. M., Thompson, L. V., and Fitts, R. H. Role of sarcolemma action potentials and excitability in muscle fatigue. *J Appl Physiol,* 76: 2157-62, 1994.

Bannai, S., and Tateishi, N. Role of membrane transport in metabolism and function of glutathione in mammals. *J Membr. Biol.,* 89: 1-8, 1986.

Barclay, J. K., and Hansel, M. Free radicals may contribute to oxidative skeletal muscle fatigue. *Can J Physiol Pharmacol* 69: 279-84, 1991.

Bejma, J., Ramires, P., and Ji, L. L. Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver. *Acta Physiol Scand,* 169: 343-51, 2000.

Bejma, J., and Ji, L. L. Aging and acute exercise enhance free radical generation in rat skeletal muscle. *J App Physiol,* 87: 465-70, 1999

Bellemare, F., and Garzaniti, N. Failure of neuromuscular propagation during human maximal voluntary contraction. *J Appl Physiol,* 64: 1084-93, 1988.

Benders, A. G., van Kuppevelt, T. H., Oosterhof, A., Wevers, R. A., and Veerkamp, J. H. Adenosine triphosphatases during maturation of cultured human skeletal muscle cells and in adult human muscle. *Biochim Biophys Acta,* 1112: 89-98, 1992.

Benrahmoune, M. Therond, P. and Abedinzadeh, Z. The reaction of superoxide radical with *N*-acetylcysteine. *Free Rad Biol Med,* 29: 775-82, 2000.

Bezanilla, F., Caputo, C., Gonzalez-Serratos, H., and Venosa, R. A. Sodium dependence of the inward spread of activation in isolated twitch muscle fibres of the frog. *J Physiol,* 223 507-23, 1972.

Bigland-Ritchie, B. EMG and fatigue of human voluntary and stimulated contractions. *Ciba Found Symp,* 82: 130-56, 1981.

Bigland-Ritchie, B., Jones, D. A., and Woods, J. J.: Excitation frequency and muscle fatigue: electrical responses during human voluntary and stimulated contractions. *Exp Neurol,* 64: 414-27, 1979.

Bigland-Ritchie, B., Kukulka, C. G., Lippold, O. C., and Woods, J. J. The absence of neuromuscular transmission failure in sustained maximal voluntary contractions. *J Physiol,* 330: 265-78, 1982.

Bjorkman, O., Sahlin, K., Hagenfeldt, L., and Wahren, J. Influence of glucose and fructose ingestion on the capacity for long-term exercise in well-trained men. *Clin Physio,* 4: 483-94, 1984.

Blum, J., and Fridovich, I. Inactivation of glutathione peroxidase by superoxide radical. *Arch Biochem Biophys* 240: 500-8, 1985.

Bogdanis, G. C., Nevill, M. E., Boobis, L. H., Lakomy, H. K., and Nevill, A. M. Recovery of power output and muscle metabolites following 30 s of maximal sprint cycling in man. *J Physiol* 482: 467-80, 1995.

Boman, G., Backer, U., Larsson, S., Melander, B., and Wahlander, L. Oral acetylcysteine reduces exacerbation rate in chronic bronchitis: report of a trial organized by the Swedish Society for Pulmonary Diseases. *Eur J Respir Dis* 64: 405-15, 1983.

Bonanomi, L., and Gazzaniga, A. Toxicological, pharmacokinetic and metabolic studies on acetylcysteine. *Eur J Resp Dis* 111: 45-51, 1980.

Bonting, S. L. Quantitative histochemical studies of the eye and the ear. *Curr Probl Clin Biochem,* 3: 231-56., 1971.

Booth, J., McKenna, M.J., Ruell, P.A., Gwinn, T.H.,, and Davis, G. M., Thompson, M.W., Harmer, A.R., Hunter, S.K., Sutton, J.R. Impaired calcium pump function does not slow relaxation in human skeletal muscle after prolonged exercise. *J Appl Physiol,* 83: 511-521, 1997.

Boraso, A., and Williams, A. J. Modification of the gating of the cardiac sarcoplasmic reticulum Ca^{2+}-release channel by H_2O_2 and dithiothreitol. *Am J Physiol* 267: H1010-6, 1994.

Borgstrom, L., Kagedal, B., and Paulsen, O. Pharmacokinetics of *N*-acetylcysteine in man. *Eur J Clin Pharmacol* 31: 217-22, 1986.

Borzone, G., Zhao, B., Merola, A. J., Berliner, L., and Clanton, T. L. Detection of free radicals by electron spin resonance in rat diaphragm after resistive loading. *J Appl Physiol,* 77: 812-8, 1994.

Bouclin, R., Charbonneau, E., and Renaud, J. M. Na^+ and K^+ effect on contractility of frog sartorius muscle: implication for the mechanism of fatigue. *Am J Physiol,* 268: C1528-36, 1995.

Boveris, A., and Chance, B. The mitochondrial generation of hydrogen peroxide: General properties and effect of hyperbaric oxygen. *Biochem J* 134: 707-16, 1973.

Brady, P. S., Brady, L. J., and Ullrey, D. E. Selenium, vitamin E and the response to swimming stress in the rat. *J Nutr,* 109: 1103-9, 1979.

Bridgeman, M. M., Marsden, M., Selby, C., Morrison, D., and MacNee, W. Effect of *N*-acetyl cysteine on the concentrations of thiols in plasma, bronchoalveolar lavage fluid, and lung tissue. *Thorax,* 49: 670-5, 1994.

Brotto, M. A., and Nosek, T. M. Hydrogen peroxide disrupts Ca^{2+} release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J Appl Physiol,* 81: 731-7, 1996.

Buono, M. J., and Yeager, J. E. Intraerythrocyte and plasma lactate concentrations during exercise in humans. 55: 326-9, 1986.

Burton, G. W., and Ingold, K. U. beta-Carotene: an unusual type of lipid antioxidant. *Science,* 224: 569-73., 1984.

Byrd, S., Bode, AK., Klug, GA. Effects of exercise of varying duration on sarcoplasmic reticulum function. *J Appl Physiol,* 66: 1383-1389, 1989.

Cadenas, E., and Davies, K. J. Mitochondrial free radical generation, oxidative stress, and aging. *Free Rad Biol Med,* 29: 222-30, 2000.

Cairns, S. P., Buller, S. J., Loiselle, D. S., and Renaud, J. M. Changes of action potentials and force at lowered $[Na^+]_o$ in mouse skeletal muscle: implications for fatigue. *Am J Physiol* 285: C1131-41. Epub 2003 Jun 25., 2003.

Cairns, S. P., Flatman, J. A., and Clausen, T. Relation between extracellular $[K^+]$, membrane potential and contraction in rat soleus muscle: modulation by the Na^+-K^+ pump. *Pflugers Arch,* 430: 909-15, 1995.

Callahan, L. A., She, Z. W., and Nosek, T. M. Superoxide, hydroxyl radical, and hydrogen peroxide effects on single-diaphragm fiber contractile apparatus. *J Appl Physiol,* 90: 45-54, 2001.

Camus, G., Felekidis, A., Pincemail, J., Deby-Dupont, G., Deby, C., Juchmes-Ferir, A., Lejeune, R., and Lamy, M. Blood levels of reduced/oxidized glutathione and plasma concentration of ascorbic acid during eccentric and concentric exercises of similar energy cost. *Arch Int Physiol Biochim Biophys,* 102: 67-70., 1994.

Cao, G., Alessio, H. M., and Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med,* 14: 303-11, 1993.

Cazzulani, P., Cassin, M., and Ceserani, R. Increased endurance to physical exercise in mice given oral reduced glutathione (GSH). *Med Sci Res* 19: 543-544, 1991.

Chance, B., Sies, H., and Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527-605., 1979.

Chin, E. R., and Green, H. J. Na^+-K^+-ATPase concentration in different adult rat skeletal muscles is related to oxidative potential. *Can J Physiol Pharmacol,* 71: 615-8., 1993.

Cho, E. S., Sahyoun, N., and Stegink, L. D. Tissue glutathione as a cyst(e)ine reservoir during fasting and refeeding of rats. *J Nutr,* 111: 914-22, 1981.

Clarkson, P. M., and Thompson, H. S. Antioxidants: what role do they play in physical activity and health? *Am J Clin Nutr* 72: 637S-46S, 2000.

Clausen, T. Na^+-K^+ pump regulation and skeletal muscle contractility. *Physiol Rev,* 83: 1269-324., 2003.

Clausen, T. Regulation of active Na^+-K^+ transport in skeletal muscle. *Physiol Rev* 66: 542-80, 1986.

Clausen, T. Significance of Na^+,K^+-pump regulation in skeletal muscle. *NIPS,* 5: 148-51, 1990.

Clausen, T., and Everts, M. E. K^+-induced inhibition of contractile force in rat skeletal muscle: role of active Na^+-K^+ transport. *Am J Physiol,* 261: C799-807, 1991.

Clausen, T. The sodium pump keeps us going. *Ann N Y Acad Sci,* 986: 595-602., 2003.

Clausen, T., Andersen, S. L., and Flatman, J. A. Na^+-K^+ pump stimulation elicits recovery of contractility in K^+-paralysed rat muscle. *J Physiol,* 472: 521-36, 1993.

Clausen, T., and Everts, M. E. Regulation of the Na,K-pump in skeletal muscle. *Kidney Int,* 35: 1-13, 1989.

Clausen, T., Everts, M. E., and Kjeldsen, K. Quantification of the maximum capacity for active sodium-potassium transport in rat skeletal muscle. *J Physiol,* 388: 163-81, 1987.

Clausen, T., Hansen, O., Kjeldsen, K., and Norgaard, A. Effect of age, potassium depletion and denervation on specific displaceable [^{3}H]ouabain binding in rat skeletal muscle in vivo. 333: 367-81, 1982.

Clough, D. Comparison of free radical sensitivity of Na^+,K^+ ATPase from rat heart, liver, kidney, brain, skeletal muscle and lung. *Proc Aust Physiol Pharmacol Soc,* 25: A-15, 1994.

Commoner, B., Townsend, J., and Pake, G. E. Free radicals in biological material. *Nature,*: 689-691, 1954.

Cooke, R., Franks, K., Luciani, G. B., and Pate, E. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J Physiol,* 395: 77-97, 1988.

Cooper, M. B., Jones, D. A., Edwards, R. H., Corbucci, G. C., Montanari, G., and Trevisani, C. The effect of marathon running on carnitine metabolism and on some aspects of muscle mitochondrial activities and antioxidant mechanisms. *J Sp Sci* 4: 79-87, 1986.

Costill, D. L., and Saltin, B. Muscle glycogen and electrolytes following exercise and thermal dehydration. In *Metabolic adaptation to prolonged physical exercise, Biochemistry of exercise II,* Howald, H., and Poortmans, J. (eds), 352-360, Basel, Birkhauser Verlag, 1975.

Cotgreave, I. A. *N*-acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol,* 38: 205-27, 1997.

Cotgreave, I. A., Eklund, A., Larsson, K., and Moldeus, P. W. No penetration of orally administered *N*-acetylcysteine into bronchoalveolar lavage fluid. *Eur J Resp Dis* 70: 73-7, 1987.

Criswell, D., Powers, S., Dodd, S., Lawler, J., Edwards, W., Renshler, K., and Grinton, S. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Med Sci Sp Exer* 25: 1135-40, 1993.

Cuzzocrea, S. Riley, D. P. Caputi, A. P. and Salvemini, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischaemia/reperfusion injury. *Pharmacol Rev,* 53: 135-59, 2001.

Davies, K. J., Quintanilha, A. T., Brooks, G. A., and Packer, L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Comm,* 107: 1198-205, 1982.

Dawson, A. H. Henry, D. A. and McEwen, J. Adverse reactions to *N*-acetylcysteine during treatment for paracetamol poisoning. *Med J Aust,* 150: 329-31, 1989.

De Caro, L., Ghizzi, A., Costa, R., Longo, A., Ventresca, G. P., and Lodola, E. Pharmacokinetics and bioavailability of oral acetylcysteine in healthy volunteers. *Arzneimittelforschung,* 39: 382-6, 1989.

De Vries, N., and De Flora, S. N-acetyl-l-cysteine. *J Cell Biochem Suppl,* 17F: 270-7, 1993.

Deneke, S. M. Thiol-based antioxidants. *Curr Top Cell Reg,* 36: 151-80, 2000.

Deneke, S. M., and Fanburg, B. L. Regulation of cellular glutathione. *Am J Physiol,* 257: L163-73, 1989.

Di Mascio, P., Murphy, M. E., and Sies, H. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am J Clin Nutr,* 53: 194S-200S, 1991.

Di Simplicio, P., Rossi, R., Falcinelli, S., Ceserani, R., and Formento, M. L. Antioxidant status in various tissues of the mouse after fasting and swimming stress. *Eur J Appl Physiol Occup Physiol,* 76: 302-7., 1997.

Diaz, P. T., Brownstein, E., and Clanton, T. L. Effects of *N*-acetylcysteine on in vitro diaphragm function are temperature dependent. *J Appl Physiol,* 77: 2434-9, 1994.

Diaz, P. T., She, Z. W., Davis, W. B., and Clanton, T. L. Hydroxylation of salicylate by the in vitro diaphragm: evidence for hydroxyl radical production during fatigue. *J Appl Physiol,* 75: 540-5, 1993.

Dillard, C. J., Litov, R. E., Savin, W. M., Dumelin, E. E., and Tappel, A. L. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol,* 45: 927-32, 1978.

Dufaux, B., Heine, O., Kothe, A., Prinz, U., and Rost, R. Blood glutathione status following distance running. *Int J Sp Med* 18: 89-93, 1997.

Duthie, G. G., Robertson, J. D., Maughan, R. J., and Morrice, P. C. Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Arch Biochem Biophys,* 282: 78-83, 1990.

Duty, S., and Allen, D. G. The distribution of intracellular calcium concentration in isolated single fibres of mouse skeletal muscle during fatiguing stimulation. *Pflugers Arch,* 427: 102-9., 1994.

Enoka, R. M., Trayanova, N., Laouris, Y., Bevan, L., Reinking, R. M., and Stuart, D. G. Fatigue-related changes in motor unit action potentials of adult cats. *Muscle Nerve,* 15: 138-50, 1992.

Evans, W. J. Vitamin E, vitamin C, and exercise. *Am J Clin Nutr,* 72: 647S-652, 2000.

Evelo, C. T., Palmen, N. G., Artur, Y., and Janssen, G. M. Changes in blood glutathione concentrations, and in erythrocyte glutathione reductase and glutathione S-

transferase activity after running training and after participation in contests. 64: 354-8, 1992.

Everts, M. E., and Clausen, T. Excitation-induced activation of the Na^+-K^+ pump in rat skeletal muscle. *Am J Physiol,* 266: C925-34, 1994.

Everts, M. E., Retterstol, K., and Clausen, T. Effects of adrenaline on excitation-induced stimulation of the sodium-potassium pump in rat skeletal muscle. *Acta Physiol Scand,* 134: 189-98, 1988.

Evertsen, F., Medbo, J. I., Jebens, E., and Nicolaysen, K.: Hard training for 5 mo increases Na^+-K^+ pump concentration in skeletal muscle of cross-country skiers. *Am J Physiol,* 272: R1417-24, 1997.

Favero, T. G. Sarcoplasmic reticulum Ca^{2+} release and muscle fatigue. *J Appl Physiol,* 87: 471-83, 1999.

Favero, T. G., Zable, A. C., and Abramson, J. J. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J Biol Chem,* 270: 25557-63, 1995.

Fenn, W. O., and Cobb, D. M. Electrolyte changes in muscle during activity. *Am J Physiol,* 115: 345-356, 1936.

Ferrari, V. Safety and drug interactions of oral acetylcysteine related to utilization data. *Eur J Resp Dis.* 111: 151-7, 1980.

Fitts, R. H. Cellular mechanisms of fatigue. *Physiol Rev,* 74: 49-93, 1994.

Fitts, R. H., Balog, E.M. Effect of intracellular and extracellular ion changes on E-C coupling and skeletal muscle fatigue. *Acta Physiologica Scandinavia,* 156: 169-181, 1996.

Flanagan, R. J., and Meredith, T. J. Use of *N*-acetylcysteine in clinical toxicology. *Am J Med,* 91: 131S-139S, 1991.

Fowles, J. R., Green, H. J., and Ouyang, J. Na^+-K^+-ATPase in rat skeletal muscle: content, isoform, and activity characteristics. *J Appl Physiol,* 96: 316-26. Epub 2003 Jul 25., 2004.

Fowles, J. R., Green, H. J., Schertzer, J. D., and Tupling, A. R.: Reduced activity of muscle Na^+-K^+-ATPase after prolonged running in rats. *J Appl Physiol,* 93: 1703-8, 2002.

Fowles, J. R., Green, H. J., Tupling, R., O'Brien, S., and Roy, B. D. Human neuromuscular fatigue is associated with altered Na^+-K^+-ATPase activity following isometric exercise. *J Appl Physiol,* 92: 1585-93., 2002.

Fraser, S. F., Li, J. L., Carey, M. F., Wang, X. N., Sangkabutra, T., Sostaric, S., Selig, S. E., Kjeldsen, K., and McKenna, M. J. Fatigue depresses maximal in vitro skeletal muscle Na^+-K^+-ATPase activity in untrained and trained individuals. *J Appl Physiol,* 93: 1650-9, 2002.

Fraser, S. F., and McKenna, M. J. Measurement of Na^+-K^+-ATPase activity in human skeletal muscle. *Anal Biochem,* 258: 63-7, 1998.

Fujimoto, T., and Nishizono, H. Involvement of membrane excitation failure in fatigue induced by intermittent submaximal voluntary contraction of the first dorsal interosseous muscle. *J Sports Med Phys Fit,* 33: 107-17, 1993.

Gaitanos, G. C., Williams, C., Boobis, L. H., and Brooks, S. Human muscle metabolism during intermittent maximal exercise. *J Appl Physiol,* 75: 712-9, 1993.

Gambelunghe, C., Rossi, R., Micheletti, A., Mariucci, G., and Rufini, S. Physical exercise intensity can be related to plasma glutathione levels. 57: 9-14, 2001.

Gandevia, S. C. Spinal and supraspinal factors in human muscle fatigue. *Physiol Rev,* 81: 1725-89., 2001.

Geering, K. The functional role of the beta-subunit in the maturation and intracellular transport of Na,K-ATPase. *FEBS Lett,* 285: 189-93, 1991.

Geering, K. Subunit assembly and functional maturation of Na,K-ATPase. *J Membr Biol,* 115: 109-21., 1990.

Gohil, K., Viguie, C., Stanley, W. C., Brooks, G. A., and Packer, L. Blood glutathione oxidation during human exercise. *J Appl Physiol,* 64: 115-9, 1988.

Goldfarb, A. H. Antioxidants: role of supplementation to prevent exercise-induced oxidative stress. *Med Sci Sp Exer,* 25: 232-6, 1993.

Gollnick, P. D. Metabolic regulation in skeletal muscle: influence of endurance training as exerted by mitochondrial protein concentration. *Acta Physiol Scand Suppl,* 93: 53-66, 1986.

Gore, M., Fiebig, R., Hollander, J., Leeuwenburgh, C., Ohno, H., and Ji, L. L. Endurance training alters antioxidant enzyme gene expression in rat skeletal muscle. *Can J Physiol Pharmacol,* 76: 1139-45, 1998.

Green HJ, Barr DJ, Fowles JR, Sandiford SD, Ouyang J. Malleability of human skeletal muscle Na^+-K^+-ATPase pump with short-term training. *J Appl Physiol* 97:143-8, 2004.

Green, H., Dahly, A., Shoemaker, K., Goreham, C., Bombardier, E., and Ball-Burnett, M. Serial effects of high-resistance and prolonged endurance training on Na^+-K^+ pump concentration and enzymatic activities in human vastus lateralis. *Acta Physiol Scand,* 165: 177-184, 1999.

Green, H. J. Cation pumps in skeletal muscle: potential role in muscle fatigue. *Acta Physiol Scand,* 162: 201-213, 1998.

Green, H. J. Mechanisms of muscle fatigue in intense exercise. *J Sp Sci,* 15: 247-56, 1997.

Green, H. J., Ball-Burnett, M., Chin, E. R., Dux, L., and Pette, D. Time-dependent increases in Na^+,K^+-ATPase content of low-frequency-stimulated rabbit muscle. *Febs Letter,* 310: 129-31, 1992.

Green, H. J., Chin, E. R., Ball-Burnett, M., and Ranney, D. Increases in human skeletal muscle Na^+-K^+-ATPase concentration with short-term training. *Am J Physiol,* 264: C1538-41, 1993.

Green, S., Bulow, J., and Saltin, B. Microdialysis and the measurement of muscle interstitial K^+ during rest and exercise in humans. *J Appl Physiol,* 87: 460-464, 1999.

Griffith, O. W., and Meister, A. Glutathione: interorgan translocation, turnover, and metabolism. *Proc Natl Acad Sci U S A,* 76: 5606-10, 1979.

Groussard, C., Machefer, G., Rannou, F., Faure, H., Zouhal, H., Sergent, O., Chevanne, M., Cillard, J., and Gratas-Delamarche, A. Physical fitness and plasma non-enzymatic antioxidant status at rest and after a wingate test. *Can J Appl Physiol,* 28: 79-92, 2003.

Groussard, C., Morel, I., Chevanne, M., Monnier, M., Cillard, J., and Delamarche, A. Free radical scavenging and antioxidant effects of lactate ion: an in vitro study. *J Appl Physiol,* 89: 169-75, 2000.

Groussard, C., Rannou-Bekono, F., Machefer, G., Chevanne, M., Vincent, S., Sergent, O., Cillard, J., and Gratas-Delamarche, A. Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. *Eur J Appl Physiol,* 89: 14-20, 2003.

Grover, A. K., Samson, S. E., Robinson, S., and Kwan, C. Y. Effects of peroxynitrite on sarcoplasmic reticulum Ca^{2+} pump in pig coronary artery smooth muscle. *Am J Physiol* , 284: C294-301, 2003.

Gul, M., Laaksonen, D. E., Atalay, M., Vider, L., and Hanninen, O. Effects of endurance training on tissue glutathione homeostasis and lipid peroxidation in streptozotocin-induced diabetic rats. *Scand J Med Sci Sp,* 12: 163-70, 2002.

Gullestad, L., Hallen, J., and Sejersted, O. M. K^+ balance of the quadriceps muscle during dynamic exercise with and without beta-adrenoceptor blockade. *J Appl Physiol,* 78: 513-23, 1995.

Gutteridge, J. M., and Halliwell, B. The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem Sci,* 15: 129-135, 1990.

Hallen, J., Gullestad, L., and Sejersted, O. M. K^+ shifts of skeletal muscle during stepwise bicycle exercise with and without beta-adrenoceptor blockade. *J Physiol,* 477: 149-59, 1994.

Hallen, J., and Sejersted, O. M. Intravasal use of pliable K^+-selective electrodes in the femoral vein of humans during exercise. *J Appl Physiol,* 75: 2318-25, 1993.

Halliwell, B., and Chirico, S. Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr,* 57: 715S-724S, 1993.

Halliwell, B., and Gutteridge, J. M. Free radicals in biology and medicine. Oxford, Clarendon Press, 2nd Edition, 1989.

Halliwell, B., and Gutteridge, J. M. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys,* 246: 501-14, 1986.

Hamada, T., Sale, D. G., MacDougall, J. D., and Tarnopolsky, M. A. Interaction of fibre type, potentiation and fatigue in human knee extensor muscles. *Acta Physiol Scand,* 178: 165-73, 2003.

Han, D., Loukianoff, S., and McLaughlin, L. Handbook of oxidants and antioxidants in exercise. In *Handbook of oxidants and antioxidants in exercise* Hanninen, O.,

Sen, C. K., and Packer, L. (eds), Amsterdam, Elsevier Science B.V., 433-484, 2000.

Hansen, O. Facilitation of ouabain binding to Na^+-K^+-ATPase by vanadate at in vivo concentrations. *Biochim Biophys Acta,* 568: 265-9, 1979.

Hansen, O. Studies on ouabain-complexed Na^+-K^+-ATPase carried out with vanadate. *Biochim Biophys Acta,* 692: 187-95, 1982.

Hansen, O., and Clausen, T. Quantitative determination of Na^+-K^+-ATPase and other sarcolemmal components in muscle cells. *Am J Physiol,* 254: C1-7, 1988.

Hargreaves, B. J., Kronfeld, D. S., Waldron, J. N., Lopes, M. A., Gay, L. S., Saker, K. E., Cooper, W. L., Sklan, D. J., and Harris, P. A. Antioxidant status of horses during two 80-km endurance races. *J Nutr,* 132: 1781S-3S, 2002.

Haramaki, N and Packer, L. Oxidative stress indices in exercise. In *Exercise and Oxygen Toxicity* Sen, S.K. and Packer, L. (eds) Amsterdam: Elseiver Science, 77-87, 1994.

Hargreaves, M., McKenna, M. J., Jenkins, D. G., Warmington, S. A., Li, J. L., Snow, R. J., and Febbraio, M. A. Muscle metabolites and performance during high-intensity, intermittent exercise. *J Appl Physiol,* 84: 1687-1691, 1998.

Harmer, A. R., McKenna, M.J., Sutton, J.R., Snow, R.J., Ruell, P.A., Booth, J, Thompson, M.W., Mackay, N.A., Stathis, C.G., Crameri, R.M., Carey, M.F. and Edgar, D.M.. Skeletal muscle metabolic and ionic adaptations during intense exercise following sprint training in humans. *J Appl Physiol,* 89: 1763 - 1803, 2000.

Harris, E. D. Regulation of antioxidant enzymes. *FASEB J.,* 6: 2675-2683, 1992.

Harrison, M. H. Effects on thermal stress and exercise on blood volume in humans. *Physiol Rev,* 65: 149-209, 1985.

Hellsten, Y., Svensson, M., Sjodin, B., Smith, S., Christensen, A., Richter, E. A., and Bangsbo, J. Allantoin formation and urate and glutathione exchange in human muscle during submaximal exercise. *Free Radic Biol Med,* 31: 1313-22., 2001.

Hellsten, Y., Apple, F. S., and Sjodin, B. Effect of sprint cycle training on activities of antioxidant enzymes in human skeletal muscle. *J Appl Physiol,* 81: 1484-7, 1996.

Hellsten, Y., Frandsen, U., Orthenblad, N., Sjodin, B., and Richter, E. Xanthine oxidase in human skeletal muscle following eccentric exercise: a role in inflammation. *J Physiol,* 498: 239-248, 1997.

Hellsten, Y., Tullson, P. C., Richter, E. A., and Bangsbo, J. Oxidation of urate in human skeletal muscle during exercise. *Free Rad Biol Med,* 22: 169-74, 1997.

Helwig, B., Schreurs, K. M., Hansen, J., Hageman, K. S., Zbreski, M. G., McAllister, R. M., Mitchell, K. E., and Musch, T. I. Training-induced changes in skeletal muscle Na^{+}-K^{+} pump number and isoform expression in rats with chronic heart failure. *J Appl Physiol,* 94: 2225-2236, 2003.

Hermansen, L., Hultman, E., and Saltin, B. Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand,* 71 129-39., 1967.

Hess, M. L., and Manson, N. H. Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J Mol Cell Cardiol,* 16: 969-85, 1984.

Heunks, L. M., Machiels, H. A., de Abreu, R., Zhu, X. P., van der Heijden, H. F., and Dekhuijzen, P. N. Free radicals in hypoxic rat diaphragm contractility: no role for xanthine oxidase. *Am J Physiol* , 281: L1402-12., 2001.

Hicks, A., and McComas, A. J. Increased sodium pump activity following repetitive stimulation of rat soleus muscles. *J Physiol,* 414: 337-49, 1989.

Higuchi, M., Cartier, L. J., Chen, M., and Holloszy, J. O. Superoxide dismutase and catalase in skeletal muscle: adaptive response to exercise. *J Gerontol,* 40: 281-6, 1985.

Hill, C. A., Thompson, M. W., Ruell, P. A., Thom, J., M., and White, M. J. Sarcoplasmic reticulum function and muscle contractile character following fatiguing exercise in humans. *J Physiol,* 531: 871-878, 2001.

Hodgkin, A. L., and Howowitz, P. Movement of Na and K in single muscle fibres. *J Physiol,* 145: 405-432, 1959.

Holdiness, M. R. Clinical pharmacokinetics of *N*-acetylcysteine. *Clin Pharmacol,* 20: 123-34, 1991.

Hollander, J., Bejma, J., Ookawara, T., Ohno, H., and Ji, L. L. Superoxide dismutase gene expression in skeletal muscle: fiber-specific effect of age. *Mech Age Devel,* 116: 33-45, 2000.

Hollander, J., Fiebig, R., Gore, M., Ookawara, T., Ohno, H., and Ji, L. L. Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. *Pflugers Arch,* 442: 426-34, 2001.

Holmberg, E., and Waldeck, B. On the possible role of potassium ions in the action of terbutaline on skeletal muscle contractions. *Acta Pharmacol Toxicol,* 46: 141-9, 1980.

Hundal, H. S., Maxwell, D. L., Ahmed, A., Darakhshan, F., Mitsumoto, Y., and Klip, A. Subcellular distribution and immunocytochemical localization of Na,K-ATPase subunit isoforms in human skeletal muscle. *Mol Mem Biol* 11: 255-62, 1994.

Inayama, T., Oka, J., Kashiba, M., Saito, M., Higuchi, M., Umegaki, K., Yamamoto, Y., and Matsuda, M. Moderate physical exercise induces the oxidation of human blood protein thiols. *Life Sciences,* 70: 2039-46, 2002.

Issels, R. D., Nagele, A., Eckert, K. G., and Wilmanns, W. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. *Biochem Pharmacol,* 37: 881-8., 1988.

Jackson, M. J., Edwards, R. H., and Symons, M. C. Electron spin resonance studies of intact mammalian skeletal muscle. *Biochimica Et Biophysica Acta,* 847: 185-90, 1985.

Jahoor, F. Jackson, A. Gazzard, B. Philips, G. Sharpstone, D. Frazer, M. E. and Heird, W. Erythrocyte glutathione deficiency in symptom-free HIV infection is associated with decreased synthesis rate. *Am J Physiol,* 276: E205-211, 1999.

James, C., Sacco, P., and Jones, D. A. Loss of power during fatigue of human leg muscles. *J Physiol,* 484: 237-46., 1995.

Jamme, I., Petit, E., Divoux, D., Gerbi, A., Maixnet, J. M., and Nouvelot, A. Modulation of mouse cerebral Na^+,K^+-ATPase activity by oxygen free radicals. *Neuroereport,* 7: 333-337, 1995.

Jenkins, R. R. Free radical chemistry. Relationship to exercise. *Sports Med,* 5: 156-70, 1988.

Jenkins, R. R., Friedland, R., and Howald, H. The relationship of oxygen uptake to superoxide dismutase and catalase activity in human skeletal muscle. *Int J Sp Med,* 5: 11-4, 1984.

Jeukendrup, A., Saris, W. H., Brouns, F., and Kester, A. D. A new validated endurance performance test. *Med Sci Sp Exerc,* 28: 266-70, 1996.

Ji, L., and Leeuwenburgh, C. Glutathione and exercise., pp. 97-123. Sen, C. K., Hanninen, O., and Packer, L. (eds.) 97-123, 1995.

Ji, L. L. Antioxidant enzyme response to exercise and aging. *Med Sci Sp Exerc,* 25: 225-31, 1993.

Ji, L. L. Antioxidants and oxidative stress in exercise. *Proc Soc Exper Biol Med,* 222: 283-92, 1999.

Ji, L. L. Exercise and oxidative stress: role of the cellular antioxidant systems. *Exer Sp Sci Rev,* 23: 135-66, 1995.

Ji, L. L. Oxidative stress during exercise: implication of antioxidant nutrients. *Free Rad Biol Med,* 18: 1079-86, 1995.

Ji, L. L., Dillon, D., and Wu, E. Alteration of antioxidant enzymes with aging in rat skeletal muscle and liver. *Am J Physiol,* 258: R918-23, 1990.

Ji, L. L., and Fu, R. Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperoxide. *J Appl Physiol,* 72: 549-54, 1992.

Ji, L. L., Fu, R., and Mitchell, E. W. Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol,* 73: 1854-9, 1992.

Ji, L. L., Katz, A., Fu, R., Griffiths, M., and Spencer, M. Blood glutathione status during exercise: effect of carbohydrate supplementation. *J Appl Physiol,* 74: 788-92, 1993.

Ji, L. L., Stratman, F. W., and Lardy, H. A. Antioxidant enzyme systems in rat liver and skeletal muscle. Influences of selenium deficiency, chronic training, and acute exercise. *Arch Biochem Biophys,* 263: 150-60, 1988.

Ji, L. L., Stratman, F. W., and Lardy, H. A. Enzymatic down regulation with exercise in rat skeletal muscle. *Arch Biochem Biophys,* 263: 137-49, 1988.

Ji, L. L., Wu, E., and Thomas, D. P. Effect of exercise training on antioxidant and metabolic functions in senescent rat skeletal muscle. *Gerontol,* 37: 317-25, 1991.

Jiang, T. X., Reid, W. D., and Road, J. D. Free radical scavengers and diaphragm injury following inspiratory resistive loading. *Am J Respir Crit Care Med,* 164: 1288-94, 2001.

Jones, D. A. High-and low-frequency fatigue revisited. *Acta Physiol Scand,* 156: 265-70, 1996.

Jorgensen, P. L., Hakansson, K. O., and Karlish, S. J. Structure and mechanism of Na,K-ATPase: functional sites and their interactions. *Annu Rev Physiol,* 65: 817-49, 2003.

Juel, C. The effect of beta 2-adrenoceptor activation on ion-shifts and fatigue in mouse soleus muscles stimulated in vitro. *Acta Physiol Scand,* 134: 209-16, 1988.

Juel, C. Muscle action potential propagation velocity changes during activity. *Muscle Nerve,* 11: 714-9, 1988.

Juel, C. Potassium and sodium shifts during in vitro isometric muscle contraction, and the time course of the ion-gradient recovery. *Pflugers Arch,* 406: 458-63, 1986.

Kanaley, J. A., and Ji, L. L. Antioxidant enzyme activity during prolonged exercise in amenorrheic and eumenorrheic athletes. *Metabolism,* 40: 88-92, 1991.

Kanter, M. Free radicals and exercise: effects of nutritional antioxidant supplementation. *Exer Sp Sci Rev,* 23: 375-97, 1995.

Kanter, M., and Williams, M. H. Antioxidants, carnitine and choline as putative ergogenic aids. *Inter J Sp Nutr,* 5: S120-S131, 1995.

Kanter, M. M., Nolte, L. A., and Holloszy, J. O. Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *J Appl Physiol,* 74: 965-9, 1993.

Karatzaferi, C., de Haan, A., Ferguson, R. A., van Mechelen, W., and Sargeant, A. J. Phosphocreatine and ATP content in human single muscle fibres before and after maximum dynamic exercise. *Pflugers Arch,* 442: 467-74, 2001.

Karelis, A. D., Marcil, M., Peronnet, F., and Gardiner, P. F. Effect of lactate infusion on M-wave characteristics and force in the rat plantaris muscle during repeated stimulation in situ. *J Appl Physiol,* 5: 5, 2004.

Katz, A., Sahlin, K., and Juhlin-Dannfelt, A. Effect of beta-adrenoceptor blockade on H^+ and K^+ flux in exercising humans. *J Appl Physiol,* 59: 336-41, 1985.

Kayatekin, B. M., Gonenc, S., Acikgoz, O., Uysal, N., and Dayi, A. Effects of sprint exercise on oxidative stress in skeletal muscle and liver. *Eur J Appl Physiol Occup Physiol,* 87: 141-4, 2002.

Kellog, E. W., and Fridovich, I. Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J Biol Chem,* 250: 8812-8817, 1975.

Kerr, M. E., Bender, C. M., and Monti, E. J. An introduction to oxygen free radicals. *Heart Lung,* 25: 200-9, quiz 210-1, 1996.

Khanna, S., Atalay, M., Laaksonen, D. E., Gul, M., Roy, S., and Sen, C. K. Alpha-lipoic acid supplementation: tissue glutathione homeostasis at rest and after exercise. *J Appl Physiol,* 86: 1191-6, 1999.

Khassaf, M., Child, R. B., McArdle, A., Brodie, D. A., Esanu, C., and Jackson, M. J. Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. *J Appl Physiol,* 90: 1031-5, 2001.

Khawli, F. A., and Reid, M. B. *N*-acetylcysteine depresses contractile function and inhibits fatigue of diaphragm in vitro. *J Appl Physiol,* 77: 317-24, 1994.

Kim, M. S., and Akera, T. O_2 free radicals: cause of ischemia-reperfusion injury to cardiac Na^+-K^+-ATPase. *Am J Physiol,* 252: H252-7, 1987.

Kjeldsen, K. Homogeneity of [^{3}H]ouabain-binding sites in rat soleus muscle. *Biochem J,* 249: 481-5, 1988.

Kjeldsen, K., Bjerregaard, P., Richter, E. A., Thomsen, P. E., and Norgaard, A. Na^+,K^+-ATPase concentration in rodent and human heart and skeletal muscle: apparent relation to muscle performance. *Cardio Res,* 22: 95-100, 1988.

Kjeldsen, K., Norgaard, A., and Hau, C. Human skeletal muscle Na,K-ATPase concentration quantified by ^{3}H-ouabain binding to intact biopsies before and after moderate physical conditioning. *Int J Sp Med,* 11: 304-7, 1990.

Kjeldsen, K., Richter, E. A., Galbo, H., Lortie, G., and Clausen, T. Training increases the concentration of [^{3}H]ouabain-binding sites in rat skeletal muscle. *Biochim Biophys Acta,* 860: 708-12, 1986.

Klitgaard, H., and Clausen, T. Increased total concentration of Na-K pumps in vastus lateralis muscle of old trained human subjects. *J Appl Physiol,* 67: 2491-4, 1989.

Knochel, J. P., Blachley, J. D., Johnson, J. H., and Carter, N. W. Muscle cell electrical hyperpolarization and reduced exercise hyperkalemia in physically conditioned dogs. *J Clin Invest,* 75: 740-5, 1985.

Kono, Y., and Fridovich, I. Superoxide radical inhibits catalase. *J Biol Chem,* 257: 5751-4, 1982.

Koska, J., Blazicek, P., Marko, M., Grna, J. D., Kvetnansky, R., and Vigas, M. Insulin, catecholamines, glucose and antioxidant enzymes in oxidative damage during different loads in healthy humans. *Physiol Res,* 49: S95-100, 2000.

Kourie, J. I. Interaction of reactive oxygen species with ion transport mechanisms. *Am J Physiol,* 275: C1-C24, 1998.

Kramer, K., Dijkstra, H., and Bast, A. Control of physical exercise of rats in a swimming basin. *Physiol Behav,* 53: 271-6, 1993.

Kukreja, R. C., and Hess, M. L. The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardio Res,* 26: 641-55, 1992.

Kukreja, R. C., Weaver, A. B., and Hess, M. L. Sarcolemmal Na^+-K^+-ATPase: inactivation by neutrophil-derived free radicals and oxidants. *Am J Physiol,* 259: H1330-6, 1990.

Kuppusamy, P., and Zweier, J. L. Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation. *J Biol Chem,* 264: 9880-4, 1989.

Laaksonen, D. E., Atalay, M., Niskanen, L., Uusitupa, M., Hanninen, O., and Sen, C. K. Blood glutathione homeostasis as a determinant of resting and exercise-induced oxidative stress in young men. *Redox Rep,* 4: 53-9, 1999.

Lamb, G. D. Excitation-contraction coupling and fatigue mechanisms in skeletal muscle: studies with mechanically skinned fibres. *J Muscle Res Cell Motil,* 23: 81-91, 2002.

Lamb, G. D. Excitation-contraction coupling in skeletal muscle: comparisons with cardiac muscle. *Clin Exp Pharmacol Physiol,* 27: 216-24, 2000.

Lamb, G. D., and Posterino, G. S. Effects of oxidation and reduction on contractile function in skeletal muscle fibres of the rat. *J Physiol,* 546: 149-163, 2003.

Lamb, G. D., and Stephenson, D. G. Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *J Physiol,* 434: 507-28., 1991.

Lands, L. C., Grey, V. L., and Smountas, A. A. Effect of supplementation with a cysteine donor on muscular performance. *J Appl Physiol,* 87: 1381-5, 1999.

Lang, J. K., Gohil, K., Packer, L., and Burk, R. F. Selenium deficiency, endurance exercise capacity, and antioxidant status in rats. *J Appl Physiol,* 63: 2532-5, 1987.

Lannergren, J., and Westerblad, H. Force and membrane potential during and after fatiguing, continuous high-frequency stimulation of single Xenopus muscle fibres. *Acta Physiol Scand,* 128: 359-68., 1986.

Laughlin, M. H., Simpson, T., Sexton, W. L., Brown, O. R., Smith, J. K., and Korthuis, R. J. Skeletal muscle oxidative capacity, antioxidant enzymes, and exercise training. *J Appl Physiol,* 68: 2337-43, 1990.

Lauterburg, B. H., Adams, J. D., and Mitchell, J. R. Hepatic glutathione homeostasis in the rat: efflux accounts for glutathione turnover. *Hepatol,* 4: 586-90, 1984.

Lawler, J. M., Cline, C. C., Hu, Z., and Coast, J. R. Effect of oxidant challenge on contractile function of the aging rat diaphragm. *Am J Physiol,* 272: E201-7, 1997.

Lawler, J. M., Cline, C. C., Hu, Z., and Coast, J. R. Effect of oxidative stress and acidosis on diaphragm contractile function. *Am J Physiol,* 273: R630-6, 1997.

Lawler, J. M., Powers, S. K., Visser, T., Van Dijk, H., Kordus, M. J., and Ji, L. L. Acute exercise and skeletal muscle antioxidant and metabolic enzymes: effects of fiber type and age. *Am J Physiol,* 265: R1344-50, 1993.

Leaf, D. A., Kleinman, M. T., Hamilton, M., and Barstow, T. J. The effect of exercise intensity on lipid peroxidation. *Med Sci Sports Exerc,* 29: 1036-1039, 1997.

Leeuwenburgh, C., Fiebig, R., Chandwaney, R., and Ji, L. L. Aging and exercise training in skeletal muscle: responses of glutathione and antioxidant enzyme systems. *Am J Physiol,* 267: R439-45, 1994.

Leeuwenburgh, C., and Ji, L. L. Alteration of glutathione and antioxidant status with exercise in unfed and refed rats. *J Nutr* 126: 1833-43, 1996.

Leeuwenburgh, C., and Ji, L. L. Glutathione depletion in rested and exercised mice: biochemical consequence and adaptation. *Arch Biochem Biophys,* 316: 941-9, 1995.

Leeuwenburgh, C. Hollander, J. Leichtweis, S. Griffiths, M.; Gore, M. and Ji, L. L. Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *Am J Physiol,* 272: R363-9, 1997.

Leivseth, G., Clausen, T., Everts, M. E., and Bjordal, E. Effects of reduced joint mobility and training on Na,K-ATPase and Ca-ATPase in skeletal muscle. *Muscle Nerve,* 15: 843-9, 1992.

Lepers, R., Maffiuletti, N. A., Rochette, L., Brugniaux, J., and Millet, G. Y. Neuromuscular fatigue during a long-duration cycling exercise. *J Appl Physiol,* 92: 1487-93, 2002.

Leppik, J. A., Aughey, R. J., Medved, I., Fairweather, I., Carey, M. F., and McKenna, M. J. Prolonged exercise to fatigue in humans impairs skeletal muscle Na^+, K^+-ATPase activity, sarcoplasmic reticulum Ca2+ release and Ca2+ uptake. *J Appl Physiol,* 97: 1414-1423, 2004.

Lew, H., Pyke, S., and Quintanilha, A. Changes in the glutathione status of plasma, liver and muscle following exhaustive exercise in rats. *Febs Letters,* 185: 262-6, 1985.

Li, J. L., Wang, X. N., Fraser, S. F., Carey, M. F., Wrigley, T. V., and McKenna, M. J. Effects of fatigue and training on sarcoplasmic reticulum Ca^{2+} regulation in human skeletal muscle. *J Appl Physiol,* 92: 912-922, 2002.

Lindinger, M. I., and Heigenhauser, G. J. Intracellular ion content of skeletal muscle measured by instrumental neutron activation analysis. *J Appl Physiol,* 63: 426-33., 1987.

Lindinger, M. I., and Heigenhauser, G. J. Ion fluxes during tetanic stimulation in isolated perfused rat hindlimb. *Am J Physiol,* 254: R117-26, 1988.

Liu, J., Yeo, H. C., Overvik-Douki, E., Hagen, T., Doniger, S. J., Chu, D. W., Brooks, G. A., and Ames, B. N. Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. *J Appl Physiol,* 89: 21-28, 2000.

Liu, M. L., Bergholm, R., Makimattila, S., Lahdenpera, S., Valkonen, M., Hilden, H., Yki-Jarvinen, H., and Taskinen, M. R. A marathon run increases the susceptibility of LDL to oxidation in vitro and modifies plasma antioxidants. *Am J Physiol,* 276: E1083-91, 1999.

Longo, V. D., Gralla, E. B., and Valentine, J. S. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. *J Biol Chem,* 271: 12275-80., 1996.

Lovlin, R., Cottle, W., Pyke, I., Kavanagh, M., and Belcastro, A. N. Are indices of free radical damage related to exercise intensity. *Eur J Appl Physiol Occup Physiol,* 56: 313-6, 1987.

Macdonald, W. A., and Stephenson, D. G. Effects of ADP on sarcoplasmic reticulum function in mechanically skinned skeletal muscle fibres of the rat. *J Physiol,* 532: 499-508, 2001.

Maddock, J. Biological properties of acetylcysteine: assay development and pharmacokinetic studies. *Eur J Resp Dis Supp,* 111: 52-8, 1980.

Madsen, K., Franch, J., and Clausen, T. Effects of intensified endurance training on the concentration of Na,K-ATPase and Ca-ATPase in human skeletal muscle. *Acta Physiol Scand,* 150: 251-8, 1994.

Mant, T. G. Tempowski, J. H. Volans, G. N. and Talbot, J. C. Adverse reactions to acetylcysteine and effects of overdose. *Br Med J,* 289: 217-9, 1984.

Margaritis, I., Tessier, F., Richard, M. J., and Marconnet, P. No evidence of oxidative stress after a triathlon race in highly trained competitors. *Int J Sp Med,* 18: 186-90, 1997.

Marin, E. Hanninen, O. Muller, D. and Klinger, W. Influence of acute physical exercise on glutathione and lipid peroxides in blood of rat and man. *Acta Physiol Hung,* 76: 71-6, 1990.

Marklund, S. L. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem J,* 222: 649-55., 1984.

Marlin, D. J., Fenn, K., Smith, N., Deaton, C. D., Roberts, C. A., Harris, P. A., Dunster, C., and Kelly, F. J. Changes in circulatory antioxidant status in horses during prolonged exercise. *J Nutr,* 132: 1622S-7S, 2002.

Marzatico, F., Pansarasa, O., Bertorelli, L., Somenzini, L., and Della Valle, G. Blood free radical antioxidant enzymes and lipid peroxides following long-distance and lactacidemic performances in highly trained aerobic and sprint athletes. *J Sp Med Phys Fit,* 37: 235-9, 1997.

Mastaloudis, A., Leonard, S. W., and Traber, M. G. Oxidative stress in athletes during extreme endurance exercise. *Free Rad Biol Med,* 31: 911-22, 2001.

McArdle, A., Pattwell, D., Vasilaki, A., Griffiths, R. D., and Jackson, M. J. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. 280: C621-7, 2001.

McArdle, A., van der Meulen, J. H., Catapano, M., Symons, M. C., Faulkner, J. A., and Jackson, M. J. Free radical activity following contraction-induced injury to the

extensor digitorum longus muscles of rats. *Free Rad Biol Med,* 26: 1085-91, 1999.

McConell, G., Snow, R. J., Proietto, J., and Hargreaves, M. Muscle metabolism during prolonged exercise in humans: influence of carbohydrate availability. *J Appl Physiol,* 87: 1083-1086, 1999.

McCord, J. M., and Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem,* 244: 6049-55, 1969.

McCutcheon, L. J., Geor, R. J., and Shen, H. Skeletal muscle Na^+-K^+-ATPase and K+ homeostasis during exercise: effects of short-term training. *Equine Vet J Suppl,* 30: 303-10, 1999.

McKenna, M. J. Mechanisms of muscle fatigue. In *Physiological Bases of Sports Performance*, Hargreaves, M., and Hawley, J. A. (eds.), 79-107, Sydney, McGraw Hill, 2003.

McKenna, M. J. Schmidt, T. A. Hargreaves, M. Cameron, L. Skinner, S. L. and Kjeldsen, K. Sprint training increases human skeletal muscle Na^+-K^+-ATPase concentration and improves K^+ regulation. *J Appl Physiol,* 75: 173-80, 1993.

McKenna, M. J. Role of skeletal muscle Na^+, K^+ -pump during exercise. In *Proceedings: International Biochemistry of Exercise X*, Hargreaves, M., and Thompson, M. (eds), 71-97, Champaighn, IL, Human Kinetics, 1999.

McKenna, M. J. The roles of ionic processes in muscular fatigue during intense exercise. *Sports Med,* 13: 134-45, 1992.

McKenna, M. J., Gissel, H., and Clausen, T. Effects of electrical stimulation on Na^+,K^+-ATPase (^{3}H-ouabain binding) in rat skeletal muscle. *J Physiol*, 547:567-580, 2003.

McKenna, M. J., Harmer, A. R., Fraser, S. F., and Li, J. L. Effects of training on potassium, calcium and hydrogen ion regulation in skeletal muscle and blood during exercise. *Acta Physiol Scand,* 156: 335-46, 1996.

McKenna, M. J., Heigenhauser, G. J., McKelvie, R. S., MacDougall, J. D., and Jones, N. L. Sprint training enhances ionic regulation during intense exercise in men. 501: 687-702, 1997.

McKenna, M. J. Heigenhouser, G. J. F. McKelvie, R. S. Obminski, G. MacDougall, J. D. and Jones, N. L. Enhanced pulmonary and active skeletal muscle gas exchange during intense exercise after sprint training in men. *J Physiol,* 501: 703-716, 1997.

McLellan, L. I. Lewis, A. D. Hall, D. J. Ansell, J. D. and Wolf, C. R. Uptake and distribution of *N*-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. *Carcinogenesis,* 16: 2099-106., 1995.

Medbø, J. I., Jebens, E., Vikne, H., Refsnes, P. E., and Gramvik, P. Effect of strenuous strength training on the NaK pump concentration in skeletal muscle of well-trained men. *Eur J Appl Physiol,* 84: 148-154, 2001.

Medbø, J. I., and Sejersted, O. M. Plasma potassium changes with high intensity exercise. 421: 105-22, 1990.

Meister, A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal, applications in research and therapy. *Pharmacol Ther,* 51: 155-94, 1991.

Michelet, F. Gueguen, R. Leroy, P. Wellman, M. Nicolas, A. and Siest, G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. *Clin Chem,* 41: 1509-17, 1995.

Millet, G. Y., Lepers, R., Maffiuletti, N. A., Babault, N., Martin, V., and Lattier, G. Alterations of neuromuscular function after an ultramarathon. *J Appl Physiol,* 92: 486-92, 2002.

Mills, P. C., Smith, N. C., Casas, I., Harris, P., Harris, R. C., and Marlin, D. J. Effects of exercise intensity and environmental stress on indices of oxidative stress and iron homeostasis during exercise in the horse. 74: 60-6, 1996.

Misra, H. P., and Fridovich, I. The generation of superoxide radical during the autoxidation of haemoglobin. *J Biol Chem,* 247: 6960-2, 1972.

Miyazaki, H., Oh-ishi, S., Ookawara, T., Kizaki, T., Toshinai, K., Ha, S., Haga, S., Ji, L. L., and Ohno, H.: Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. *Eur J Appl Physiol Occup Physiol,* 84: 1-6, 2001.

Morales, C. F., Anzueto, A., Andrade, F., Levine, S. M., Maxwell, L. C., Lawrence, R. A., and Jenkinson, S. G. Diethylmaleate produces diaphragmatic impairment after resistive breathing. *J Appl Physiol,* 75: 2406-2411, 1993.

Murphy, K. T., Snow, R. J., Petersen, A. C, Murphy, R. M., Mollica, J., Lee, J. S., Garnham, A. P., Aughey, R. J., Leppik, J. A., Medved, I., Cameron-Smith, D., McKenna, M. J. Intense exercise upregulates Na^+,K^+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *J Physiol,* 30: 30, 2004.

Murrant, C. L. Andrade, F. H. and Reid, M. B. Exogenous reactive oxygen and nitric oxide alter intracellular oxidant status of skeletal muscle fibres. *Acta Physiol Scand,* 166: 111-21, 1999.

Nakajima, S., Nakajima, Y., and Bastian, J. Effects of sudden changes in external sodium concentration on twitch tension in isolated muscle fibers. *J Gen Physiol,* 65: 459-82., 1975.

Nakao, C., Ookawara, T., Kizaki, T., Oh-Ishi, S., Miyazaki, H., Haga, S., Sato, Y., Ji, L. L., and Ohno, H. Effects of swimming training on three superoxide dismutase isoenzymes in mouse tissues. *J Appl Physiol,* 88: 649-54, 2000.

Nashawati, E., Dimarco, A., and Supinski, G. Effects produced by infusion of a free radical-generating solution into the diaphragm. *Am Rev Resp Dis,* 147: 60-5, 1993.

Nielsen, J. J., Mohr, M., Klarskov, C., Kristensen, M., Krustrup, P., Juel, C., and Bangsbo, J. Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *J Physiol,* 554: 857-70. Epub 2003 Nov 21., 2004.

Nielsen, O. B., and Clausen, T. The Na^+/K^+-pump protects muscle excitability and contractility during exercise. *Exerc Sport Sci Rev,* 28: 159-64, 2000.

Nielsen, O. B., and Clausen, T. The significance of active Na^+,K^+ transport in the maintenance of contractility in rat skeletal muscle. *Acta Physiol Scand,* 157: 199-209, 1996.

Nielsen, O. B., de Paoli, F., and Overgaard, K. Protective effects of lactic acid on force production in rat skeletal muscle. *J Physiol,* 536: 161-166, 2001.

Nielsen, O. B., Harrison, A.P. The regulation of the Na^+, K^+ pump in contracting skeletal muscle. *Acta Physiol Scand,* 162: 191-200, 1998.

Nielsen, O. B., and Overgaard, K. Ion gradients and contractility in skeletal muscle: the role of active Na^+, K^+ transport. *Acta Physiol Scand,* 156: 247 - 256, 1996.

Nohl, H., and Hegner, D. Do mitochondria produce oxygen radicals in vivo? *Eur J Biochem,* 82: 563-7., 1978.

Nørgaard, A. Quantification of the Na,K-pumps in mammalian skeletal muscle. *Acta Pharmacol Toxicol,* 58: 1-34, 1986.

Novelli, G. P., Falsini, S., and Bracciotti, G. Exogenous glutathione increases endurance to muscle effort in mice. *Pharmacol Res,* 23: 149-55, 1991.

Oba, T., Koshita, M., and Yamaguchi, M. H_2O_2 modulates twitch tension and increases P_o of Ca^{2+} release channel in frog skeletal muscle. *Am J Physiol,* 271: C810-8, 1996.

Oba, T., Kurono, C., Nakajima, R., Takaishi, T., Ishida, K., Fuller, G. A., Klomkleaw, W., and Yamaguchi, M. H_2O_2 activates ryanodine receptor and has little effect on recovery of releasable Ca^{2+} content after fatigue. *J Appl Physiol,* 93: 1999-2008, 2002.

Oh-ishi, S., Kizaki, T., Ookawara, T., Sakurai, T., Izawa, T., Nagata, N., and Ohno, H. Endurance training improves the resistance of rat diaphragm to exercise-induced oxidative stress. *Am J Resp Crit Care Med,* 156: 1579-85, 1997.

Oh-ishi, S., Kizaki, T., Ookawara, T., Toshinai, K., Haga, S., Karasawa, F., Satoh, T., Nagata, N., Ji, L. L., and Ohno, H. The effect of exhaustive exercise on the antioxidant enzyme system in skeletal muscle from calcium-deficient rats. *Pflugers Archiv.* 435: 767-74, 1998.

Ohkuwa, T. Sato, Y. and Naoi, M. Glutathione status and reactive oxygen generation in tissues of young and old exercised rats. *Acta Physiol Scand,* 159: 237-44, 1997.

Ohno, H., Yahata, T., Sato, Y., Yamamura, K., and Taniguchi, N. Physical training and fasting erythrocyte activities of free radical scavenging enzyme systems in sedentary men. *Eur J Appl Physiol Occup Physiol,* 57: 173-6, 1988.

Okamura, K., Doi, T., Hamada, K., Sakurai, M., Yoshioka, Y., Mitsuzono, R., Migita, T., Sumida, S., and Sugawa-Katayama, Y. Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. *Free Rad Res,* 26: 507-14, 1997.

Olsson, B., Johansson, M., Gabrielsson, J., and Bolme, P. Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. *Eur J Clin Pharmacol,* 34: 77-82, 1988.

O'Neill, C. A., Stebbins, C. L., Bonigut, S., Halliwell, B., and Longhurst, J. C. Production of hydroxyl radicals in contracting skeletal muscle of cats. *J Appl Physiol,* 81: 1197-206, 1996.

Ono, K., Inui, K., Hasegawa, T., Matsuki, N., Watanabe, H., Takagi, S., Hasegawa, A., and Tomoda, I. The changes of antioxidative enzyme activities in equine erythrocytes following exercise. *Nippon Juigaku Zasshi,* 52: 759-65, 1990.

Oostenbrug, G. S. Mensink, R. P. Hardeman, M. R. De Vries, T. Brouns, F. and Hornstra, G. Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E. *J Appl Physiol,* 83: 746-52, 1997.

Ørtenblad, N., Madsen, K., and Djurhuus, M. S. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. *Am J Physiol,* 272: R1258-63, 1997.

Overgaard, K., Lindstrom, T., Ingemann-Hansen, T., and Clausen, T. Membrane leakage and increased content of Na+-K+ pumps and Ca++ in human muscle following a 100 km run. *J Appl Physiol,* 92: 1891-1898, 2002.

Overgaard, K., and Nielsen, O. B. Activity-induced recovery of excitability in K^+-depressed rat soleus muscle. *Am J Physiol* 280: R48-55, 2001.

Overgaard, K., Nielsen, O. B., and Clausen, T. Effects of reduced electrochemical Na+ gradient on contractility in skeletal muscle: role of the Na^+-K^+ pump. *Pflugers Arch,* 434: 457-65, 1997.

Overton, E.: Beitrage zur allgermeinen Muskel und Nervenphysiologie. *Pflugers Arch,* 92: 346-386, 1902.

Oyanagui, Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal Biochem,* 142: 290-6, 1984.

Packer, L.: Oxidants, antioxidant nutrients and the athlete. *J Sp Sci,* 15: 353-63, 1997.

Parks, D. A., Williams, T. K., and Beckman, J. S. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation. *Am J Physiol,* 254: G768-74, 1988.

Pate, E., Bhimani, M., Franks-Skiba, K., Cooke, R. Reduced effect of pH on skinned rabbit psoas muscle mechanics at high temperatures: implications for fatigue. *J Physiol,* 486: 689 - 694., 1995.

Plant, D. R., Gregorevic, P., Williams, D. A., and Lynch, G. S. Redox modulation of maximum force production of fast-and slow-twitch skeletal muscles of rats and mice. *J Appl Physiol,* 90: 832-8, 2001.

Posterino, G. S., Cellini, M. A., and Lamb, G. D. Effects of oxidation and cytosolic redox conditions on excitation-contraction coupling in rat skeletal muscle. *J Physiol,* 547: 807-823, 2003.

Posterino, G. S., and Lamb, G. D. Effects of reducing agents and oxidants on excitation-contraction coupling in skeletal muscle fibres of rat and toad. *J Physiol,* 496: 809-25, 1996.

Powers, S., and Sen, C. K. Physiological antioxidants and exercise training. In *Handbook of oxidants and antioxidants in exercise*, Sen, C. K., Packer, L., and Hänninen, O. (eds.), 221-242, Amsterdam, Elsevier Science B.V., 2000.

Powers, S. K., Criswell, D., Lawler, J., Ji, L. L., Martin, D., Herb, R. A., and Dudley, G. Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *Am J Physiol,* 266: R375-80, 1994.

Powers, S. K., Ji, L. L., and Leeuwenburgh, C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. *Med Sci Sp Exerc,* 31 987-97, 1999.

Powers, S. K., Lawler, J., Criswell, D., Lieu, F. K., and Martin, D. Aging and respiratory muscle metabolic plasticity: effects of endurance training. *J Appl Physiol,* 72: 1068-73, 1992.

Prescott, L. F. Donovan, J. W. Jarvie, D. R. and Proudfoot, A. T. The disposition and kinetics of intravenous *N*-acetylcysteine in patients with paracetamol overdosage. *Eur J Clin Pharmacol,* 37: 501-6, 1989.

Pyke, S., Lew, H., and Quintanilha, A. Severe depletion in liver glutathione during physical exercise. *Biochem Biophys Res Comm,* 139: 926-31, 1986.

Rabinovich, R. A., Ardite, E., Troosters, T., Carbo, N., Alonso, J., Gonzalez de Suso, J. M., Vilaro, J., Barbera, J. A., Polo, M. F., Argiles, J. M., Fernandez-Checa, J. C., Roca, J. Reduced muscle redox capacity after endurance training in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 164: 1114-8., 2001.

Radak, Z., Kaneko, T., Tahara, S., Nakamoto, H., Ohno, H., Sasvari, M., Nyakas, C., and Goto, S. The effect of exercise training on oxidative damage of lipids, proteins, and DNA in rat skeletal muscle: evidence for beneficial outcomes. *Free Rad Biol Med,* 27: 69-74, 1999.

Reid, M. Reactive Oxygen and Nitric Oxide in Skeletal Muscle. *NIPS,* 11(3): 114-119, 1996.

Reid, M. B. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol,* 90(2): 724-31, 2001.

Reid, M. B. Nitric oxide, reactive oxygen species, and skeletal muscle contraction. *Med Sci Sp Exer,* 33(3): 371-6, 2001.

Reid, M. B. Plasticity in Skeletal, Cardiac, and Smooth Muscle: Invited Review: Redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol,* 90(2): 724-731, 2001.

Reid, M. B. Redox modulation of skeletal muscle contraction by reactive oxygen and nitric oxide. In *Biochemistry of Exercise X*, pp. 155-166. Hargreaves, M., and Thompson, M. (eds), 155-166, Champaign, IL., Human Kinetics, 1999.

Reid, M. B. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand,* 162(3): 401-9, 1998.

Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L. and West, M. S. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol,* 73(5): 1797-804, 1992.

Reid, M. B., Khawli, F. A. and Moody, M. R. Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. *J Appl Physiol,* 75(3): 1081-7, 1993.

Reid, M. B., and Li, Y. P. Cytokines and oxidative signalling in skeletal muscle. *Acta Physiol Scand,* 171(3): 225-32, 2001.

Reid, M. B., and Moody, M. R.: Dimethyl sulfoxide depresses skeletal muscle contractility. *J Appl Physiol,* 76(5): 2186-90, 1994.

Reid, M. B., Shoji, T., Moody, M. R. and Entman, M. L. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol,* 73(5): 1805-9, 1992.

Reid, M. B., Stokic, D. S., Koch, S. M. Khawli, F. A. and Leis, A. A. *N*-acetylcysteine inhibits muscle fatigue in humans. *Journal of Clinical Investigation,* 94(6): 2468-74, 1994.

Renaud, J. M. Modulation of force development by Na^+, K^+, Na^+ K^+ pump and K_{ATP} channel during muscular activity. *Can J Appl Physiol,* 27(3): 296-315., 2002.

Rice-Evans, C. and Burdon, R. Free radical-lipid interactions and their pathological consequences. *Prog Lipid Res,* 32(1): 71-110, 1993.

Roberts, D. and Smith, D. J.: Biochemical aspects of peripheral muscle fatigue. A review. *Sports Med,* 7(2): 125-38, 1989.

Rodenstein, D. DeCoster, A. and Gazzaniga, A. Pharmacokinetics of oral acetylcysteine: absorption, binding and metabolism in patients with respiratory disorders. *Clin Pharm,* 3: 247-54, 1980.

Rokitzki, L. Logemann, E. Sagredos, A. N. Murphy, M. Wetzel-Roth, W. and Keul, J. Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta Physiol Scand,* 151: 149-58, 1994.

Sahlin, K., Cizinsky, S., Warholm, M., and Hoberg, J. Repetitive static muscle contractions in humans--a trigger of metabolic and oxidative stress? 64: 228-36, 1992.

Sahlin, K. Ekberg, K. and Cizinsky, S. Changes in plasma hypoxanthine and free radical markers during exercise in man. *Acta Physiol Scand,* 142: 275-81, 1991.

Sahlin, K. Fernstrom, M. Svensson, M. and Tonkonogi, M. No evidence of an intracellular lactate shuttle in rat skeletal muscle. *J Physiol,* 541: 569-574, 2002.

Sahlin, K. Tonkonogi, M. and Soderlund, K. Energy supply and muscle fatigue in humans. *Acta Physiol Scand,* 162: 261-6, 1998.

Sandiford, S. D. Green, H. J. Duhamel, T. A. Perco, J. G. Schertzer, J. D. and Ouyang, J. Inactivation of human muscle Na^+-K^+-ATPase in vitro during prolonged exercise is increased with hypoxia. *J Appl Physiol,*: 01273.2003, 2004.

Saraiva, P. A. Giovani, L. Giordan, E. A. and Leitao, F. B.: Prolonged use of *N*-acetyl-cysteine by oral route. *Revista Do Hospital Das Clinicas; Faculdade de Medicina Da Universidade de Sao Paulo,* 35: 56-9, 1980.

Sastre, J. Asensi, M. Gasco, E. Pallardo, F. V. Ferrero, J. A. Furukawa, T. and Vina, J. Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. *Am J Physiol,* 263: R992-5, 1992.

Schauer, J. E. Schelin, A. Hanson, P. and Stratman, F. W. Dehydroepiandrosterone and a beta-agonist, energy transducers, alter antioxidant enzyme systems: influence of chronic training and acute exercise in rats. *Arch Biochem Biophys,* 283: 503-11, 1990.

Scherer, N. M., and Deamer, D. W. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca^{2+}-ATPase. *Arch Biochem Biophys,* 246: 589-601, 1986.

Sejersted, O. M., and Hallèn, J. Na,K homeostasis of skeletal muscle during activation. *Med Sp Sci,* 26: 1-11, 1985.

Sejersted, O. M. Medbo, J. I. and Hermansen, L. Metabolic acidosis and changes in water and electrolyte balance after maximal exercise. *Ciba Found Symp,* 87: 153-67, 1982.

Sejersted, O. M., and Sjogaard, G. Dynamics and Consequences of Potassium Shifts in Skeletal Muscle and Heart During Exercise. *Physiol. Rev.,* 80: 1411-1481, 2000.

Sen, C. K. Antioxidants in exercise nutrition. *Sports Med,* 31: 891-908, 2001.

Sen, C. K. Oxidants and antioxidants in exercise. *J Appl Physiol,* 79: 675-86, 1995.

Sen, C. K. Update on thiol status and supplements in physical exercise. *Can J Appl Physiol,* 26: S4-12, 2001.

Sen, C. K., Atalay, M., and Hanninen, O. Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J Appl Physiol,* 77: 2177-87, 1994.

Sen, C. K., Kolosova, I., Hanninen, O., and Orlov, S. N. Inward potassium transport systems in skeletal muscle derived cells are highly sensitive to oxidant exposure. *Free Rad Biol Med,* 18: 795-800, 1995.

Sen, C. K., Marin, E., Kretzschmar, M., and Hanninen, O. Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J Appl Physiol,* 73: 1265-72, 1992.

Sen, C. K., and Packer, L. Thiol homeostasis and supplements in physical exercise. *Am J Clin Nutr,* 72: 653S-69S, 2000.

Sen, C. K., Rankinen, T., Vaisanen, S., and Rauramaa, R. Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *J Appl Physiol,* 76: 2570-7, 1994.

Shindoh, C., DiMarco, A., Thomas, A., Manubay, P., and Supinski, G. Effect of N-acetylcysteine on diaphragm fatigue. *J Appl Physiol,* 68: 2107-13, 1990.

Simon-Schnass, I. Risk of oxidative stress during exercise at high-altitude. In *Exercise and oxygen toxicity*. Sen, C. K., Packer, L., and Hanninen, O. (eds.), Elsevier Science, 1994.

Sjødin, B., Hellsten-Westing, Y., and Apple, F. S. Biochemical mechanisms for oxygen free radical formation during exercise. *Sports Med,* 10: 236-54, 1990.

Sjøgaard, G. Electrolytes in slow and fast muscle fibers of humans at rest and with dynamic exercise. *Am J Physiol,* 245: R25-31, 1983.

Sjøgaard, G. Potassium and fatigue: the pros and cons. *Acta Physiol Scand,* 156: 257-264, 1996.

Sjøgaard, G. Water and electrolyte fluxes during exercise and their relation to muscle fatigue. *Acta Physiol Scand*, 556: 129-36, 1986.

Sjøgaard, G., Adams, R. P., and Saltin, B. Water and ion shifts in skeletal muscle of humans with intense dynamic knee extension. *Am J Physiol,* 248: R190-6, 1985.

Skou, J. The Na-K Pump. *NIPS,* 7: 95-100, 1992.

Smolka, M. B., Zoppi, C. C., Alves, A. A., Silveira, L. R., Marangoni, S., Pereira Da-Silva, L., Novello, J. C., and Macedo, D. V. HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats. 279: R1539-45, 2000.

Somani, S. M., Frank, S., and Rybak, L. P. Responses of antioxidant system to acute and trained exercise in rat heart subcellular fractions. *Pharmacol Biochem Behav,* 51: 627-34, 1995.

Spodaryk, K., Szygu,a, Z., Dabrowski, Z., and Miszta, H. The activity of erythrocyte enzymes in rats subjected to running exercises. *Eur J Appl Physiol Occup Physiol,* 54: 533-7, 1985.

Stephens, T. J., McKenna, M. J., Canny, B. J., Snow, R. J., and McConell, G. K. Effect of sodium bicarbonate on muscle metabolism during intense endurance cycling. *Med Sci Sports Exerc,* 34: 614-21., 2002.

Stephenson, D. G., Lamb, G. D., and Stephenson, G. M. Events of the excitation-contraction-relaxation (E-C-R) cycle in fast- and slow-twitch mammalian muscle fibres relevant to muscle fatigue. *Acta Physiol Scand,* 162: 229-45, 1998.

Stephenson, D. G., Nguyen, L. T., and Stephenson, G. M. Glycogen content and excitation-contraction coupling in mechanically skinned muscle fibres of the cane toad. *J Physiol,* 519: 177-187, 1999.

Strojnik, V., and Komi, P. V. Neuromuscular fatigue after maximal stretch-shortening cycle exercise. *J Appl Physiol,* 84: 344-50, 1998.

Subudhi, A. W., Fu, M., Strothkamp, K. G., and Murray, D. M. Effect of graded exercise on blood glutathione status in trained and untrained humans. *Int Sp J,* 7: 82-87, 2003.

Supinski, G., Nethery, D., Stofan, D., and DiMarco, A. Effect of free radical scavengers on diaphragmatic fatigue. *Am J Resp Crit Care Med,* 155: 622-9, 1997.

Supinski, G. S. Stofan, D. Ciufo, R. and DiMarco, A.: *N*-acetylcysteine administration and loaded breathing. *J Appl Physiol,* 79: 340-7, 1995.

Supinski, G., Nethery, D., Stofan, D., and DiMarco, A. Extracellular calcium modulates generation of reactive oxygen species by the contracting diaphragm. *J Appl Physiol,* 87: 2177-85, 1999.

Supinski, G., Nethery, D., Stofan, D., Szweda, L., and DiMarco, A. Oxypurinol administration fails to prevent free radical-mediated lipid peroxidation during loaded breathing. *J Appl Physiol,* 87: 1123-31, 1999.

Supinski, G., Stofan, D., and DiMarco, A. Effect of ischemia-reperfusion on diaphragm strength and fatigability. *J Appl Physiol,* 75: 2180-2187, 1993.

Suwannachot, P., Verkleij, C. B., Weijs, W. A., van Weeren, P. R., and Everts, M. E. Effects of training on the concentration of Na^+, K^+-ATPase in foal muscle. *Equine Vet J Suppl,* 31: 101-5, 1999.

Svensson, M. B., Ekblom, B., Cotgreave, I. A., Norman, B., Sjoberg, B., Ekblom, O., Sjodin, B., and Sjodin, A. Adaptive stress response of glutathione and uric acid metabolism in man following controlled exercise and diet. *Acta Physiol Scand,* 176: 43-56., 2002.

Tateishi, N., Higashi, T., Naruse, A., Nakashima, K., and Shiozaki, H. Rat liver glutathione: possible role as a reservoir of cysteine. *J Nutr,* 107: 51-60, 1977.

Tauler, P., Aguilo, A., Cases, N., Sureda, A., Gimenez, F., Villa, G., Cordova, A., and Biescas, A. P. Acute phase immune response to exercise coexists with decreased neutrophil antioxidant enzyme defences. *Free Radic Res,* 36: 1101-7, 2002.

Tauler, P., Aguilo, A., Gimeno, I., Fuentespina, E., Tur, J. A., and Pons, A. Influence of vitamin C diet supplementation on endogenous antioxidant defences during exhaustive exercise. *Pflugers Arch,*446: 658-664, 2003.

Tauler, P., Gimeno, I., Aguilo, A., Guix, M. P., and Pons, A. Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. *Pflugers Arch,* 438: 782-7, 1999.

Tessier, F., Margaritis, I., Richard, M. J., Moynot, C., and Marconnet, P. Selenium and training effects on the glutathione system and aerobic performance. *Med Sci Sp Exerc,* 27: 390-6, 1995.

Tiidus, P. M., and Houston, M. E. Antioxidant and oxidative enzyme adaptations to vitamin E deprivation and training. *Med Sci Sports Exerc,* 26: 354-9, 1994.

Tiidus, P. M., Pushkarenko, J., and Houston, M. E. Lack of antioxidant adaptation to short-term aerobic training in human muscle. *Am J Physiol,* 271: R832-6, 1996.

Tonkonogi, M., Walsh, B., Svensson, M., and Sahlin, K. Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress. *J Physiol,* 528: 379-88, 2000.

Travaline, J. M., Sudarshan, S., Roy, B. G., Cordova, F., Leyenson, V., and Criner, G. J. Effect of *N*-Acetylcysteine on Human Diaphragm Strength and Fatigability. *Am. J. Respir. Crit. Care Med.,* 156: 1567-1571, 1997.

Tupling, A. R., Green, H. J., Roy, B. D., Grant, S., and Ouyang, J. Paradoxical effects of prior activity on human sarcoplasmic reticulum Ca^{2+}-ATPase response to exercise. *J Appl Physiol,* 95: 138-144, 2003.

Tyler, D. D. Role of superoxide radicals in the lipid peroxidation of intracellular membranes. *FEBS Lett,* 51: 180-3., 1975.

Urso, M. L., and Clarkson, P. M. Oxidative stress, exercise, and antioxidant supplementation. *Toxicol,* 189: 41-54, 2003.

Vani, M., Reddy, G. P., Reddy, G. R., Thyagaraju, K., and Reddanna, P. Glutathione-S-transferase, superoxide dismutase, xanthine oxidase, catalase, glutathione peroxidase and lipid peroxidation in the liver of exercised rats. *Biochem Int,* 21: 17-26, 1990.

Veeneklaas, R. J., Verkleij, C. B., van Schie, B., Harun, M. A., and Everts, M. E. Preliminary studies on the concentration of Na^+,K^+-ATPase in skeletal muscle of draught cattle in Mozambique: effect of sex, age and training. *Trop Anim Health Prod,* 34: 431-47, 2002.

Verburg, E., Hallen, J., Sejersted, O. M., and Vollestad, N. K. Loss of potassium from muscle during moderate exercise in humans: a result of insufficient activation of the Na^+-K^+-pump? *Acta Physiol Scand,* 165: 357-67, 1999.

Viguie, C. A., Frei, B., Shigenaga, M. K., Ames, B. N., Packer, L., and Brooks, G. A. Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J Appl Physiol,* 75: 566-72, 1993.

Vina, J., Gimeno, A., Sastre, J., Desco, C., Asensi, M., Pallardo, F. V., Cuesta, A., Ferrero, J. A., Terada, L. S., Repine, J. E. Mechanism of free radical production in exhaustive exercise in humans and rats, role of xanthine oxidase and protection by allopurinol. *IUBMB Life,* 49: 539-44, 2000.

Vinnikova, A. K., Kukreja, R. C., and Hess, M. L. Singlet oxygen-induced inhibition of cardiac sarcolemmal Na^+K^+-ATPase. *J Mol Cell Cardiol,* 24: 465-70, 1992.

Vrabas, I. S., Dodd, S. L., Powers, S. K., Hughes, M., Coombes, J., Fletcher, L., Demirel, H., and Reid, M. B. Endurance training reduces the rate of diaphragm fatigue in vitro. *Med Sci Sp Exerc,* 31: 1605-12, 1999.

Weisiger, R. A., and Fridovich, I. Mitochondrial superoxide dismutase: Site of synthesis and intramitochondrial localization. *J Biol Chem,* 248: 4793-6., 1973.

Weiss, C., Bierhaus, A., Kinscherf, R., Hack, V., Luther, T., Nawroth, P. P., and Bartsch, P. Tissue factor-dependent pathway is not involved in exercise-induced formation of thrombin and fibrin. *J Appl Physiol,* 92: 211-218, 2002.

Westerblad, H., Allen, D. G., and Lannergren, J. Muscle fatigue: lactic acid or inorganic phosphate the major cause? *NIPS,* 17: 17-21., 2002.

Westerblad, H., Bruton, J. D., and Lannergren, J. The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. *J Physiol,* 500: 193-204, 1997.

Wilson, J. R., McCully, K. K., Mancini, D. M., Boden, B., and Chance, B. Relationship of muscular fatigue to pH and diprotonated P_i in humans: a ^{31}P-NMR study. *J Appl Physiol,* 64: 2333-9, 1988.

Wolitzky, B. A., and Fambrough, D. M. Regulation of the Na^+,K^+-ATPase in cultured chick skeletal muscle. Modulation of expression by the demand for ion transport. *J Biol Chem,* 261: 9990-9, 1986

Xu, K. Y., Zweier, J. L., and Becker, L. C. Hydroxyl radical inhibits sarcoplasmic reticulum Ca^{2+}-ATPase function by direct attack on the ATP binding site. *Circ Res,* 80: 76-81, 1997.

Yamashita, H., Sata, M., Sugiura, S., Momomura, S., Serizawa, T., and Iizuka, M. ADP inhibits the sliding velocity of fluorescent actin filaments on cardiac and skeletal myosins. *Circ Res,* 74: 1027-33, 1994.

Yu, B. P. Cellular defenses against damage from reactive oxygen species. *Physiol Rev,* 74: 139-62, 1994.

CHAPTER 11: APPENDICES

Attached are the following:

Appendix 1: Malcolm Brown, Andrew Bjorksten, Ivan Medved and Michael McKenna. Pharmacokinetics of intravenous *N*-acetylcysteine in men at rest and during exercise. *European Journal of Clinical Pharmacology* In Press 2004

Appendix 2: Subject informed consent form Study 1

Appendix 3: Approval of ethics application involving human subjects

Appendix 4: Calculation of NAC dose

Appendix 5: Subject informed consent form Study 2

Appendix 6: Standard food packages consumed by subjects

Appendix 7: Subject informed consent form Study 3 – Part I and II

Appendix 8: Raw data Study 1

Appendix 9: Raw data Study 2

Appendix 10: Raw data Study 3 – Part I

Appendix 11: Raw data Study 3 – Part II

Appendix 12: Photos from experimental days

APPENDIX 1:

Malcolm Brown, Andrew Bjorksten, Ivan Medved and Michael McKenna. **Pharmacokinetics of intravenous *N*-acetylcysteine in men at rest and during exercise**. *European Journal of Clinical Pharmacology* In Press 2004

Title:

Pharmacokinetics of intravenous N-acetylcysteine in men at rest and during exercise.

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Abstract:

Objective: We aimed to determine the pharmacokinetics (PK) of N-acetylcysteine (NAC) at rest and during exercise when given by continuous intravenous infusion intended to maintain relatively constant plasma concentrations.

Methods: Plasma concentrations of NAC were measured in 24 healthy male subjects during and after a two-stage intravenous infusion designed to provide constant NAC concentrations during cycling exercise, including intense exercise to fatigue.

Results: A three compartment open PK model was the best fit using population PK analysis with NONMEM. Whole body clearance (CL) was 0.58 $l.kg^{-1}.h^{-1}$ (95% c.i. 0.44-0.72) for reduced NAC (NACR) and 0.16 (0.13-0.20) $l.kg^{-1}.h^{-1}$ for total NAC (NACT). The central volume of distribution (V1) was 0.064 (0.008-0.12) $l.kg^{-1}$ for NACR and 0.037 (0.02-0.06) $l.kg^{-1}$ for NACT. Exercise was a significant covariate in the model resulting in 25% and 23% reduction in CL of NACR and NACT respectively. V1 in our subjects was smaller than expected, resulting in higher than anticipated initial concentrations of NAC. Despite these findings, the incidence of adverse effects attributable to NAC was minimal, without using prophylactic or concomitant drug therapy.

Conclusions: NAC can be given to healthy exercising men by intravenous infusion and to the plasma concentrations seen in this study with minimal adverse effects due to the drug. The PK parameters of NAC at rest in volunteers are consistent with previously reported values and are

significantly altered by vigorous cycling exercise.

Introduction:

Antioxidant compounds have been used in many investigations of skeletal muscle fatigue. The use of N-acetylcysteine (NAC) to attenuate muscle fatigue in humans was first reported by Reid et al [1] who also observed a number of significant adverse effects attributable to the drug. We have recently reported the effects of NAC on exercise and blood redox status [2,3,4] during short term intense, and prolonged cycling exercise in healthy men. In those studies we aimed to infuse NAC to achieve a relatively constant plasma concentration assuming that if the effects of NAC were concentration-dependent the effects should also be relatively constant during the exercise phases. The target plasma concentration of NAC was chosen based on available data from published studies. In particular we were assisted by the report of adverse effects [1] in a study which showed that the reactions to NAC infusion showed a steady increase in incidence during infusion at a constant rate over one hour.

A limited number of pharmacokinetic studies have been published in which the NAC was given by intravenous infusion. Borgstrom et al. [5] infused 600 mg of NAC over 5 min, approximating a bolus dose, and observed rapidly changing plasma concentrations. Olsson et al. [6] gave 200 mg intravenously, also as a bolus, and measured both total and reduced NAC over 12 h in healthy subjects. This was the first report distinguishing the differing pharmacokinetics of NAC in both the reduced form and as total NAC. Prescott et al [7] determined the pharmacokinetics of intravenous NAC in patients with paracetamol overdose. Although not all authors reported adverse effects during the use of NAC, those that did generally related their occurrence to higher concentrations, or to times when the higher concentration would be expected [1,8,9]. The most common adverse effects, sometimes termed "anaphylactoid" include flushing, urticaria, bronchospasm, pruritis, angioedema and nausea and vomiting. On the assumption that

histamine release may underlie some of these effects, the use of antihistamines has been recommended, though we planned to avoid concomitant drug administration, choosing to treat adverse effects if and as they occurred. We had three main aims in this study. Firstly to design an infusion protocol which would achieve relatively constant plasma NAC concentrations during the exercise phase while avoiding adverse effects; secondly to determine the pharmacokinetics of both reduced and total N-acetylcysteine in our subjects at rest and during exercise; and thirdly to either confirm our original protocol or revise it based on the pharmacokinetic analysis.

Methods:

The overall study design has been published previously [2,3,4]. Twenty four male subjects (age: 23.5 (4.5) mean (S.D.) yr; body mass: 77.7 (9.9) kg; height: 179.1 (4.7) cm volunteered for the study after being informed of all known risks and giving written informed consent. Subjects refrained from vigorous activity and avoided ingesting alcohol, caffeine or other drugs in the 24 h before the studies. They were required to have no history of asthma, bronchospasm or atopy, no previous exposure to NAC and not to be taking medication at the time of the study. Females were specifically excluded from the studies with NAC. Eight subjects participated in a study of the effects of intermittent high intensity exercise comprising three bouts of 45 s followed by a final bout to fatigue, each bout at a power output corresponding to 130% of $\dot{V}O_{2peak}$ as detailed previously [2]. Sixteen other subjects took part in studies of lower intensity but more prolonged exercise comprising 45 min at 70% $\dot{V}O_{2peak}$ then to fatigue at 90% $\dot{V}O_{2peak}$ [3,4]. The protocol complies with the World Medical Association Declarations of Helsinki and revisions (2002) regarding ethical principles for medical research involving human subjects. Approval for the studies was obtained from the Victoria University of Technology Human Research Ethics Committee.

Design of the infusion protocol:

The aim was to achieve a target plasma concentration of NAC sufficient to achieve a pharmacological effect while avoiding concentration-dependent adverse effects. Based on published data it was assumed that there is a plasma concentration of NAC below which adverse effects are absent or minimal, and above which frequency is either dose- or concentration-dependent. The time course of cumulative adverse effects during a one hour constant rate infusion of NAC [1] are consistent with the time course of predicted plasma concentrations. Using pharmacokinetic data derived from prolonged NAC infusions in overdose patients [7] we simulated plasma NAC concentrations for the previously reported studies [1,5,6,8,9]. On the basis of this model we estimated that Reid's [1] subjects could have been expected to have plasma NAC concentrations below 100 $mg.l^{-1}$ for the first 15 min of the infusion used. Thereafter, we estimated that the NAC concentration would eventually exceed 100 $mg.l^{-1}$, peaking at approximately 350 $mg.l^{-1}$ by 60 min.

The avoidance of adverse effects has to be counterbalanced by the need to achieve drug concentrations that would be expected to be effective in terms of the experimental outcomes. Unfortunately, there are no data on the minimally effective concentrations of NAC in experimental studies of muscle fatigue in intact humans. We therefore chose a target of 100 $mg.l^{-1}$ as being sufficient to avoid adverse effects and having the expectation of pharmacological effectiveness.

The general theory and method of rapidly achieving and maintaining a desired plasma concentration of a drug by the use of consecutive constant rate infusions has been described [10,11]. An initial infusion is given to achieve the target concentration quickly, with or without some overshoot depending on the rate and duration of the infusion. One or more subsequent infusions are used to maintain a relatively constant

plasma concentration. Estimates of pharmacokinetic parameters are required for this method,and we used the same parameter set as for our simulations. With linear pharmacokinetic models, plasma drug concentrations following any input are a function of the input and disposition functions [12]. That is, a pharmacokinetic model derived from plasma concentration-time data will be independent of the input apart from scaling. Using simulations based on our PK model we designed an infusion protocol of two consecutive constant rate infusions, the first of 125 $mg.kg^{-1}.h^{-1}$ for 15 min followed by 25 $mg.kg^{-1}.h^{-1}$ with the subjects at rest for 35 min, thereafter continuing the infusion during exercise until this phase was terminated by subject fatigue [2,3,4]. N-acetylcysteine (Parvolex®, Faulding Pharmaceuticals) was diluted with 0.9% Sodium Chloride Injection to a concentration of either 80 or 120 $mg.ml^{-1}$. The solution was infused into a forearm vein via a 22-gauge canula (Terumo) using a syringe pump (Graseby 3400, Graseby Medical, Watford, UK) programmed to deliver the two infusions sequentially. The infusion site was chosen to allow the subject to have relatively free use of both arms and to enable constant inspection of the infusion site for local drug related effects. Each subject acted as his own control by receiving on a separate occasion 0.9% Sodium Chloride Injection infusion at the same rate in the placebo phase of the study. A different vein was used on each occasion.

Arterialised venous blood [2] was withdrawn from the contralateral arm for determination of the concentrations of total and reduced thiols, plasma haemoglobin concentration, haematocrit and plasma concentrations of K^+, Na^+, Cl^- and Ca^{++} ions. In each subject samples were taken before commencing the NAC infusion and then at 1, 2, 5, 10, 15, 25 and 35 min. In the intense exercise group, samples were taken at the beginning and end of each 45 s bout, and at fatigue. Post fatigue samples were taken at 1, 2, 5, 10, 30, 60, 120 and 240 min. In the prolonged exercise group, samples were

taken each 15 min during exercise and at fatigue. Post fatigue sampling was as for the previous study. Blood processing and analysis for total and reduced thiols have been detailed previously [2].

Stability and form of the infused NAC solution.

There are no data available on the relative amounts of NAC in the reduced or oxidized forms as infused, or on the stability of these over time. This was determined by diluting the Parvolex® to 120 $mg.ml^{-1}$ and storing the solution at room temperature for up to 120 min. At times 0, 30, 60 and 120 min an aliquot was further diluted in phosphate buffered saline and 10 samples of each were immediately assayed for both reduced and total NAC using the method employed for the analysis of plasma. Difference between batches was tested using analysis of variance.

Pharmacokinetic Analysis:

Concentration-time data for both reduced NAC (NACR) and total NAC (NACT) were modelled using the nonlinear mixed effects program NONMEM [13]. The pharmacokinetics of NACR and NACT were modelled separately, and since the drug input was virtually entirely in the reduced form (Figure 1), the same input data were used for each analysis. We estimated population PK parameters with NONMEM. Although a pooled data approach could be used, particularly with subject groups with similar physical characteristics, the population method allows estimation of both between- and within-subject variability. This method assumes that there is a typical population value for each parameter and that each individual subject has a parameter value which differs from the population value by the between subject variability which can be estimated. Structural PK models were fitted to the concentration-time data using individual values and all data were weighted equally. Both two and three compartment models were fitted to the data. The effect of addition or removal of a parameter was

tested by the change in the reduction in the NONMEM objective function (-2 x log likelihood). Covariates such as exercise, $\dot{V}O_2$, $\dot{V}O_{2peak}$ and age were added to the model or removed according to the reduction in the objective function. A reduction of the objective function by 4 or more with inclusion was accepted as an improved fit.

Between-subject variability was modelled as an exponential error model.

$$\theta_i = TV\theta_i \bullet \exp(\eta_i) \qquad (1)$$

in which θ_i is the value of the parameter θ in the ith subject, $TV\theta$ is the typical or mean population value of this parameter and η_i is a random variable with mean zero and variance ω_η^2.

The remaining variability, which includes within subject variability and measurement errors, was modelled using a proportional error model

$$Cmeas_{ij} = Cpred_{ij} \bullet (1 + \epsilon_{ij}) \qquad (2)$$

where $Cmeas_{ij}$ is the jth measured concentration in the ith individual, $Cpred_{ij}$ is the model prediction for that value and ϵ_{ij} is a random variable with mean zero and variance σ_ϵ^2. NONMEM can provide estimates for the typical population parameter values, the between individual variances and the within individual variances. The NONMEM subroutines ADVAN3 and and ADVAN 11 with TRANS4 were used for building two and three compartment models respectively. This reparameterisation allowed the model parameters to be included as volumes of distribution, whole body clearance and intercompartmental clearances. The NONMEM first order conditional estimates method (FOCE) was used.

Further information on the accuracy of a model can be obtained by calculating the median weighted residuals (MWR) and the median absolute weighted residuals (MAWR) as:

$$MWR = \text{median}\,[(Cmeas_{ij} - Cpred_{ij}) / Cpred_{ij}] \qquad (3)$$

$$MAWR = \text{median}\ \{ABS[(Cmeas_{ij} - Cpred_{ij}) / Cpred_{ij}]\} \qquad (4)$$

with MWR as a measure of bias and MAWR as a measure of precision.

Results are presented as the typical population values for the PK parameters with their between subject variability. Body weight was not used as a covariate since all drug was infused on a per kg body weight basis. Weight was included in the model as a scaling factor. As a covariate, the effect of exercise on parameters was examined both as a two point proportional effect between the two levels of high intensity and the lesser prolonged intensity [2,3,4] and also as a single additive effect.

Results:

Stability and form of the infused NAC solution.

As infused, the NAC in solution was almost entirely in the reduced form with the mean value in the 40 samples being 96.5 (6.8)%. The change of NAC in the reduced form measured over time are shown in Figure 1. There was no significant change between samples with time; p=0.57.

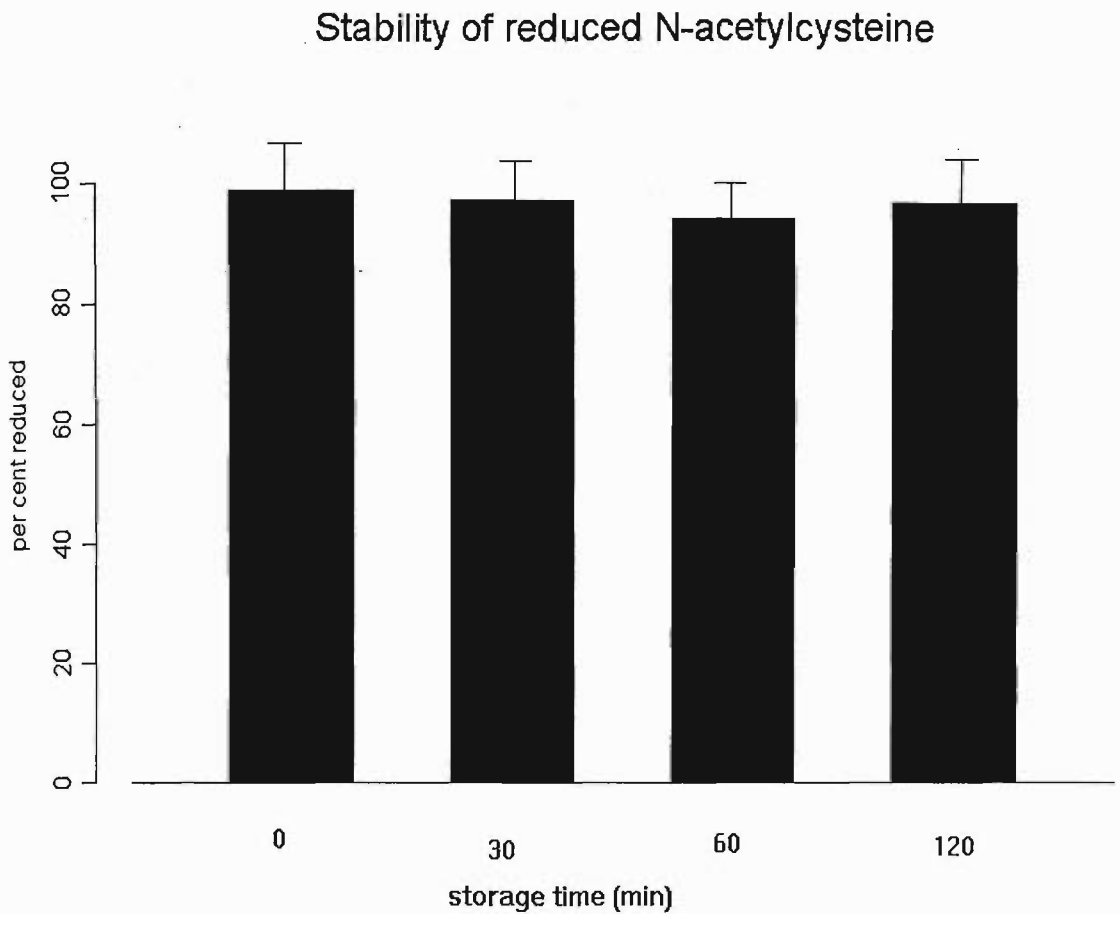

Figure 1. Stability of reduced N-acetylcysteine after dilution of the Parvolex® to 120 $mg.ml^{-1}$ with saline and storage in a syringe at room temperature for the time shown. Each bar shows the percent of the N-acetylcysteine in the reduced form and is the mean(SD) of ten samples.

Pharmacokinetic analysis.

Both total and reduced NAC concentration-time data were best fitted to three compartment open models with covariate effects. The final parameter estimates obtained from the NONMEM analysis are shown in Table 1. The only significantly influential covariate in either model was exercise. This was modelled as an additive effect since including exercise in the model as a graded variable did not improve the fit as determined by the objective function. For clearance, exercise was modelled as:

$$TVCL = \theta_1 + \theta_5 * exer \qquad (5)$$

Where TVCL is the population estimate for clearance, θ_1 and θ_5 are parameters to be estimated and exer is an indicator variable which is 1 during exercise and zero otherwise.

Clearance (CL) of NACT was reduced by 23.2% (6.1 – 40.3) mean (95% confidence limits) during exercise. The clearance of NACR was also reduced during exercise 24.7% (11.1 – 38.2). The small influence of exercise on the central volume of distribution (V1) for NACT was not significant, with the confidence limits including zero. The other covariates $\dot{V}O_2$, $\dot{V}O_{2peak}$ and age did not significantly influence the PK parameter estimates.

Considerable improvements in both MWR and MAWR were seen with the three compartment model as compared with the simpler model. For NACR, MWR was reduced from 17.7% to –8.1% and MAWR was reduced from 47.6% to 27.5%. Improvements of similar magnitude were

seen with NACT.

Pharmacokinetic and model parameters

Total N-acetylcysteine

Model parameter		**value**	**%CV**
CL (whole body clearance)		θ_1 - (exer*0.038)	10.7
Q2 (intercompartmental clearance)		θ_2	23.9
Q3 (intercompartmental clearance)		θ_3	10.9
V1 (central distribution volume)		θ_4	31.2
V2 (peripheral distribution volume)		θ_5	10.3
V3 (peripheral distribution volume)		θ_6	21.8
Parameter estimates		**value**	**SE**
θ_1		0.164 $l^{-1}.kg^{-1}.h^{-1}$	0.018
θ_2		0.123 $l^{-1}.kg^{-1}.h^{-1}$	0.014
θ_3		0.43 $l^{-1}.kg^{-1}.h^{-1}$	0.013
θ_4		0.037 $l^{-1}.kg^{-1}$	0.009
θ_5		0.21 $l^{-1}.kg^{-1}$	0.022
θ_6		0.035 $l^{-1}.kg^{-1}$	0.008
θ_7		-0.038 $l^{-1}.kg^{-1}.h^{-1}$	0.014
Within individual variability	19.6%		
MWR	4.18%		
MAWR	24.5%		

Reduced N-acetylcysteine

Model parameter		**value**	**%CV**
CL (whole body clearance)		θ_1 - (exer*0.14)	12.3
Q2 (intercompartmental clearance)		θ_2	46.2
Q3 (intercompartmental clearance)		θ_3	19.5
V1 (central distribution volume)		θ_4	44.0
V2 (peripheral distribution volume)		θ_5	23.8
V3 (peripheral distribution volume)		θ_6	16.8
Parameter estimates		**value**	**SE**
θ_1		0.58 $l^{-1}.kg^{-1}.h^{-1}$	0.07
θ_2		1.01 $l^{-1}.kg^{-1}.h^{-1}$	0.46
θ_3		0.063 $l^{-1}.kg^{-1}.h^{-1}$	0.012
θ_4		0.064 $l^{-1}.kg^{-1}$	0.028
θ_5		0.125 $l^{-1}.kg^{-1}$	0.03
θ_6		0.14 $l^{-1}.kg^{-1}$	0.023
θ_7		-0.14 $l^{-1}.kg^{-1}.h^{-1}$	0.039
Within individual variability	24.3%		
MWR	-8.1%		
MAWR	27.5%		

Table 1. Population pharmacokinetic and model parameters for reduced and total N-acetylcysteine from 24 subjects. Within and between individual variability are given as CV%. Exer is an indicator variable; exer = 1 during exercise and exer = 0 otherwise.

Drug concentrations and time course:

In all subjects, peak plasma concentrations were seen at the termination of the initial loading infusion (125 $mg.kg^{-1}.h^{-1}$ for 15 min). The mean peak concentration of NACR was 105.1 (31.1) $mg.l^{-1}$ (mean (S.D.)) and for NACT was 205.1 (68.3) $mg.l^{-1}$. The highest concentrations seen in all subjects were 181.9 and 310.5 $mg.l^{-1}$ respectively. Following the first infusion, plasma concentrations fell rapidly and reached a relatively stable plateau within 15 – 20 min during the second infusion (25 $mg.kg^{-1}.h^{-1}$) maintaining concentrations of more than 50 $mg.l^{-1}$ for NACR and more than 150$mg.l^{-1}$ for NACT (Figure 2). During the exercise phase, there was a transient rise in plasma NAC concentration in all subjects. Following the termination of the longer drug infusion, plasma concentrations declined approximately exponentially.

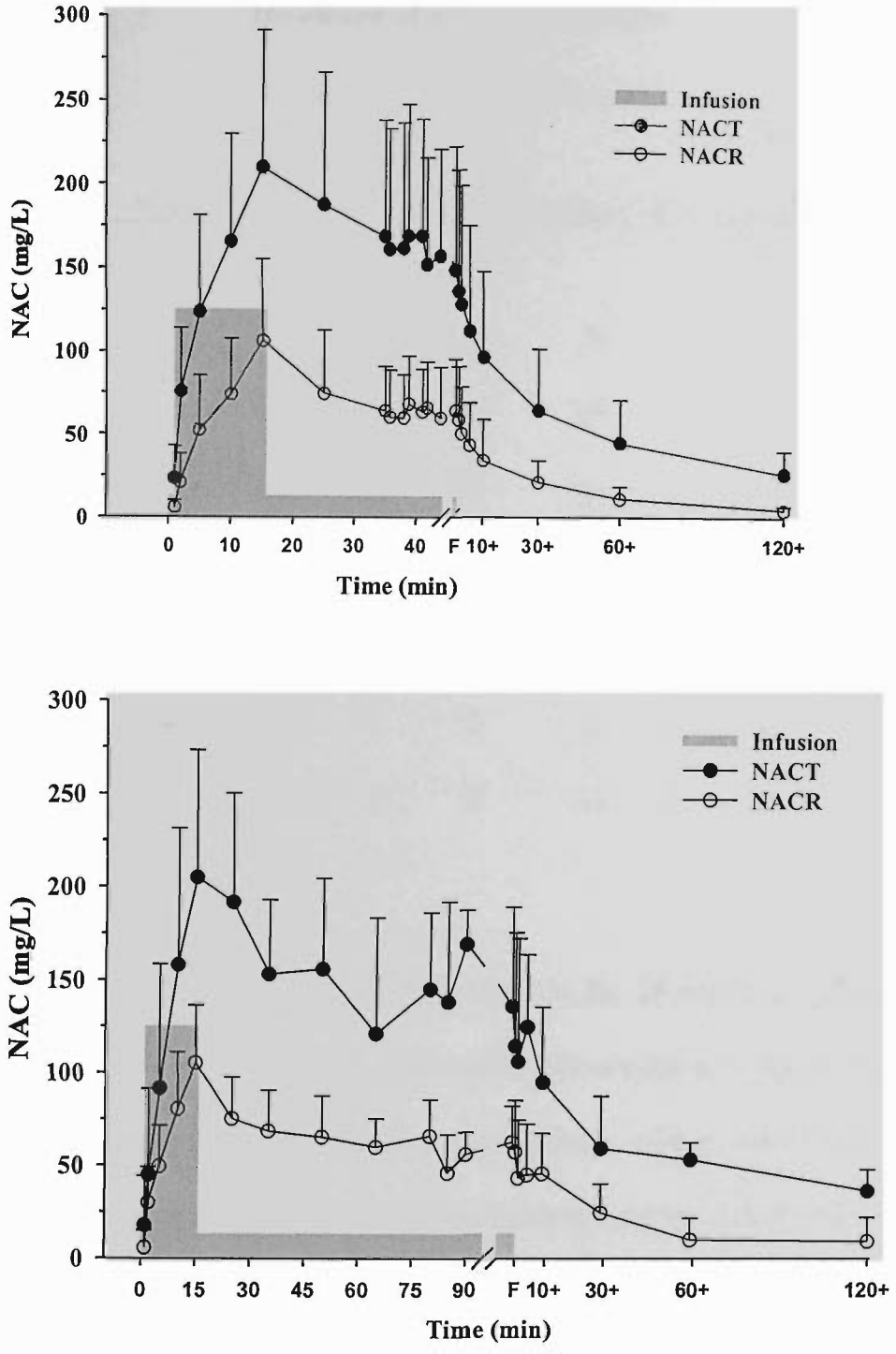

Figure 2. Plasma concentrations of N-acetylcysteine: reduced (NACR) and total (NACT). The upper plot shows the data from Study 1 (high intensity, N = 8) and the lower from Studies 2 and 3 (prolonged, N = 16) exercise. Each point is the mean with S.D. The NAC infusions are shown as hatched areas proportional to the infusion rate. F is the time of fatigue. Duration of exercise (not shown) was 11.4(1.5) min for Study 1 and 62.5(8.1) min for Studies 2 and 3.

Incidence of adverse reactions.

	Type of Reaction and Severity							
	NAC				saline control			
	None	mild	moderate	severe	None	mild	moderate	severe
Type								
Nausea/vomiting	22	1	1	0	23	1	0	0
Local erythema	14	10	0	0	17	7	0	0
Local edema	10	14	0	0	24	0	0	0
Flushing	20	4	0	0	23	1	0	0
Rash	22	2	0	0	22	2	0	0
Coughing	23	1	0	0	23	1	0	0
Bronchospasm	24	0	0	0	24	0	0	0

Table 2. Adverse effects to both NAC and saline in the 24 subjects. Effects have been graded into mild, moderate or severe. Mild and moderate did not require interruption of the protocol or treatment. There were no severe adverse effects seen which would have required intervention or stopping the infusion. Each subject received NAC and saline on different occasions.

Discussion:

Our original intention to evaluate an infusion protocol for NAC suitable for use in high intensity and/or prolonged lower intensity exercise has been achieved with the additional benefit of deriving PK data for the intra-infusion and immediate post-infusion periods. The data originally chosen for use as the basis for designing an infusion protocol was taken from a study of the pharmacokinetics of NACT in the treatment of paracetamol overdose [7]. Since the drug was infused in the reduced form and measured as both the reduced and total NAC it was possible to derive PK data for both forms of the drug. Total NAC is not a single species, but is a mixture of oxidized and reduced dimers and monomers. For the PK analysis we considered the NACT as an entity since NACT was determined after back reduction of the oxidized species [2].

For NACR we estimated CL to be 0.58 $l.kg^{-1}.h^{-1}$ compared to 0.84 $l.kg^{-1}.h^{-1}$ [6]. The three-compartment fit gave half-lives of 1.3 min, 15.9 min and 101.6 min, compared to 8.74 and 117 min [6]. The value we found for NACT clearance was 0.164 $l.kg^{-1}.h^{-1}$ compared to the previously reported values of 0.11[6] and 0.191[7]. The three half lives for NACT were 1.5 min, 11.6 min and 132 min. The latter two half lives are consistent with previous results [6]. We failed to find a longer terminal half life for either species, and we consider that this is probably due to our short post-infusion observation period. It is possible that the inclusion of more data from the elimination phase post-infusion could have resulted in defining this parameter. However, our aim was to obtain data for use during drug infusion, and as with any model, it is strictly applicable only to the subjects and circumstances under which the data were obtained. The finding that both species have very short initial half lives of 1 to 2 min may indicate rapid uptake or redistribution into well perfused tissues, including lung.

We infused a mean total amount of 4473 mg in our subjects and obtained mean peak concentrations of 105.1 $mg.l^{-1}$ for NACR and 205.1 $mg.l^{-1}$ for NACT, this compares with a 200 mg intravenous bolus achieving corresponding concentrations of 12.2 and 19.7 $mg.l^{-1}$ [6]. Despite these marked differences in mode of administration and dose, the comparable values obtained for the PK parameters are consistent with the pharmacokinetics of NAC being essentially linear over a wide range and that comparable plasma concentrations within this range should be achievable by scaling dosage and infusion rates between subjects.

Changes during exercise:

The clearances of both NACR and NACT were reduced during exercise. The mechanism, although unclear, may be related at least in part to changes in hepatic blood flow. Hepatosplanchnic blood flow measured using indocyanine green is reduced by more than 50% from resting values during cycling exercise of greater intensity than 70% $\dot{V}O_{2max}$ and under conditions comparable to this study [14,15]. We found the clearance of both NACR and NACT to be reduced about 20% by exercise at 70% and 90% $\dot{V}O_{2peak}$ which is less than the expected reduction in hepatic blood flow. Approximately 70% of NAC clearance is non-renal [5], and if most of this is hepatic an estimate of hepatic extraction ratio can be made. In our average 78 kg subject, assuming hepatic plasma flow of 880 $ml.min^{-1}$ at rest, extraction ratios of 0.6 and 0.17 can be estimated for NACR and NACT respectively. The latter value is consistent with the estimate of 0.26 [5] based on a smaller estimate of hepatic plasma flow. During vigorous exercise, the reduction of hepatic blood flow would be expected to have a greater effect on the more highly cleared NACR than on NACT [11]. The relatively small reduction is consistent with an increase in extrahepatic clearance of NACR during exercise. We have recently examined the effects of NAC infusion on skeletal muscle NAC and glutathione status

during exercise [4]. Our observations support the intramuscular action of NAC as evidenced by both an increase in muscle NAC content, and also elevated total and reduced muscle glutathione during exercise. We are unable to evaluate the contribution of these effects to the whole body clearance of NAC.

Infusion protocol:

The intention of using the original infusion protocol was to achieve a target concentration by means of a constant rate loading infusion and to maintain this target by means of a second constant rate infusion with NACT being the marker drug. The NAC concentrations were considerably higher than expected during the initial loading infusion. This is due almost certainly to our overestimating the value chosen for the initial volume of distribution. The original estimate derived from published data [7] was for a V1 of 0.283 $l.kg^{-1}$ and a CL of 0.191 $l.kg^{-1}.h^{-1}$; the final PK estimates of 0.037 $l.kg^{-1}$ and 0.164 $l.kg^{-1}.h^{-1}$ indicated that we were close with the clearance estimate but markedly overestimated V1 giving NACT concentrations over our intended target of 100 $mg.l^{-1}$. For future use of this infusion technique, reducing the rate of the loading infusion should proportionately reduce the target overshoot. A useful outcome of these results is that despite the higher than expected NAC concentrations, there were minimal adverse effects of the drug seen (Table 2) as previously reported [2,3,4], although our data demonstrate that adverse effects graded 'mild' were more than twice as common in the NAC phase as compared with the control. It is probable that our small cohort of healthy males do not represent an adequate sample from which to extrapolate, particularly given our selection criteria which were intended to exclude subjects considered to be at increased risk of adverse effects. However we consider that we have achieved a reasonably safe and reproducible method of administering NAC for exercise and related studies. In conclusion, we have estimated the PK of both reduced and total NAC at rest

and during vigorous exercise. The plasma concentrations achieved were sufficient to cause pharmacological effects [2,3,4] without causing severe adverse effects due to the drug. We have also shown that exercise has major effects on the pharmacokinetics of NAC.

References:

1. **Reid MB, Grubweiser GJ, Stokic DS, Koch SM, Reis AA** (1994) N-acetylcysteine inhibits muscle fatigue in humans. J clin Invest 94: 2468-2474.

2. **Medved I, Brown MJ, Bjorksten AR, Leppik JA, Sostaric S, McKenna MJ** (2003) N-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. J Appl Physiol 94: 1572-1582.

3. **Medved I, Brown MJ, Bjorksten AR, McKenna MJ** (2004) Effects of intravenous N-acetylcysteine on time to fatigue and potassium regulation during prolonged cycling exercise. J Appl Physiol 96: 211-217.

4. **Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X, McKenna MJ** (2004) N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. J Appl Physiol 97: 1477-85.

5. **Borgstrom L, Kagedal B, Paulsen O** (1986) Pharmacokinetics of N-acetylcysteine in man. Eur J Clin Pharmacol 31: 217-222.

6. **Olsson B, Johansson M, Gabrielsson J, Bolme P** (1988) Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. Eur J Clin Pharmacol 34: 77-82.

7. **Prescott LF, Donovan JW, Jarvie DR, Proudfoot AT** (1989) The disposition kinetics of intravenous N-acetylcysteine in patients with paracetamol overdosage. Eur J Clin Pharmacol 37: 501-506.

8. **Dawson AH, Henry DA, McEwan J** (1989) Adverse reactions to N-acetylcysteine during treatment for paracetamol poisoning. Med J Aust 151:329-31.

9. **Mant TG, Tempowski JH, Volans GN, Talbot JC** (1984) Adverse reactions to N-acetylcysteine and effects of overdose. Br Med J 289: 217-9.

10. **Vaughan DP, Tucker GT** (1975) General theory for rapidly establishing steady state drug concentrations using two consecutive constant rate intravenous infusions. Eur J Clin Pharmacol 9: 235-238.

11. **Gibaldi M. Perrier D.** (1982) Pharmacokinetics. 2nd edition Marcel Dekker New York.

12. **Benet LZ.** (1972) General treatment of linear mammillary models with elimination from any compartment as used in pharmacokinetics. J Pharm Sci 61:536-41.

13. **Beal SL, Sheiner LB** (1992) NONMEM user's guide; NONMEM project group, University of California, San Francisco.

14. **Bergeron R, Kjaer M, Simonson L, Bulow J, Skovgaard D, Howlett H, Galbo H** (2001) Splanchnic blood flow and hepatic glucose production in exercising humans: role of renin-angiotensin system. Am J Physiol Regul Integr Comp Physiol 281:R1854-61.

15. **Neilsen HB, Clemmesen JO, Skak C, Ott P, Secher NH** (2002) Attenuated hepatosplanchnic uptake of lactate during intense exercise in humans. J Appl Physiol 92: 1677-1683.

APPENDIX 2:

SUBJECT INFORMED CONSENT FORM STUDY 1

Victoria University of Technology
PO Box 14428
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Australia
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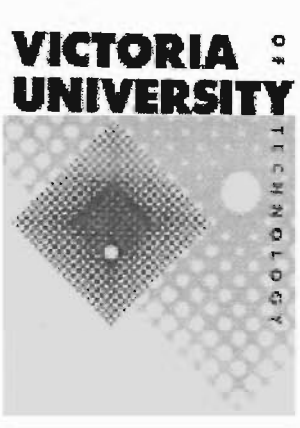
(03) 9688 4432
Facsimile:
(03) 9688 4891

School of Human Movement, Recreation and Performance
Footscray Park Campus



INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study into the causes of muscle fatigue during exercise. The study aims to determine whether giving an "anti-oxidant" improves your exercise performance. Full details are given on the subject information sheet.

CERTIFICATION BY SUBJECT
I,

of

certify that I am at least 17 years old* and that I am voluntarily giving my consent to participate in the experiment entitled:

"The effects of antioxidants on skeletal muscle fatigue and ion regulation during exercise"

being conducted at Victoria University of Technology by:

Dr Michael McKenna, Dr Malcolm Brown, Dr. Andrew Bjorksten and Ivan Medved
Departments of Human Movement, Recreation and Performance, Victoria University of Technology; Anaesthesia, Austin and Repatriation Medical Centre; Anaesthesia, Royal Melbourne Hospital.

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by:

and that I freely consent to participation involving the use on me of these procedures.

Procedures:
1. VO_2 peak test
2. Intermittent, high intensity exercise test
3. Infusion of *N*-acetyl cysteine
4. Infusion of saline
5. Catheterisation and blood sampling

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: ..... }

Witness other than the experimenter: } Date: .....

Any queries about your participation in this project may be directed to the researcher (Dr. Michael McKenna ph. W: (03) 9688 4499; H (03) [redacted]). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MCMC, Melbourne, 8001 (telephone no: 03-9688 4710).

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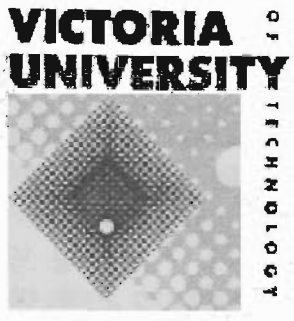

SUBJECT INFORMATION SHEET

The effects of anti-oxidants on skeletal muscle fatigue and ion regulation during exercise.

Investigators:
Dr Michael McKenna and Ivan Medved (Masters student)
Department of Human Movement, Recreation and Performance, Victoria University of Technology;
Dr Malcolm Brown, Department of Anaesthesia, Austin and Repatriation Medical Centre, Heidelberg.
Dr Andrew Bjorksten, Department of Anaesthesia, Royal Melbourne Hospital.

Aims of the study:
Your muscles normally get energy by breaking down oxygen. However, during this process, some of this oxygen forms compounds that are highly reactive and believed to impair normal muscular function. This study investigates whether these reactive oxygen compounds contribute to muscle fatigue, by giving an "anti-oxidant" compound, *N*-acetyl cysteine (NAC), which prevents the build up of these reactive oxygen compounds. A secondary aim is to see whether these compounds affect changes in potassium in the blood during exercise. Through this study we hope to provide a better understanding of the causes of fatigue of your muscles during exercise.

Subject participation:
As a volunteer, you are free to withdraw from the study at any time, without any adverse effects or reactions.

Overview
You will be required to attend the Exercise Physiology Laboratory (Room L305, Building L, Victoria University of Technology-Footscray campus) on six occasions, separated by a 1-week period. During first visit you will be asked to complete a test on a cycle ergometer, to measure your fitness level (VO_2peak). This lasts for approximately 12-15 minutes. During the next three tests you will be asked to complete the exercise protocol that is outlined below. On your final two visits to the laboratory a qualified medical practitioner will infuse a fluid containing either NAC or saline into your arm and you will again perform the exercise test. Blood samples will also be taken during

these two visits by the method outlined below. Details of all procedures are given in the sections below.

Exercise testing procedures:

Test session #1. During your visit to the Laboratory, we will take several measurements of your body dimensions, including weight and height. You will then perform an incremental exercise test on a bicycle ergometer. This test involves continuous exercise with the exercise intensity (effort) becoming progressively greater. The test is completed when you have reached volitional exhaustion (wish to stop), or unless we stop the test due to you having an abnormal response to exercise. We will closely monitor you and your heart's electrical activity (ECG) during exercise to ensure your safety.

Test session #2. This session will be to familiarise you with the experimental trial. You will be required to complete four bouts of high intensity exercise on a cycle ergometer. The first three exercise bouts will last for 45 seconds, with 135 seconds of recovery. The fourth and final exercise bout will involve you cycling to volitional fatigue (until you can go no further).

Test session #3 & #4. These tests will be repeats of the protocol in the previous visit and used to determine how variable your exercise performance is.

Test sessions #5 & #6. The remaining two trials will be the experimental trials. During these two visits you will be required to complete the same exercise described above. During this time either *N*-acetyl cysteine (NAC), or a placebo (saline) solution will be infused into your arm. The order in which these are given to you will not be revealed until after completion of the experiment, to minimise any bias. Blood sampling (see below) will also be completed during these trials. You will also be requested to return to the laboratory at 4, 6 and 24 hours post-exercise. During each of these times a single blood sample will be taken.

Catheterisation and Blood Sampling

At specific intervals throughout the experimental trials a small blood sample will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the teflon tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed into the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein).

Each time a blood sample is taken, a small volume (1-2ml) of sterile heparinised saline will be injected to clear the catheter and keep it patent. Catheterisation of subjects can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. No more than 160ml. of blood will be withdrawn during each of the two experimental trials. For comparison, the normal donation at the Blood Bank is 400 ml.

What is *N*-acetyl cysteine and how will it be given?

N-acetyl cysteine (NAC) is a compound that neutralises reactive oxygen compounds. It is used for a number of clinical situations, including treatment of paracetamol overdose. *N*-acetyl cysteine will be infused into a vein in your arm. The infusion protocol that we will employ will elevate NAC in your blood to a steady concentration much lower than that used in other studies.

Are there any risks with NAC Infusion?

When NAC is infused at very high doses to healthy human volunteers, adverse reactions have been reported and include nausea, diarrhoea, vomiting, rash, altered moods, sleepiness, dizziness and coughing. When given in smaller doses to healthy volunteers no side effects have been reported. NAC administered during varying treatment programs (10 days - 6 months) and in varying daily doses (300 - 600mg) has been well tolerated and is generally safe and without major adverse reactions.

Our modified infusion protocol avoids the very high levels of NAC in the blood known to induce adverse reactions. The estimated levels of NAC will be less than ¼ of that used clinically. Thus, we anticipate the risks of adverse reactions to NAC will be greatly reduced with our infusion protocol.

In patients being treated for paracetamol overdose, an accidental overdose of NAC, 10 times the recommended (clinical) dose, has resulted in some deaths. An accidental overdose of NAC cannot occur in this study due to a number of important and clearly defined procedures. First, the amount of NAC made up in the syringe to be infused will be limited by the use of a defined number of NAC ampoules and a standard low dose will be used for all subjects. Second, a standard infusion pump will be used to ensure that the correct amount of NAC is administered. It should be noted that the dose given in this study is approximately 40 times less than that reported in overdose situations.

A medical practitioner will be present to treat any adverse reactions should they eventuate. The most likely treatment is to simply stop the NAC infusion. This should eliminate the adverse reaction.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

Contact Numbers:

Ivan Medved	W: 9688 4883	H: (STD 03) [redacted]
Dr Michael McKenna	W: 9688 4499	H: (STD 03)
Dr Malcolm Brown	W: 9496 5077	

APPENDIX 3:

APPROVAL OF ETHICS APPLICATION INVOLVING HUMAN SUBJECTS

University of Technology

14428 Telephone
ne City (03) 9688 4456
Australia Facsimile
(03) 9688 4803

ry Park Campus
Human Development
Food

Faculty Human Research Ethics Committee

MEMORANDUM

:
Michael McKenna
Principal Investigator
HMRP
Faculty of Human Development

OM:
Dr Dennis Hemphill
Chair
Human Research Ethics Committee
Faculty of Human Development

TE:
July 24, 2001

JECT:
Application HRETH.FHD.053/01 involving human subjects

Faculty Human Research Ethics Committee, at its meeting on -date, assessed your cation for the project titled ***The Effects of Intravenous N-acetyl cysteine on Skeletal le Regulation and Fatigue During Exercise.*** The Committee thanks you for this sting research project application.

s resolved to **approve** application HRETH.FHD.053/01 on the **condition** that the ving amendments are made to the research protocol:

Please attend to spelling errors (Venep*un*turist needs to be venep*unc*turist.)

Section 8	Omits mention of heparinised saline flush following each blood sampling. Total dose (i.e., units) of heparin should be shown.
Section 12	To comply with the current NHMRC requirements, the FHREC must view the advertisements/posters to be used for recruiting.
Section 15	(a) Venous catheterisation and blood sampling pose the risk of exposure of the researchers to blood-borne infection. Risks associated with the use of heparin should also be acknowledged.

(f) (i) The use of standard precautions minimises the above and needs to be shown here. Measures to minimise risks associated with the use of heparinized saline need to be shown here.

As the supply and administration of drugs requiring a prescription (i.e., S4 drugs) are subject to the requirements of the Drugs, Poisons and Controlled Substances Regulations (available at http://www.dms.dpc.vic.gov.au), the researchers would need to show that they are complying with the law in this regard. The University HREC may be able to provide advice about this.

It is noted that in the proposed study (and that conducted previously by the researchers) the NAC is mixed with isotonic NaCl and not 5% glucose (as approved by the TGA for use of NAC in Australia – see manufacturer's information and other sources of information about NAC infusions). Please clarify this variation with regard to the safety of participants.

Consent Form

Procedure 3 needs to be "venous cannulation and blood sampling"; and 5 needs to be "infusion of N-acetyl cysteine and a placebo"

Information to Participants

Blood Samples. Can show that "The researchers use of standard precautions minimises the risk of their exposure to blood-borne infections".
Muscle Biopsies ...(last par) The signs of infection are presently shown as signs of altered sensation. These can be moved to follow infection and "numbness and tingling" could be included as signs of altered sensation.

ase be aware that amendments made in one Item of the application may need to be lected at other Items also. It is your responsibility to ensure that this occurs.

e submission of the revised application may occasion further comment from the mmittee if the information provided in the revised application results in unanticipated nges in the ethical merit of the research protocol.

ase note that Beth Bennett has been nominated by the Committee to assist you with these endments. Please contact Beth Bennett on 9365 2177 to discuss the requirements of the mmittee.

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icray Park Campus
y of Human Development
at Road
ray

Faculty Human Research Ethics Committee

MEMORANDUM

TO: Michael McKenna
Principal Investigator
HMRP

FROM: Dr Jim Kiatos
Chair
Human Research Ethics Committee
Faculty of Human Development

DATE: December 18, 2001

SUBJECT: **Approval of application involving human subjects**

Thank you for your submission detailing amendments to the research protocol for the project titled, ***The Effects of Intravenous N-acetyl cysteine on Skeletal Muscle Regulation and Fatigue During Exercise*** (HRETH.FHD.053/01).

The proposed amendments have been accepted by the Faculty Human Research Ethics Committee and approval for application HRETH.FHD.053/01 has been granted from 01/12/01 to 01/12/03.

Please note that, the Faculty Human Research Ethics Committee must be informed of the following: any changes to the approved research protocol, project timelines, any serious or unexpected adverse effects on participants, and unforeseen events that may effect continued ethical acceptability of the project. In these unlikely events, researchers must immediately cease all data collection until the Committee has approved the changes.

If you have any queries, please do not hesitate to contact me on ext 4486.

The Committee wishes you all the best for the conduct of the project.

[redacted]

Dr Jim Kiatos
Chair
Human Research Ethics Committee
Faculty of Human Development

E:\ethics\correspondence\memo\finalapproval.doc

)nce the amendments are made to the satisfaction of Beth Bennett, you must:

a) Submit a revised application to the Faculty Ethics Officer and,

b) Attach a covering letter/memo detailing each amendment and its corresponding item within the application.

LEASE NOTE:
ubmission of draft revisions to the nominated Committee member is not ıfficient for gaining approval. You must submit a complete, signed copy of your evised application to the Faculty Ethics Officer. Recruitment of participants nd data collection activities may only commence when you have received official pproval advice from the Faculty Ethics Officer.

you have any queries, please do not hesitate to contact me on ext 4486.

ıe Committee wishes you all the best for the conduct of the project.

[redacted]

r Dennis Hemphill
ıair
uman Research Ethics Committee
ıculty of Human Development

APPENDIX 4:

CALCULATION OF NAC DOSE

High, intensity intermittent exercise

Drug Dose.

Priming Phase

$= 125\ (mg.kg^{-1}.h^{-1}) \times \frac{15\ (h)}{60} \times$ body mass (kg)

$= \frac{125 \times 15}{60} \times$ body mass (mg)

$= 31.25 \times$ body mass (mg)

Maintenance Phase.

$= 25\ mg.kg^{-1}.h^{-1} \times$ {20minutes (equilibrium) + 20minutes (exercise)}

$= \frac{25 \times 40}{60} \times$ body mass

$= 16.66 \times$ body mass (mg)

Total Dose = a + b

= (31.25 + 16.66) x body mass

= 47.91 x body mass (mg)

= 0.04791 x body mass (g)

eg. 70 kg = 0.04791 x 70 = 3.353 g

80 kg = 3.832 g

90 kg = 4.311 g

100 kg = 4.791 g

Thus, no more than 6g of NAC will be required per syringe for the study. This will prevent an accidental overdose. Therefore, make up 3 ampoules (2g NAC) Parvolex™ to give a standard dose of 6g NAC.

Volume Delivered.

Priming Phase.

$= \frac{\text{amount given}}{\text{concentration}}$

$= \frac{125 \times 15\ mg}{60} \times \frac{1}{120\ mg.ml^{-1}}$

$= \frac{125 \times 15}{60 \times 120}$

$= 0.26 \times$ body mass (ml)

b) Maintenance Phase

$= \frac{25 \times 40}{60 \times 120}$

$= 0.138 \times$ body mass (ml)

Prolonged Exercise

Drug Dose.

Priming Phase

$= 125\ (mg.kg^{-1}.h^{-1}) \times \frac{15\ (h)}{60} \times$ body mass (kg)

$= \frac{125 \times 15}{60} \times$ body mass (mg)

$= 31.25 \times$ body mass (mg)

Maintenance Phase

$= 25\ mg.kg^{-1}.h^{-1} \times$ {20minutes (equilibrium) + 60minutes (exercise)}

$= \frac{25 \times 80}{60} \times$ body mass

$= 33.33 \times$ body mass (mg)

Total Dose = a + b
= (31.25 + 33.33) x body mass
= 64.58 x body mass (mg)
= 0.06458 x body mass (g)

eg. 60 kg = 3.874 g
70 kg = 4.521 g
80 kg = 5.166 g
90 kg = 5.812 g

Thus, no more than 6g of NAC will be required per syringe for the study. This will prevent an accidental overdose. Therefore, make up 3 ampoules (2g NAC) Parvolex™ to give a standard dose of 6g NAC.

Volume Delivered.

Priming Phase

$= \frac{125 \times 15\ mg}{60} \times \frac{1}{120\ mg.ml^{-1}}$

$= \frac{125 \times 15}{60 \times 120}$

$= 0.26 \times$ body mass (ml)

Maintenance Phase

$= \frac{25 \times 80}{60 \times 120}$

$= 0.277 \times$ body mass (ml)

APPENDIX 5:

SUBJECT INFORMED CONSENT FORM STUDY 2

Victoria University of Technology
PO Box 14428
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Facsimile:
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School of Human Movement, Recreation and Performance
Footscray Park Campus

Consent Form for Subjects Involved in Research

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study into the causes of muscle fatigue during exercise. The study aims to determine whether giving an "anti-oxidant" improves your exercise performance. Full details are given on the subject information sheet.

CERTIFICATION BY SUBJECT

I,

of

certify that I am at least 17 years old* and that I am voluntarily giving my consent to participate in the experiment entitled:
"The effects of antioxidants on skeletal muscle fatigue and ion regulation during exercise"

being conducted at Victoria University of Technology by:
Dr Michael McKenna, Dr Malcolm Brown, Dr Andrew Bjorksten and Ivan Medved
Departments of Human Movement, Recreation and Performance, Victoria University of Technology; Anaesthesia, Austin and Repatriation Medical Centre; Anaesthesia, Royal Melbourne Hospital.

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by:

and that I freely consent to participation involving the use on me of these procedures.

Procedures:
1. VO_2 peak test
2. Prolonged, sub-maximal exercise test
3. Infusion of *N*-acetyl cysteine
4. Infusion of saline
5. Catheterisation and blood sampling

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: .....

Witness other than the experimenter: Date: .....

Any queries about your participation in this project may be directed to the researcher (Dr. Michael McKenna ph. W: (03) 9688 4499; H (03) [redacted]). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MCMC, Melbourne, 8001 (telephone no: 03-9688 4710).

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SUBJECT INFORMATION SHEET

THE EFFECTS OF ANTI-OXIDANTS ON SKELETAL MUSCLE FATIGUE AND ION REGULATION DURING EXERCISE.

Investigators:
Dr Michael McKenna and Ivan Medved (Masters student)
Department of Human Movement, Recreation and Performance, Victoria University of Technology;
Dr Malcolm Brown, Department of Anaesthesia, Austin and Repatriation Medical Centre, Heidelberg
Dr Andrew Bjorksten, Department of Anaesthesia, Royal Melbourne Hospital.

Aims of the study:
Your muscles normally get energy by breaking down oxygen. However, during this process, some of this oxygen forms compounds that are highly reactive and thought to impair normal muscular function. This study investigates whether these reactive oxygen compounds contribute to muscle fatigue, by giving an "anti-oxidant" compound, *N*-acetyl cysteine (NAC), which prevents the build up of these reactive oxygen compounds. A secondary aim is to see whether these compounds affect changes in potassium in the blood during exercise. Through this study we hope to provide a better understanding of the causes of fatigue of your muscles during exercise.

Subject participation:
As a volunteer, you are free to withdraw from the study at any time, without any adverse effects or reactions.

Overview
You will be required to attend the Exercise Physiology Laboratory (Room L305, Building L, Victoria University of Technology-Footscray campus) on six occasions, separated by a 1-week period. During first visit you will be asked to complete a test on a cycle ergometer, to measure your fitness level (VO_2peak). During the next three tests you will be asked to complete the exercise protocol that is outlined below. On your final two visits to the laboratory a qualified medical practitioner will infuse a fluid containing either NAC or saline into your arm and you will again perform the exercise test. Blood samples will also be taken during these two visits by the method outlined below. For the final four visits to the laboratory you will be given food packages to consume on the day before the tests. Details of all procedures are given in the sections below.

Exercise testing procedures:

Test session #1. During your visit to the Laboratory, we will take several measurements of your body dimensions, including weight and height. You will then perform an incremental exercise test on a bicycle ergometer. This test involves continuous exercise with the exercise intensity (effort) becoming progressively greater. The test is completed when you have reached volitional exhaustion (wish to stop), or unless we stop the test due to you having an abnormal response to exercise. We will closely monitor you and your heart's electrical activity (ECG) during exercise to ensure your safety.

Test session #2. This session will be to familiarise you with the experimental trial. You will be required to cycle at a workload corresponding to 70% VO_2peak for 45 minutes. The intensity will then be increased to 90% VO_2peak. The test will stop when you wish to stop or can go no longer.

Test session #3 & #4. These tests will be repeats of the protocol in the previous visit and used to determine how variable your exercise performance is.

Test sessions #5 & #6. The remaining two trials will be the experimental trials. During these two visits you will be required to complete the same exercise described above. During this time either *N*-acetyl cysteine (NAC), or a placebo (saline) solution will be infused into your arm. Blood sampling (see below) will also be completed during these trials. You will also be requested to return to the laboratory at 2 and 4 hours post-exercise. During each of these times a single blood sample will be taken.

Catheterisation and Blood Sampling

At specific intervals throughout the experimental trials a small blood sample will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the teflon tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed into the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein).

Each time a blood sample is taken, a small volume (1-2ml) of sterile heparinised saline will be injected to clear the catheter and keep it patent. Catheterisation of subjects can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. No more than 160ml. of blood will be withdrawn during each of the two experimental trials. For comparison, the normal donation at the Blood Bank is 400 ml.

What is *N*-acetyl cysteine and how will it be given?

N-acetyl cysteine (NAC) is a compound that neutralises reactive oxygen compounds. It is used for a number of clinical situations, including treatment of paracetamol overdose. *N*-acetyl cysteine will be infused into a vein in your arm. The infusion protocol that we will employ will elevate NAC in your blood to a steady concentration much lower than that used in other studies.

Are there any risks with NAC Infusion?

When NAC is infused at very high doses to healthy human volunteers, adverse reactions have been reported and include nausea, diarrhoea, vomiting, rash, altered moods, sleepiness, dizziness and coughing. When given in smaller doses to healthy volunteers no side effects have been reported. NAC administered during varying treatment programs (10 days - 6 months) and in varying daily doses (300 - 600mg) has been well tolerated and is generally safe and without major adverse reactions.

Our modified infusion protocol avoids the very high levels of NAC in the blood known to induce adverse reactions. The estimated levels of NAC will be less than ¼ of that used clinically. Thus, we anticipate the risks of adverse reactions to NAC will be greatly reduced with our infusion protocol.

In patients being treated for paracetamol overdose, an accidental overdose of NAC - 10 times the recommended (clinical) dose, has resulted in some deaths. An accidental overdose of NAC cannot occur in this study due to a number of important and clearly defined procedures. First, the amount of NAC made up in the syringe to be infused will be limited by the use of a defined number of NAC ampoules and a standard low dose will be used for all subjects. Second, a standard infusion pump will be used to ensure that the correct amount of NAC is administered. It should be noted that the dose given in this study is approximately 40 times less than that reported in overdose situations.

A medical practitioner will be present to treat any adverse reactions should they eventuate. The most likely treatment is to simply stop the NAC infusion. This should eliminate the adverse reaction.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

Contact Numbers:

Ivan Medved	W: 9688 4883	H: (STD 03) [redacted]
Dr Michael McKenna	W: 9688 4499	H: (STD 03) [redacted]
Dr Malcolm Brown	W: 9496 5077	

APPENDIX 6:

STANDARD FOOD PACKAGES CONSUMED BY SUBJECTS

EFFECT OF ANTI-OXIDANTS

ON SKELETAL MUSCLE FATIGUE

NO EXERCISE, CAFFEINE, TOBACCO OR CAFFEINE IN THE 24 HOURS BEFORE THE TRIAL!!!!

BREAKFEAST:

Cereal	90g
Low fat milk	333ml
4 slices of toast (with jam or honey)	1 tablespoon
1 tetra pack of orange juice	250ml

LUNCH:

1 can of lift	375ml
2 bread rolls	180g
Half a tomato	50g
Lettuce	30g
Cheese	40g
Ham (lean)	60g
Margarine / butter	15g
1 apple	140g

DINNER:

Half a pack of pasta	175g
Half a jar of pasta sauce	200ml
1 can of lift	375ml
2 fruity snacks	280g

SNACKS:

Please record any snacks eaten (type and amount) so that food intake is the same for both trials. Water may be consumed at any time on the day before the trial, as you feel necessary.

Possible snacks include:

1 banana	140g
2 muesli bars	70g
2 packets of sultanas	60g
4 slices of bread with jam or honey	1 tablespoon

MORNING OF THE TRIAL:

Upon waking on the morning of the trial consume 5ml of water per kilogram of body weight.

eg. 70 kilogram person consumes 350ml of water

If you any queries please do not hesitate to contact Ivan on (03) 9688 4160 or (03) [redacted] Thankyou for participating in this study and we trust that you will enjoy your meals.

APPENDIX 7:

SUBJECT INFORMED CONSENT FORM STUDY 3 – PART I AND II

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MELBOURNE CITY MC VIC 8001
Australia

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Facsimile:
(03) 9688 4891

School of Human Movement, Recreation and Performance
Footscray Park Campus

Consent Form for Subjects Involved in Research

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study into the effects of intravenous *N*-acetyl cysteine on skeletal muscle ion regulation and fatigue during exercise.

CERTIFICATION BY SUBJECT
I,

of

certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the experiment entitled:

The effects of intravenous *N*-acetyl cysteine on skeletal muscle ion regulation and fatigue during exercise.

being conducted at Victoria University of Technology by:

Associate Professor Michael McKenna, Dr. Malcolm Brown, Mr. Ivan Medved

I certify that the objectives of the experiment, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by:

Mr. Ivan Medved and Associate Professor Michael McKenna

and that I freely consent to participation involving the use on me of these procedures.

1. Maximal Oxygen Uptake test.
2. Prolonged sub-maximal exercise test
3. Venous cannulation and blood sampling
4. Muscle biopsies
5. Infusion of *N*-acetyl cysteine and a placebo

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed of the risks involved and am freely participating in this study.

Signed: Date: .....

.....

Witness other than the experimenter: Date: .....

.....

Any queries about your participation in this project may be directed to the researcher (Assoc. Prof. McKenna; ph. 9688 4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

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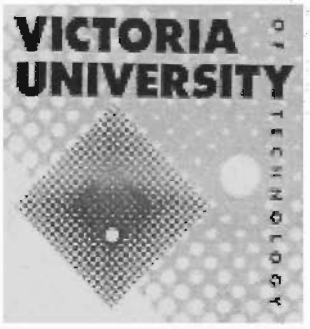

SUBJECT INFORMATION SHEET

THE EFFECTS OF INTRAVENOUS *N*-ACETYL CYSTEINE ON SKELETAL MUSCLE ION REGULATION AND FATIGUE DURING EXERCISE.

Investigators:
Associate Professor Michael McKenna and Ivan Medved (PhD student)
School of Human Movement, Recreation and Performance, Victoria University of Technology;
Dr Malcolm Brown, Department of Anaesthesia, Austin and Repatriation Medical Centre, Heidelberg

Aims of the study:
Your muscles normally get energy by breaking down oxygen. However, during this process, some of this oxygen forms compounds that are highly reactive and thought to impair normal muscular function. This study investigates whether these reactive oxygen compounds contribute to muscle fatigue, by giving an anti-oxidant compound, *N*-acetyl cysteine (NAC). Anti-oxidants are important since they prevent the build up of these reactive oxygen compounds. Through this study we hope to provide a better understanding of the causes of fatigue of your muscles during exercise.

Subject participation:
Should you decide to participate in this study, you are free to withdraw from the study at any time, without any adverse effects or reactions.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Campus of Victoria University on six separate occasions for an exercise test. Whilst each test is tiring, you will recover from this very quickly. For the final two visits to the laboratory you will be given food packages to consume on the day before the tests. Full details of all procedures are given in the sections below. Each exercise test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an abnormal response to exercise, such as unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain or severe shortness of breath. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. The most common event associated with maximal exercise testing is fainting. This will be prevented using our standard laboratory procedures. In the unlikely event of emergency situations, a medical practitioner will be in attendance, two members of the research

team have current CPR (cardio pulmonary resuscitation) qualifications and the Western Hospital is minutes away by ambulance.

Test Session # 1 (VO_2 peak): This test is used to determine your aerobic fitness, by measurement of the peak oxygen consumption (VO_2 peak). This test involves continuous exercise on a cycle ergometer with the exercise intensity (effort) being progressively increased.

Test session #2 (Familiarisation). This session will be to familiarise you with the experimental trial. You will be required to cycle at a workload corresponding to 70% of your VO_2peak for 45 minutes. The intensity will then be increased to 90% of your VO_2peak.

Test session #3 & #4 (Variability Trials). These tests will be repeats of the protocol in the previous visit and used to determine how variable your exercise performance is.

Test sessions #5 & #6. The remaining two trials will be the experimental trials. During these two visits you will be required to complete the same prolonged submaximal exercise trial described above. During this trial either *N*-acetyl cysteine (NAC), or a placebo (saline) solution will be infused into your arm. Blood sampling and muscle biopsies (see below) will also be completed during these trials. You will also be requested to return to the laboratory at 2 and 4 hours post-exercise for a further blood sample to be taken.

Blood Samples:

Should you decide to participate in this study, at specific intervals throughout the exercise test a small blood sample (each 5 ml) will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the teflon tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed into the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein). A total of approximately one hundred and sixty (160) ml of blood will be taken during each of the two final exercise trials. The total overall volume of blood sampled will not exceed 320 ml. Normally 400 ml is taken when you donate at the Blood Bank. Each time a blood sample is taken, a small volume of sterile heparinised saline will be injected to clear the catheter. Catheterisation of subjects can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small (for example puss, tenderness and/or redness), if by chance it does eventuate, inform us immediately and then consult your doctor. Furthermore, the researchers use of standard precautions minimises the risk of their exposure to blood-borne infections. Blood samples will be analysed for metabolites, electrolytes and pH.

Muscle Biopsies and Muscle Fatigue Testing:

Should you decide to participate in this study, on your final two visits to the Exercise Physiology Laboratory, a muscle biopsy will be taken from your thigh muscle, at rest, 45 minutes into the exercise period and immediately after you stop the fatigue test. Thus a total of six biopsies will be taken during your final two visits. Three biopsies will be

performed on each leg. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of enzymes and energy sources. An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable, but will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. Many people experience pain during this part of the procedure but this lasts only for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a grain of rice. This poses no long-term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days.

You should not exercise for 24 hours after biopsies and you should avoid heavy knocks. It is common for subjects to experience some mild soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. A qualified medical practitioner will perform the whole procedure under sterile conditions. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation (for example puss, tenderness, numbness, tingling and/or redness) is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy.

What is *N*-acetyl cysteine and how will it be given?

N-acetyl cysteine (NAC) is an anti-oxidant compound that neutralises reactive oxygen compounds. It is used for a number of clinical situations, including treatment of paracetamol overdose. Should you decide to participate in this study, NAC will be infused into a vein in your arm. The infusion protocol that we will employ will elevate NAC in your blood to a steady concentration much lower than that used in other studies.

Are there any risks with NAC Infusion?

When NAC is infused at very high doses to healthy human volunteers, adverse reactions have been reported and include nausea, diarrhoea, vomiting, rash, altered moods, sleepiness, dizziness and coughing. When given in smaller doses to healthy volunteers no side effects have been reported. NAC administered during varying treatment programs (10 days - 6 months) and in varying daily doses (300 - 600mg) has been well tolerated and is generally safe and without major adverse reactions. We have previously found in two separate studies conducted with volunteers in our laboratory, that there

were no serious adverse reactions to NAC infusion. Importantly, our modified infusion protocol avoids the very high levels of NAC in the blood known to induce adverse reactions. The estimated levels of NAC in your blood will be less than ¼ of that used clinically. Thus, we anticipate the risks of adverse reactions to NAC will be low with our infusion protocol.

In patients being treated for paracetamol overdose, an accidental overdose of NAC (10 times the recommended clinical dose) has resulted in some deaths. An accidental overdose of NAC cannot occur in this study due to a number of important and clearly defined procedures. First, the amount of NAC made up in the syringe to be infused will be limited by the use of a defined number of NAC ampoules and a standard low dose will be used for all subjects. Second, a standard infusion pump will be used to ensure that the correct amount of NAC is administered. It should be noted that the dose given in this study is approximately 40 times less than that reported in overdose situations. Should any adverse reactions eventuate a medical practitioner will be present at all times. The most likely treatment is to simply stop the NAC infusion. This should eliminate the adverse reaction.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

Contact Numbers:

Mr. Ivan Medved	M: 0402-219-332	H: (03) [redacted]
Assoc. Prof. Michael McKenna	W: 9688 4499	H: (03) [redacted]
Dr. Malcolm Brown	W: 9496 5077	Mob. [redacted]

Any queries about your participation in this project may be directed to the researcher (Assoc. Prof. McKenna; ph. 9688 4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

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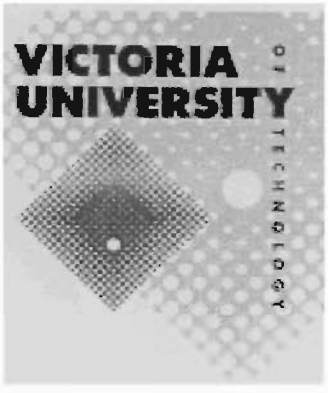

CARDIOVASCULAR RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:
"The effects of intravenous *N*-acetyl cysteine on skeletal muscle ion regulation and fatigue during exercise" you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: _____ Date: _____

Age: _____ years Weight: _____ kg Height: _____ cms

Give a brief description of your average activity pattern in the past 2 months:

_____

_____

Circle the appropriate response to the following questions.

1. Are you overweight? Yes No Don't know
2. Do you smoke? Yes No Social
3. Does your family have a history of premature cardiovascular problems (eg. heart attack, stroke)? Yes No Don't Know
4. Are you an asthmatic Yes No Don't Know
5. Are you a diabetic? Yes No Don't Know
6. Do you have a high blood cholesterol level? Yes No Don't Know
7. Do you have high blood pressure? Yes No Don't Know
8. Are you on any medication? Yes No
   If so, what is the medication?

_____

9. Do you think you have any medical complaint or any other reason that you know of which you think may prevent you from participating in strenuous exercise? Yes No

If Yes, please elaborate _____

I, _____, believe that the answers to these questions are true and correct.

Signed: _____ Date: _____

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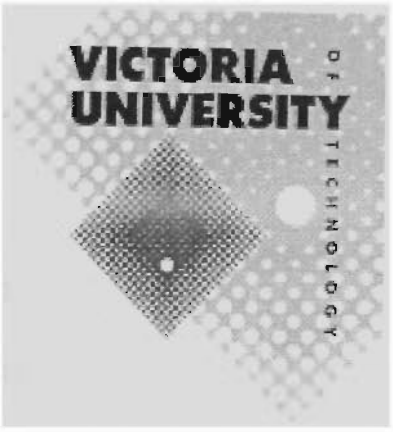


MUSCLE BIOPSY QUESTIONNAIRE

NAME: _____
ADDRESS: _____
DATE: _____ AGE: _____ years

1. Have you or your family suffered from any tendency to bleed excessively ? (eg. haemophilia) or bruise very easily ?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

2. Are you allergic to local anaesthetic?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

3. Do you have any skin allergies?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

4. Have you any allergies that should be made known?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

5. Are you currently on any medication?

   Yes No Don't Know

   If yes, what is the medication?

   _____

6. Do you have any other medical problem that should be made known?

   Yes No

   If yes, please elaborate... _____
   _____

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: _____ Date: _____

APPENDIX 8:

RAW DATA STUDY 1

Attached a raw data spreadsheets for the following:

- General Information
- Time to fatigue
- Blood total glutathione (TGSH)
- Blood reduced glutathione (GSH)
- Blood calculated oxidised glutathione (GSSG)
- Plasma K^+
- $\Delta[K^+]$ to work ratio
- Change in Plasma volume
- Plasma Hydrogen (H^+)
- Plasma Sodium (Na^+)
- Plasma Calcium (Ca^{2+})
- Partial pressure oxygen (PO_2)
- Haemoglobin (Hb)
- Haematocrit (Hct)
- Bicarbonate (HCO_3^-)
- Partial pressure carbon dioxide (PCO_2)
- Plasma Chloride (Cl^-)

General Information

Subjects	Age	Weight	VO2	VO2	W/load 130%
	(yr)	(kg)	($l.min^{-1}$)	($kg.min^{-1}$)	(W)
Subject 1	23	86.4	3.57	41.34	366
Subject 2	23	77.9	3.10	39.86	282
Subject 3	27	95.5	3.37	35.37	311
Subject 4	24	82.5	4.21	51.19	391
Subject 5	20	66.1	3.10	37.04	288
Subject 6	20	73.4	3.71	50.65	365
Subject 7	20	64.6	3.06	47.44	292
Subject 8	23	76.1	3.14	41.38	324
n	**8**	**8**	**8**	**8**	**8**
Mean	**22.5**	**77.8**	**3.4**	**43.0**	**327**
SD	**2.4**	**10.3**	**0.4**	**6.0**	**42**
SEM	**0.9**	**3.6**	**0.1**	**2.1**	**14.7**

Time to fatigue

Subjects	Var 1	Var 2	NAC	CON	Trial 1	Trial 2	Famil.
	(s)	(s)	(s)	(s)	(s)	(s)	(s)
Subject 1	91	92	100	102	100	102	89
Subject 2	208	215	192	224	192	224	206
Subject 3	126	125	134	126	126	134	119
Subject 4	56	59	64	68	68	64	51
Subject 5	79	77	81	74	81	74	71
Subject 6	89	87	91	89	91	89	75
Subject 7	53	49	51	51	51	51	55
Subject 8	120	125	115	117	117	115	115
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**102.75**	**104.19**	**103.50**	**106.38**	**103.25**	**106.63**	**96.78**
SD	**49.95**	**51.56**	**44.54**	**53.77**	**43.44**	**54.65**	**50.04**
SEM	**17.66**	**19.23**	**15.75**	**19.01**	**15.36**	**19.32**	**17.69**

Blood TGSH NAC (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.93	0.90	0.87	0.85	0.74	0.97	0.77	0.72	0.81	0.83	0.87	0.85	0.89	0.80	0.95	0.89	0.83	0.85	0.85	0.83
Subject 2	0.76	0.83	0.77	0.95	0.88	0.94	0.84	0.85	0.74	0.91	0.83	0.93	0.98	0.88	0.89	0.89	0.77	0.73	0.73	0.89
Subject 3	0.86	0.91	0.86	0.89	0.87	0.79	0.77	0.89	0.84	0.94	0.87	0.79	0.91	0.83	0.95	0.78	0.75	0.81	0.85	0.91
Subject 4	0.97	1.08	1.07	0.95	0.00	0.92	0.87		0.87	1.03	1.12	0.93	0.00	0.41	0.81	0.81	0.84	0.78		1.35
Subject 5	0.90	0.89	0.98	0.91	1.12	1.05	1.04	1.03	0.77	0.90	0.84	0.83	0.97	0.93	1.08		0.95	0.93	0.97	0.82
Subject 6	0.89	0.89	0.86	0.86	0.81	0.85	0.92	0.91	0.90	1.04	1.07	1.07	1.10	1.01	0.93	0.89		0.79	0.80	0.81
Subject 7	0.87	0.86	0.74	0.95	1.03	1.00	1.04	1.02	1.19	1.15	1.09	1.02	1.06	0.99	0.95				0.85	0.87
Subject 8	0.88	0.92	0.94	0.95	1.04	1.05	0.90	0.87	0.89	0.84	0.87	1.03	0.99	1.09	0.95	0.86	0.82			0.84
n	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**6**	**6**	**6**	**6**	**7**
Mean	**0.88**	**0.91**	**0.89**	**0.91**	**0.81**	**0.95**	**0.89**	**0.90**	**0.77**	**0.95**	**0.95**	**0.93**	**0.86**	**0.87**	**0.93**	**0.84**	**0.83**	**0.81**	**0.85**	**0.91**
SD	**0.07**	**0.08**	**0.11**	**0.05**	**0.35**	**0.09**	**0.10**	**0.11**	**0.37**	**0.11**	**0.13**	**0.12**	**0.36**	**0.21**	**0.09**	**0.06**	**0.07**	**0.06**	**0.12**	**0.19**
SEM	**0.03**	**0.03**	**0.04**	**0.02**	**0.12**	**0.03**	**0.04**	**0.04**	**0.14**	**0.04**	**0.05**	**0.05**	**0.13**	**0.07**	**0.04**	**0.03**	**0.03**	**0.02**	**0.07**	**0.07**

Blood TGSH CON (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.91	0.98	0.87	0.85	0.87	0.84	0.89	1.27	1.21	1.31	1.22	1.43	1.38	1.39	0.88	1.11	1.08	1.01	0.85	0.89
Subject 2	0.81	0.88	0.88	0.86	0.79	0.81	0.79	0.80	0.98	0.80	0.82	0.84	0.89	0.82	1.0				0.90	0.99
Subject 3	0.80	0.82	0.84	0.85	0.82	0.84	0.81	0.85	0.94	0.99	1.00	0.95	0.87	0.96	0.91	0.83	0.80	0.71		0.85
Subject 4	0.95	0.91	0.94	0.83	0.80	0.76	0.82	0.83	1.01	1.04	1.02	1.22	1.25	1.27	1.08	1.34	1.04	1.00	0.93	0.92
Subject 5	0.76	0.85	0.86	0.87	0.81	0.75	0.73		0.69	0.78	0.87	0.93	1.08	0.95	1.06			1.02		0.82
Subject 6	0.88	0.84	0.87	0.73	0.69	0.78	0.57	0.67	0.6	0.87	0.95	1.09	0.85	0.85	0.91	0.90	0.86	0.76	0.72	0.89
Subject 7	0.80	0.90	0.87	0.80	0.79	1.00	0.93	0.96	0.98	1.11	1.15	1.04	1.08	1.15	1.43	1.34	1.21	1.04	1.21	0.87
Subject 8	0.92	0.88	0.91	0.81	0.77	0.73	0.77	0.80	0.99	1.00	1.14	1.19	1.23	1.25	1.30	1.20	0.99		0.91	0.89
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	7	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**6**	**6**	**6**	**8**
Mean	**0.85**	**0.88**	**0.88**	**0.82**	**0.79**	**0.81**	**0.79**	**0.88**	**0.98**	**0.99**	**1.02**	**1.09**	**1.08**	**1.08**	**1.08**	**1.07**	**0.99**	**0.93**	**0.92**	**0.89**
SD	**0.07**	**0.05**	**0.03**	**0.05**	**0.06**	**0.09**	**0.12**	**0.19**	**0.21**	**0.19**	**0.15**	**0.20**	**0.22**	**0.21**	**0.21**	**0.24**	**0.13**	**0.13**	**0.16**	**0.06**
SEM	**0.02**	**0.02**	**0.01**	**0.02**	**0.02**	**0.03**	**0.04**	**0.07**	**0.11**	**0.07**	**0.06**	**0.08**	**0.08**	**0.08**	**0.08**	**0.09**	**0.05**	**0.04**	**0.07**	**0.02**

Blood GSH NAC (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.83	0.79	0.72	0.71	0.55	0.76	0.58	0.58	0.62	0.62		0.60	0.58	0.48		0.46	0.41	0.49	0.71	0.54
Subject 2	0.66	0.71	0.68	0.78	0.74	0.83	0.72		0.51	0.68	0.52	0.79	0.63	0.52		0.32		0.44	0.52	0.44
Subject 3	0.83	0.79	0.72	0.71	0.55	0.76	0.58	0.58	0.62	0.62		0.60	0.58	0.48		0.46	0.41	0.49	0.71	0.54
Subject 4	0.94	0.89	0.86	0.80	0.81		0.83	0.77	0.76		0.76	0.76	0.76	0.77	0.52		0.40	0.47	0.62	0.59
Subject 5	0.86	0.80	0.84		0.82	0.84	0.85	0.84		0.66	0.60	0.57	0.59	0.57	0.61		0.54	0.63	0.65	0.58
Subject 6	0.76	0.76	0.73	0.78	0.71	0.74	0.80	0.78	0.72	0.86	0.84	0.85	0.83	0.72	0.62	0.71	0.79	0.49	0.43	0.59
Subject 7	0.83	0.78		0.85	0.90	0.84	0.89	0.90	0.94	0.88	0.87	0.76	0.77	0.71	0.72	0.95	0.98	0.67	0.53	0.71
Subject 8		0.84	0.82	0.75	0.76	0.76	0.78	0.74	0.71	0.74	0.72	0.71	0.72	0.72	0.49	0.40	0.38	0.45	0.55	0.54
n	**7**	**8**	**7**	**7**	**8**	**7**	**8**	**7**	**7**	**7**	**6**	**8**	**8**	**8**	**5**	**6**	**7**	**8**	**8**	**8**
Mean	**0.82**	**0.80**	**0.77**	**0.77**	**0.73**	**0.79**	**0.75**	**0.74**	**0.70**	**0.72**	**0.72**	**0.71**	**0.68**	**0.62**	**0.59**	**0.55**	**0.56**	**0.52**	**0.59**	**0.57**
SD	**0.09**	**0.06**	**0.07**	**0.05**	**0.12**	**0.04**	**0.12**	**0.12**	**0.14**	**0.11**	**0.14**	**0.10**	**0.10**	**0.12**	**0.09**	**0.23**	**0.24**	**0.08**	**0.10**	**0.07**
SEM	**0.03**	**0.02**	**0.03**	**0.02**	**0.04**	**0.02**	**0.04**	**0.05**	**0.05**	**0.04**	**0.06**	**0.04**	**0.04**	**0.04**	**0.04**	**0.10**	**0.09**	**0.03**	**0.04**	**0.03**

Blood GSH CON (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.83	0.87	0.69	0.71	0.71	0.69	0.69	0.89	0.42	0.56	0.43	0.66	0.53	0.44	0.45	0.40	0.42	0.43	0.33	0.56
Subject 2	0.72	0.78	0.75	0.76	0.74	0.74	0.74	0.70	0.48	0.49	0.50	0.46	0.41	0.31	0.45					0.65
Subject 3	0.76	0.77	0.69	0.71	0.71	0.69	0.69	0.89	0.42	0.56	0.43	0.66	0.53	0.44	0.49	0.40	0.42	0.43		0.56
Subject 4	0.92	0.78	0.80	0.79	0.81	0.78	0.77	0.78		0.61	0.60	0.57	0.56	0.63	0.45	0.38	0.35	0.42	0.50	0.43
Subject 5	0.71	0.77	0.76	0.75	0.66	0.63	0.54	0.42	0.40	0.50	0.39	0.46	0.51	0.43	0.39				0.39	0.41
Subject 6	0.80	0.74	0.76	0.65	0.54	0.61	0.43	0.56	0.64	0.65	0.61	0.64	0.49	0.51	0.49	0.47	0.49	0.42	0.41	0.52
Subject 7	0.75	0.83	0.78	0.94	0.98	0.86	0.77	0.85	0.81	0.53	0.51	0.54	0.53	0.63	0.65	0.66	0.59	0.45	0.60	0.38
Subject 8	0.87	0.73	0.75	0.74	0.76	0.69	0.72	0.71	0.58	0.55	0.55	0.56	0.56	0.58	0.40	0.36	0.32	0.38	0.46	0.40
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**6**	**6**	**6**	**8**
Mean	**0.80**	**0.78**	**0.75**	**0.76**	**0.74**	**0.71**	**0.66**	**0.73**	**0.54**	**0.56**	**0.50**	**0.57**	**0.51**	**0.50**	**0.47**	**0.44**	**0.44**	**0.45**	**0.43**	**0.49**
SD	**0.08**	**0.05**	**0.04**	**0.09**	**0.14**	**0.09**	**0.13**	**0.17**	**0.15**	**0.05**	**0.09**	**0.09**	**0.05**	**0.11**	**0.10**	**0.11**	**0.08**	**0.07**	**0.10**	**0.10**
SEM	**0.03**	**0.02**	**80.02**	**0.03**	**0.05**	**0.03**	**0.05**	**0.06**	**0.06**	**0.02**	**0.03**	**0.03**	**0.02**	**0.04**	**0.05**	**0.04**	**0.03**	**0.02**	**0.04**	**0.03**

Blood GSSG NAC (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.10	0.11	0.15	0.14	0.18	0.21	0.20	0.14	0.19	0.21		0.25	0.30	0.32		0.43	0.42	0.35		0.29
Subject 2	0.10	0.12	0.09	0.17	0.14	0.11	0.12	0.16	0.23	0.23	0.31	0.25	0.35	0.35				0.29	0.21	0.44
Subject 3	0.03	0.12	0.14	0.17	0.32	0.04	0.19	0.31	0.22	0.32	0.21	0.19	0.32	0.34		0.32	0.35	0.32	0.13	0.27
Subject 4	0.01	0.06	0.11	0.17	0.21	0.28	0.12	0.13	0.16	0.22	0.14	0.25	0.27	0.35	0.44		0.42	0.33		0.25
Subject 5	0.04	0.09	0.14	0.15	0.29	0.21	0.19	0.19		0.24	0.24	0.26	0.39	0.37	0.47		0.41	0.31	0.31	0.24
Subject 6	0.13	0.14	0.14	0.08	0.10	0.11	0.12	0.12	0.18	0.17	0.23	0.22	0.26	0.29	0.31			0.30		0.21
Subject 7	0.05	0.08	0.11	0.10	0.14	0.16	0.15	0.12	0.25	0.27	0.22	0.25	0.29	0.28						0.16
Subject 8	0.06	0.08	0.13	0.20	0.28	0.29	0.13	0.13	0.17	0.10	0.15	0.31	0.27	0.36	0.46	0.46	0.44	0.35		0.30
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**6**	**6**	**8**	**8**	**4**	**3**	**5**	**7**	**3**	**7**
Mean	**0.06**	**0.10**	**0.13**	**0.15**	**0.21**	**0.18**	**0.15**	**0.16**	**0.20**	**0.22**	**0.21**	**0.25**	**0.31**	**0.33**	**0.42**	**0.40**	**0.41**	**0.32**	**0.22**	**0.27**
SD	**0.04**	**0.03**	**0.02**	**0.04**	**0.09**	**0.09**	**0.03**	**0.07**	**0.03**	**0.07**	**0.06**	**0.04**	**0.04**	**0.03**	**0.07**	**0.07**	**0.04**	**0.02**	**0.09**	**0.09**
SEM	**0.02**	**0.01**	**0.01**	**0.02**	**0.03**	**0.03**	**0.01**	**0.03**	**0.01**	**0.03**	**0.03**	**0.02**	**0.02**	**0.01**	**0.04**	**0.04**	**0.02**	**0.01**	**0.05**	**0.03**

Blood GSSG CON (mM)

	0	1	2	5	10	15	25	PE1	E1	PE2	E2	PE3	E3	PE4	F	+1	+2	+5	+10	+30
Subject 1	0.09	0.10	0.18	0.14	0.16	0.14	0.21	0.39	0.79	0.75		0.77	0.86	0.95		0.71	0.67	0.58	0.51	0.44
Subject 2	0.08	0.10	0.13	0.10	0.11	0.12	0.13	0.10		0.31	0.52	0.38	0.48	0.51		0.46	0.43	0.34		0.34
Subject 3	0.04	0.05	0.15	0.14	0.11	0.15	0.12	0.14		0.41	0.57	0.30	0.34	0.52		0.44	0.38	0.27	0.43	0.29
Subject 4	0.03	0.14	0.14	0.05	0.05	0.07	0.05	0.05		0.43	0.53	0.51	0.69	0.64		0.95	0.69	0.59	0.43	0.49
Subject 5	0.05	0.08	0.10	0.11	0.15	0.12	0.19	0.14	0.29	0.28	0.49	0.47	0.51	0.51	0.68		0.59	0.52		0.42
Subject 6	0.08	0.10	0.12	0.08	0.15	0.17	0.14	0.11		0.22	0.34	0.24	0.36	0.34	0.42	0.43	0.37	0.34	0.31	0.40
Subject 7	0.04	0.07	0.08	0.10	0.11	0.13	0.15	0.11		0.58	0.64	0.51	0.55	0.52	0.78	0.68	0.62	0.59	0.61	0.48
Subject 8	0.05	0.16	0.17	0.07	0.01	0.04	0.05	0.09	0.41	0.45	0.58	0.63	0.67	0.66	0.90		0.67	0.59	0.45	0.49
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**3**	**8**	**6**	**6**	**7**	**8**	**4**	**6**	**8**	**8**	**6**	**6**
Mean	**0.06**	**0.10**	**0.13**	**0.10**	**0.11**	**0.12**	**0.13**	**0.14**	**0.50**	**0.43**	**0.52**	**0.51**	**0.56**	**0.58**	**0.69**	**0.61**	**0.55**	**0.48**	**0.46**	**0.42**
SD	**0.02**	**0.04**	**0.04**	**0.04**	**0.06**	**0.05**	**0.06**	**0.12**	**0.26**	**0.17**	**0.10**	**0.17**	**0.19**	**0.18**	**0.20**	**0.21**	**0.14**	**0.13**	**0.10**	**0.09**
SEM	**0.01**	**0.01**	**0.01**	**0.02**	**0.03**	**0.02**	**0.02**	**0.05**	**0.15**	**0.06**	**0.04**	**0.07**	**0.07**	**0.06**	**0.10**	**0.09**	**0.05**	**0.05**	**0.04**	**0.03**

Plasma K+ NAC (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	4.13	4.29		4.25	4.99	4.13	4.76	4.01	6.48	5.15	4.38	3.94	3.77	4.04
Subject 2	4.84	4.73	4.83	4.18	4.87	4.04	4.40	4.17	6.42	5.11	4.29	3.77	3.63	3.95
Subject 3	3.75	3.85	4.51	3.94	4.39	3.91	4.58	3.95	6.50	5.65	4.71	3.83	3.72	3.79
Subject 4	3.72	3.80	4.77	3.81	4.53	3.86	4.60	4.01	6.24	5.42	4.14	3.48	3.41	3.65
Subject 5	3.97	4.03	4.83	4.11	4.51	3.98	4.68	4.13	5.84	5.01	4.44	3.84	3.74	3.91
Subject 6	4.12	4.12	4.94	3.87	4.47	3.77	4.38	3.72	6.14	4.20	3.83	3.42	3.58	4.03
Subject 7	3.68	4.08	4.69	3.86	4.47	3.82	4.36	3.86	4.94	4.37	3.95	3.68	3.64	3.80
Subject 8	3.92	3.82	4.46	4.00	4.63	3.85	4.97	3.82	6.93	4.99	4.05	3.49	3.68	3.92
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**4.02**	**4.09**	**4.72**	**4.00**	**4.61**	**3.92**	**4.59**	**3.96**	**6.19**	**4.99**	**4.22**	**3.68**	**3.65**	**3.89**
SD	**0.38**	**0.31**	**0.18**	**0.16**	**0.21**	**0.12**	**0.21**	**0.15**	**0.59**	**0.53**	**0.29**	**0.21**	**0.11**	**0.13**
SEM	**0.13**	**0.11**	**0.07**	**0.06**	**0.08**	**0.04**	**0.07**	**0.05**	**0.21**	**0.20**	**0.10**	**0.08**	**0.04**	**0.05**

Plasma K+ CON (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	4.10	3.84	4.75	4.08	4.40	4.03	4.43	3.98	5.82	5.06	4.45	4.14	3.83	3.93
Subject 2	4.74	4.26	4.69	4.13	4.74	4.12	4.73	4.12	7.74	6.13	3.99	3.65	3.62	3.68
Subject 3	3.68	3.92	4.52	3.82	3.92	3.90	4.43	3.98	6.56	5.74	4.83	3.76	3.70	3.90
Subject 4	3.76	4.09		3.84	4.44	3.80	4.40	3.94	6.46	4.97	4.35	3.33	3.15	3.48
Subject 5	4.05	3.86	4.53	4.27	4.58	4.00	4.49	4.20	5.40	5.40	4.52	3.79	3.65	4.00
Subject 6	4.14	4.14	4.67	3.98	4.43	3.94	4.27	4.05	4.83	4.53	4.34	3.72	3.64	3.82
Subject 7	3.91	4.16	4.51	4.08	4.35	3.93	4.31	3.92	5.01	4.35	4.00	3.64	3.74	3.76
Subject 8	4.27	4.47	5.09	4.38	4.53	4.21	4.54	4.19	6.02	4.99	4.26	3.74	3.78	4.02
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**4.08**	**4.09**	**4.65**	**4.07**	**4.42**	**3.99**	**4.45**	**4.05**	**5.98**	**5.15**	**4.34**	**3.72**	**3.64**	**3.82**
SD	**0.33**	**0.23**	**0.21**	**0.19**	**0.24**	**0.13**	**0.14**	**0.12**	**0.95**	**0.59**	**0.30**	**0.26**	**0.23**	**0.20**
SEM	**0.12**	**0.09**	**0.08**	**0.07**	**0.08**	**0.05**	**0.05**	**0.05**	**0.34**	**0.21**	**0.11**	**0.11**	**0.09**	**0.07**

Delta [K+] to work ratio CON ($nmol.l^{-1}.J^{-1}$)

	EB1	EB2	EB3	EB4
Subject 1	51.7	18.19	22.73	46.136
Subject 2	30.7	43.59	43.59	51.964
Subject 3	46.3	7.716	40.9	71.098
Subject 4		36.53	36.53	101.53
Subject 5	40.7	18.82	29.75	44.307
Subject 6	41.8	35.46	26	192.45
Subject 7	24	18.52	26.06	65.965
Subject 8	47.2	11.42	25.11	53.565
n	**7**	**8**	**8**	**8**
Mean	**40.3**	**23.78**	**31.33**	**78.376**
SD	**9.77**	**13.02**	**7.927**	**49.668**
SEM	**3.69**	**4.602**	**2.803**	**17.56**

Delta [K+] to work ratio NAC ($nmol.l^{-1}.J^{-1}$)

	EB1	EB2	EB3	EB4
Subject 1		42.057	35.806	63.171
Subject 2	7.1454	49.303	25.723	37.681
Subject 3	50.926	34.722	51.698	66.076
Subject 4	59.056	43.836	45.053	95.462
Subject 5	48.573	24.287	42.502	57.681
Subject 6	64.618	47.281	48.069	94.303
Subject 7	41.838	41.838	37.037	65.359
Subject 8	48.706	47.945	85.236	92.615
n	7	**8**	**8**	**8**
Mean	**45.838**	**41.409**	**46.39**	**71.544**
SD	**18.628**	**8.3144**	**17.662**	**20.741**
SEM	**7.0409**	**2.9396**	**6.2443**	**7.3329**

Change in Plasma Volume (%) NAC

	PE1	E1	PE2	E2	PE3	E3	PE4	FAT	R+1	R+2	R+5	R+10	R+30
Subject 1	-3.878	-8.713	-9.967	-13.29	-11.19	-13.65	-12.79	-19.85	-19.33	-15.86	-16.25	-9.976	2.6722
Subject 2	3.0757	-7.621	-9.014	-1.136	-12.28	-11.54	-14.8	-2.717	-4.359	-0.735	-1.91	-20.54	12.603
Subject 3	-5.407	-9.38	-11.84	-14.66	-16.17	-17.37	-15.78	-23.68	-23.04	-23.06	-21.22	-16.53	-6.996
Subject 4	-3.407	-4.379	-7.785	-10.89	-12.14	-13.7	-15.83	-17.42		-15.46	-13.75	-8.73	-1.061
Subject 5	-3.109	-8.01	-11	-12.97	-12.55	-15.98	-14.8	-17.23	-19.83	-21.69	-19.63	-15.11	-4.279
Subject 6	3.2643	2.2321	1.5991	5.4548	1.9173	2.2668	1.4203	7.9619	8.3507	8.8524	7.6421	4.852	7.6261
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**5**	**6**	**6**	**6**	**6**
Mean	**-2.545**	**-7.621**	**-9.921**	**-10.59**	**-12.87**	**-14.45**	**-14.8**	**-16.18**	**-16.64**	**-15.36**	**-14.55**	**-14.18**	**0.5879**
SD	**4.24**	**7.35**	**6.56**	**7.95**	**8.57**	**9.32**	**10.01**	**12.13**	**11.69**	**11.57**	**10.87**	**8.55**	**8.35**
SEM	**1.4598**	**1.116**	**0.7152**	**2.4394**	**0.8574**	**1.0137**	**0.7102**	**3.5607**	**4.1754**	**3.9589**	**3.4177**	**2.1699**	**3.4105**

Change in Plasma Volume (%) CON

	PE1	E1	PE2	E2	PE3	E3	PE4	FAT	R+1	R+2	R+5	R+10	R+30
Subject 1	-4.054	2.3547	-2.502	-5.084	-4.389	-4.643	-7.074	-12.35	-12.14	-13.51	-10.69	-6.301	11.602
Subject 2	-4.769	-5.639	-6.51	-7.603	-8.636	-8.098	-8.141	-15.56	-13.91	-12.57	-6.336	-4.333	11.48
Subject 3	-9.961	-14.4	-13.34	-15.93	-15.76	-17.27	-17.77	-25.23	-24.85	-24.04	-19.91	-15.93	-6.626
Subject 4	-5.895	-7.251	-11.6	-14.42	-15.96	-15.79	-20.49	-21.91	-21.54	-21.08	-19.17	-12.13	-0.752
Subject 5													
Subject 6	2.4152	5.9671	4.3011	4.8331	5.3311	5.9776	6.1847	5.8664	6.0123	5.9101	6.6763	5.6837	8.8298
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Mean	**-6.519**	**-6.403**	**-8.709**	**-11.51**	**-12.12**	**-12.73**	**-14.26**	**-18.76**	**-19.41**	**-19.29**	**-15.56**	**-11.16**	**2.1562**
SD	**3.76**	**4.81**	**4.92**	**8.17**	**6.27**	**7.12**	**7.36**	**12.16**	**13.31**	**12.66**	**11.35**	**8.90**	**7.40**
SEM	**1.0801**	**2.6686**	**1.9235**	**2.1614**	**2.3842**	**2.6732**	**2.7659**	**2.9332**	**2.6888**	**2.6431**	**2.9857**	**2.5418**	**3.9488**

Plasma H^+ NAC (nM)

	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	39.0	36.3	36.0	47.2	45.1	51.6	49.6	56.3	56.8	65.46	70.7	69.6	67.7	46.3
Subject 2	39.9	39.3	38.6	46.6	43.4	48.0	44.6	46.9	58.6	65.6	63.5	63.2	55.7	34.5
Subject 3	39.1	39.1	50.6	45.4	54.3	49.0	48.8	55.3	61.9	70.3	66.3	73.9	77.6	58.3
Subject 4	37.9	37.1		46.8	44.6	53.0	49.7	55.7	50.2	60.8	58.6	61.2	62.6	44.3
Subject 5	38.6	37.2	37.4	47.3	45.7	51.2	50.2	54.3	53.2	59.5	60.5	62.0	64.8	46.6
Subject 6	38.8	37.3	38.9	46.8	46.2	50.5	48.5	53.4	54.6	63.4	63.0	64.9	64.8	44.6
Subject 7	37.4	37.7	38.1	46.5	44.7	50.5	48.7	52.2	49.8	56.6	54.8	56.6	56.6	41.7
Subject 8	39.8	34.4	36.0	47.8	45.3	50.1	47.6	52.8	52.1	65.7	66.8	68.0	69.0	40.2
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**38.8**	**37.3**	**38.9**	**46.8**	**46.2**	**50.5**	**48.5**	**53.4**	**54.6**	**63.4**	**63.0**	**64.9**	**64.8**	**44.6**
SD	**0.9**	**1.6**	**5.2**	**0.7**	**3.6**	**1.6**	**1.8**	**3.2**	**4.5**	**4.6**	**5.4**	**5.8**	**7.5**	**7.3**
SEM	**0.3**	**0.6**	**1.9**	**0.2**	**1.3**	**0.6**	**0.7**	**1.2**	**1.7**	**1.7**	**2.0**	**2.2**	**2.8**	**2.7**

Plasma H+ CON (nM)

	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	38.9	39.3	39.7	51.6	49.0	53.3	50.1	55.3	52.8	66.2	70.6	72.1	69.6	44.5
Subject 2	40.3	41.5	40.1	47.5	49.4	48.1	46.4	48.5	64.7	65.1				48.1
Subject 3	38.9	40.1	37.8	53.9	49.3	52.1	53.0	52.7	64.4	76.7	73.9	70.3	78.8	56.6
Subject 4	37.6	36.1		45.7	44.7	53.8	50.2	56.7	49.6	62.5	65.9	68.2	63.6	52.3
Subject 5	38.4	38.1	37.6	47.3	45.9	51.8	50.0	53.5	54.5	60.8	63.5	67.2	71.1	48.5
Subject 6	39.1	39.0	38.5	49.3	47.8	52.1	49.9	53.5	55.7	64.2	65.6	66.8	67.5	48.3
Subject 7	39.1	38.9	39.0	48.4	47.8	52.1	50.1	54.8	52.0	53.9	54.4	54.9	56.4	42.5
Subject 8	40.4	39.2	38.9	50.6	48.7	53.5	49.4	53.2	51.8	64.5	65.4	68.0	65.3	45.8
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	7	7	7	**8**
Mean	**39.1**	**39.0**	**38.5**	**49.3**	**47.8**	**52.1**	**49.9**	**53.56**	**55.7**	**64.2**	**65.6**	**66.8**	**67.5**	**48.3**
SD	**0.9**	**1.6**	**1.2**	**2.8**	**1.8**	**1.9**	**1.9**	**2.6**	**6.2**	**6.8**	**6.6**	**6.0**	**7.5**	**5.8**
SEM	**0.3**	**0.6**	**0.4**	**1.0**	**0.6**	**0.7**	**0.7**	**0.9**	**2.3**	**2.5**	**2.7**	**2.4**	**3.0**	**2.6**

Plasma Na+ NAC (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	142.8	145.7	140.4	141.8	145.9	146.0	145.2	151.2	154.2	151.6	156.4	144.2	138.7	141.9
Subject 2	140.2	142.7	138.5	149.0	143.8	148.4	145.0	146.1	153.2	152.9	149.8	146.1	144.7	142.4
Subject 3	136.8	140.0	143.7	141.2	145.2	139.1	138.1	145.9	150.6	151.8	152.0	145.8	143.6	140.2
Subject 4	142.6	142.2		146.7	146.5	147.7	147.4	149.0	151.3	149.6	155.1	152.0	149.3	145.2
Subject 5	140.6	139.3	141.1	144.9	143.1	145.7	144.6	146.1	149.3	149.6	148.3	144.8	142.9	140.1
Subject 6	143.0	141.7	143.0	146.7	144.5	146.6	146.1	147.5	151.0	148.1		144.2	141.7	138.6
Subject 7	140.5	142.7	143.0	146.7	145.0	147.9	147.0	148.9	150.4	151.5	149.4	146.9	144.5	142.2
Subject 8	141.5	141.1	141.7	144.8	143.6	144.8	143.4	145.1	147.9	150.0	149.0	146.0	143.5	140.4
n	**8**	**8**	7	**8**	**8**	**8**	**8**	**8**	**8**	**8**	7	**8**	**8**	**8**
Mean	**141.0**	**141.9**	**141.8**	**145.2**	**144.7**	**145.8**	**144.6**	**147.5**	**151.0**	**150.6**	**151.4**	**146.3**	**143.6**	**141.4**
SD	**2.0**	**2.0**	**1.8**	**2.6**	**1.2**	**3.0**	**2.9**	**2.2**	**2.2**	**1.6**	**3.2**	**2.5**	**3.0**	**2.0**
SEM	**0.7**	**0.7**	**0.6**	**0.9**	**0.4**	**1.0**	**1.0**	**0.8**	**0.8**	**0.6**	**1.2**	**0.9**	**1.1**	**0.7**

Plasma Na+ CON (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	139.6	137.5	141.6	147.9	144.7	144.1	139.2	148.2	145.2	156.7	149.9	151.8	145.7	138.8
Subject 2	132.8	147.9		148.9	151.7	141.5	144.9	145.6	151.5	147.1	150.0	147.6	146.2	142.6
Subject 3	143.6	147.0	130.9	154.0	146.0	135.8	146.6	134.0	141.4	168.3	153.5	138.6	134.2	140.5
Subject 4	139.9	142.1	141.5	145.8	144.8	146.5	145.9	148.7	149.2	152.3	151.4	149.2	145.0	142.6
Subject 5	135.0	137.0	135.0	139.0	139.0	141.0	141.0	142.0	144.0	145.0	146.0	143.0	140.0	141.2
Subject 6	138.3	142.2	139.4	146.1	144.2	142.2	143.4	144.7	147.3	152.9	149.9	145.1	142.2	141.7
Subject 7	138.4	141.8	140.3	146.0	145.0	147.0	145.5	146.3	149.4	151.7	150.5	145.6	144.4	142.6
Subject 8	140.5	140.8	141.3	144.6	142.9	144.3	143.6	144.2	149.3	149.6	148.4	144.6	142.4	139.8
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**138.5**	**142.0**	**139.1**	**146.6**	**144.9**	**142.9**	**143.8**	**144.1**	**147.1**	**153.0**	**150.0**	**145.8**	**142.6**	**141.2**
SEM	**1.4**	**1.6**	**1.7**	**1.7**	**1.4**	**1.5**	**1.0**	**1.9**	**1.4**	**2.9**	**0.9**	**1.6**	**1.6**	**0.7**

Plasma Ca^{2+} NAC (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	1.22	1.22	1.20	1.19	1.21	1.23	1.23	1.24	1.35	1.32	1.32	1.19	1.25	1.20
Subject 2	1.23	1.22		1.27	1.26	1.24	1.25	1.25	1.34	1.34	1.30	1.23	1.21	1.18
Subject 3	1.20	1.21	1.25	1.20	1.25	1.21	1.18	1.25	1.30	1.38	1.35	1.26	1.22	1.19
Subject 4	1.26	1.24	1.27	1.26	1.29	1.27	1.31	1.29	1.34	1.36	1.32	1.27	1.25	1.23
Subject 5	1.22	1.20	1.24	1.24	1.25	1.25	1.25	1.24	1.29	1.31	1.29	1.25	1.21	1.18
Subject 6	1.24	1.20	1.25	1.24	1.26	1.26	1.26	1.25	1.31	1.30		1.21	1.21	1.19
Subject 7	1.22	1.25	1.27	1.26	1.26	1.21	1.27	1.23	1.28	1.30	1.27	1.23	1.23	1.23
Subject 8	1.21	1.18	1.22	1.21	1.23	1.21	1.23	1.23	1.28	1.30	1.30	1.25	1.20	1.20
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
Mean	**1.23**	**1.22**	**1.24**	**1.23**	**1.25**	**1.24**	**1.25**	**1.25**	**1.31**	**1.33**	**1.31**	**1.24**	**1.22**	**1.20**
SD	**0.02**	**0.02**	**0.03**	**0.03**	**0.02**	**0.02**	**0.04**	**0.02**	**0.03**	**0.03**	**0.03**	**0.03**	**0.02**	**0.02**
SEM	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**

Plasma Ca^{2+} CON (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	1.18	1.20	1.21	1.26	1.26	1.22	1.20	1.27	1.25	1.32	1.27	1.26	1.21	1.13
Subject 2	1.18	1.30		1.30	1.34	1.24	1.28	1.27	1.38	1.31	1.24	1.22	1.17	1.19
Subject 3	1.24	1.27	1.15	1.34	1.31	1.14	1.32	1.14	1.24	1.52	1.40	1.16	1.32	1.23
Subject 4	1.24	1.24	1.26	1.25	1.28	1.27	1.29	1.25	1.32	1.37	1.35	1.33	1.22	1.25
Subject 5	1.26	1.17	1.22	1.23	1.25	1.22	1.26	1.23	1.30	1.33	1.33	1.23	1.21	1.20
Subject 6	1.20	1.22	1.21	1.26	1.26	1.24	1.25	1.22	1.29	1.33	1.32	1.23	1.21	1.21
Subject 7	1.20	1.23	1.28	1.27	1.29	1.29	1.27	1.26	1.30	1.27	1.25	1.20	1.17	1.20
Subject 8	1.17	1.22	1.23	1.22	1.23	1.26	1.24	1.22	1.29	1.30	1.32	1.26	1.24	1.21
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**1.21**	**1.23**	**1.23**	**1.27**	**1.28**	**1.23**	**1.27**	**1.23**	**1.30**	**1.35**	**1.31**	**1.24**	**1.22**	**1.20**
SD	**0.04**	**0.04**	**0.05**	**0.04**	**0.04**	**0.05**	**0.04**	**0.05**	**0.05**	**0.08**	**0.06**	**0.05**	**0.05**	**0.04**
SEM	**0.01**	**0.02**	**0.02**	**0.02**	**0.01**	**0.02**	**0.01**	**0.02**	**0.02**	**0.03**	**0.02**	**0.02**	**0.02**	**0.02**

pO2 NAC (Torr)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	83.0	90.3	84.1	106.4	103.1	111.6	94.2	114.0	73.8	99.4	106.7	106.7	126.4	94.9
Subject 2	82.7	88.2	74.3	79.1	80.6	93.6	87.9	99.4	88.9	100.1	115.8	104.0	97.6	102.5
Subject 3	79.1	90.2		101.8	98.0	95.3	106.2	99.1	85.0	88.2	94.8	117.9	114.3	102.9
Subject 4	92.2	90.2	94.1	106.8	111.2	107.3	109.8	115.1	125.1	111.8	139.4	123.9	127.7	100.6
Subject 5	87.5	94.2	82.9	89.1	96.0	97.0	96.9	101.7	92.1	100.2	106.0	111.2	114.1	102.5
Subject 6	87.0	83.3	87.4	89.4	98.3	84.7	88.5		96.8	78.1		107.7	107.9	95.2
Subject 7	90.2	101.0	98.4	120.9	112.6	127.6	97.5	125.6	114.2	125.0	119.6	116.2	107.5	89.5
Subject 8	83.5	97.8	94.8	112.2	100.9	101.0	98.3	106.5	98.8	101.6	113.7	112.0	117.6	108.2
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**7**	**8**	**8**	**8**
Mean	**85.7**	**91.9**	**89.9**	**100.7**	**100.1**	**102.3**	**97.4**	**108.8**	**96.8**	**100.6**	**113.7**	**112.5**	**114.1**	**99.5**
SD	**4.3**	**5.6**	**9.4**	**13.8**	**10.0**	**13.2**	**7.7**	**9.9**	**17.6**	**14.1**	**13.9**	**6.6**	**10.0**	**5.9**
SEM	**1.5**	**2.0**	**3.3**	**4.9**	**3.5**	**4.7**	**2.7**	**3.7**	**6.7**	**5.0**	**5.3**	**2.3**	**3.5**	**2.1**

pO2 CON (Torr)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	95.9	88.4	78.8	97.5	87.7	89.7	87.3	92.3	71.5	96.9	107.5	108.9	110.9	105.2
Subject 2	69.9	78.1		98.1	88.0	106.0	83.1	108.9	93.1	110.2	110.4	105.5	91.7	79.7
Subject 3	80.2	86.2	86.2	107.5	99.4	104.5	97.0	103.9	91.7	95.4	101.0	107.7	107.1	101.8
Subject 4	98.4	92.5	98.0	110.8	109.0	117.1	109.8	111.9	114.4	112.1	113.1	122.5	119.0	97.4
Subject 5	91.6	86.9	85.1	97.7	92.1	95.6	91.9	93.2	84.4	90.1	101.1	109.3	108.4	94.7
Subject 6	85.2	86.5	84.9	102.5	98.6	101.1	93.6	102.4	92.7	103.2	110.7	116.2	109.5	95.1
Subject 7	74.9	88.4	86.4	100.4	94.3	104.3	99.7	108.9	93.6	110.5	121.3	128.9	113.0	93.2
Subject 8	85.8	85.2	81.8	105.3	119.7	90.3	86.5	97.5	100.0	106.9	119.9	130.6	116.2	91.8
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**85.2**	**86.5**	**84.9**	**102.5**	**98.6**	**101.1**	**93.6**	**102.4**	**92.7**	**103.2**	**110.7**	**116.2**	**109.5**	**94.9**
SD	**10.8**	**4.4**	**6.7**	**5.4**	**11.9**	**9.8**	**9.3**	**8.0**	**13.2**	**8.8**	**8.9**	**10.8**	**8.9**	**9.0**
SEM	**4.1**	**1.7**	**2.5**	**2.0**	**4.5**	**3.7**	**3.5**	**3.0**	**5.0**	**3.3**	**3.6**	**4.1**	**3.4**	**3.7**

Hb NAC (g/dl)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	16.1	16.4	16.8	17	17.3	17.2	17.3	17.2	17.8	17.8	17.5	17.4	16.9	15.8
Subject 2	16.8	16.7	15.7	17.8	16.8	17.9	18.2	16.9	17.4	17.1	16.7	16.9	19.1	15.4
Subject 3	14.7	15.2	15.6	15.7	16	16	16.2	16.1	16.8	16.8	16.7	16.6	16.1	15.2
Subject 4	13.9	14.3	14.5	14.8	15	15.1	15.2	15.3	15.8	15.7	15.5	15.4	15.1	14.4
Subject 5	16	16.4	16.6	16.8	17.1	17.2	17.4	17.5	17.8	17.6	17.5	17.3	16.8	16.3
Subject 6	14	14.3	14.8	15	15.2	15.1	15.4	15.3	15.4	15.6	16	15.8	15.3	14.5
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**15.3**	**15.6**	**15.7**	**16.2**	**16.2**	**16.4**	**16.6**	**16.4**	**16.8**	**16.8**	**16.7**	**16.6**	**16.6**	**15.3**
SD	**1.2**	**1.1**	**1.0**	**1.2**	**1.0**	**1.2**	**1.2**	**1.0**	**1.0**	**0.9**	**0.8**	**0.8**	**1.5**	**0.7**
SEM	**0.5**	**0.4**	**0.5**	**0.5**	**0.4**	**0.5**	**0.5**	**0.4**	**0.4**	**0.4**	**0.3**	**0.3**	**0.6**	**0.3**

Hb CON (g/dl)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	16.2	14.8	14.9	15.2	15.5	15.5	15.4	15.6	16.2	16.1	16.2	15.9	15.5	14.1
Subject 2	16.3	16.9	16.9	16.9	17.1	17.1	17	17.2	17.7	17.6	17.5	16.9	16.7	15.4
Subject 3	14.9	15.9	16.4	16.2	16.6	16.5	16.7	16.7	17.4	17.2	17.2	16.8	16.4	15.6
Subject 4	15.3	15.8	16	16.3	16.5	16.7	16.7	17	17.2	17.3	17.2	16.9	16.3	15.3
Subject 5	14.2	14.8	14.8	15.1	15.5	15.6	15.8	15.8	16.8	16.4	16.5	16.1	15.6	14.7
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Mean	**15.4**	**15.6**	**15.8**	**15.9**	**16.2**	**16.3**	**16.3**	**16.5**	**17.1**	**16.9**	**16.9**	**16.5**	**16.1**	**15.0**
SD	**0.9**	**0.9**	**0.9**	**0.8**	**0.7**	**0.7**	**0.7**	**0.7**	**0.6**	**0.6**	**0.5**	**0.5**	**0.5**	**0.6**
SEM	**0.4**	**0.4**	**0.4**	**0.3**	**0.3**	**0.3**	**0.3**	**0.3**	**0.3**	**0.3**	**0.2**	**0.2**	**0.2**	**0.3**

Hct NAC (%)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	47.3	48.4	49.8	49.9	50.9	50	51.1	50.9	53.3	53	51.8	52.3	50.2	46.9
Subject 2	47.2	45.9	47.4	49.1	47.8	50.65	49.4	46.5	46.8	48.6	47.9	47.9	52.3	45.5
Subject 3	45.2	46.4	47.3	48.4	49.1	50	50.1	49.45	52.2	51.8	52.1	51.25	49.9	47.3
Subject 4	45.6	45.7	46.6	47.4	48	48.9	49.2	49.1	50.8	50.3	50.1	50.1	49.4	46.4
Subject 5	49.6	50.1	50	51.2	52	52.4	52.7	53.6	53.7		53.4	53	51.7	49.2
Subject 6	41.9	42.5	43.5	44.6	45.1	45.2	46.3	45.9	47.1	48.1	48	47.3	46.1	42.4
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**5**	**6**	**6**	**6**	**6**
Mean	**46.1**	**46.5**	**47.4**	**48.4**	**48.8**	**49.5**	**49.8**	**49.2**	**50.7**	**50.4**	**50.6**	**50.3**	**49.9**	**46.3**
SD	**2.6**	**2.6**	**2.7**	**2.3**	**2.5**	**2.4**	**2.1**	**2.8**	**3.0**	**2.1**	**2.3**	**2.3**	**2.2**	**2.3**
SEM	**1.1**	**1.1**	**1.2**	**0.9**	**1.0**	**1.0**	**0.9**	**1.2**	**1.2**	**0.9**	**0.9**	**0.9**	**0.9**	**0.9**

Hct CON (%)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	47.3	43.7	43.4	45	45.4	45	45.5	46.2	47.3	47.5	48	47.3	46.1	41.6
Subject 2	44.6	45.3	45.8	46.3	46.3	46.9	46.9	46.3	49.2	48.5	48	46.2	45.7	41.65
Subject 3	46.4	48.5	49.5	49.5	49.8	50	50.3	50.6	53.2	53.5	53	51.6	50.4	47.6
Subject 4	46.8	48.3	48.4	49.9	50.9	51.2	51.1	53	53.3	52.8	52.8	52.5	50.2	47.2
Subject 5	42.9	45.2	44.7	45.1	46.7	47.2	47.8	47.8	50.8	50.3	50.4	49.3	48	43.8
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Mean	**46.1**	**46.2**	**46.4**	**47.2**	**47.8**	**48.1**	**48.3**	**48.8**	**50.8**	**50.5**	**50.4**	**49.4**	**48.1**	**44.4**
SD	**1.8**	**2.1**	**2.5**	**2.4**	**2.4**	**2.5**	**2.3**	**3.0**	**3.0**	**2.6**	**2.5**	**2.7**	**2.2**	**2.9**
SEM	**0.8**	**0.9**	**1.1**	**1.1**	**1.1**	**1.1**	**1.0**	**1.3**	**1.5**	**1.2**	**1.1**	**1.2**	**1.0**	**1.3**

HCO_3^- NAC (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	28.2	26.2	27.5	19.6	20.1	16.6	17.0	13.7	13.7					16.7
Subject 2	25.3	23.5	24.6	20.3	31.2	18.2	19.4	18.0	14.6	11.8	8.1	8.4	9.2	14.1
Subject 3	27.4	24.6	19.5	20.7	17.2	17.8	18.0	15.6	16.6	14.1	11.8	9.7	8.9	13.1
Subject 4	36.5	27.6	26.3	20.6	19.8	16.3	16.1	14.3	13.0	12.0				16.3
Subject 5	26.5	25.5	25.8	21.1	20.3	17.6	17.1	15.1	14.8	12.8	11.3	11.3	10.8	17.2
Subject 6	26.9	25.7	25.3	20.7	21.4	17.2	17.4	15.7	14.5					16.5
Subject 7	26.7	25.3	25.1	20.3	21.8	17.6	17.8	15.3	14.9			11.3	12.5	19.6
Subject 8	26.2	25.8	26.7	20.4	20.7	17.7	18.0	16.1	15.5	13.4	11.9	11.3	10.5	18.7
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**5**	**4**	**5**	**5**	**8**
Mean	**26.7**	**25.5**	**25.1**	**20.5**	**21.6**	**17.4**	**17.6**	**15.5**	**14.7**	**12.8**	**10.8**	**10.4**	**10.4**	**16.5**
SD	**1.1**	**1.6**	**2.9**	**0.5**	**4.9**	**0.7**	**1.1**	**1.5**	**1.3**	**1.0**	**1.8**	**1.3**	**1.4**	**2.8**
SEM	**0.5**	**0.7**	**1.2**	**0.2**	**2.0**	**0.3**	**0.5**	**0.6**	**0.5**	**0.4**	**0.9**	**0.6**	**0.6**	**1.3**

HCO_3^- CON (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	24.7	25.5	24.3	19.0	19.5	16.6	17.7	15.2	16.8	12.2	9.8	9.3	9.7	19.3
Subject 2	24.2	23.1	23.6	19.8	19.3	18.3	18.1	16.5	12.0	9.7				17.5
Subject 3	25.7	22.6	26.6	17.8	19.0	17.3	16.3	16.0	14.9	11.7	10.6	9.9	8.9	13.2
Subject 4	25.6	24.2		19.6	19.9	17.1	17.3	15.7	14.7					17.3
Subject 5	26.7	25.4	26.1	21.1	21.8	18.1	18.8	15.9	16.0	13.9	11.9	10.6	10.1	17.9
Subject 6	25.4	24.1	25.1	19.6	19.6	17.2	17.5	15.3	15.1					17.1
Subject 7	25.7	24.2	25.4	19.2	18.6	15.7	15.8	13.1	14.0	13.9	12.1	12.5	13.2	19.8
Subject 8	25.9	24.9	25.3	20.0	19.1	17.8	17.9	16.3	15.8	13.3	11.9	11.7	12.3	19.3
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**5**	**5**	**5**	**8**
Mean	**25.5**	**24.3**	**25.2**	**19.5**	**19.6**	**17.3**	**17.4**	**15.5**	**14.9**	**12.5**	**11.3**	**10.8**	**10.8**	**17.9**
SD	**0.9**	**1.2**	**1.1**	**1.1**	**1.1**	**1.0**	**1.1**	**1.3**	**1.7**	**1.6**	**1.0**	**1.3**	**1.8**	**3.1**
SEM	**0.4**	**0.5**	**0.5**	**0.5**	**0.5**	**0.4**	**0.5**	**0.5**	**0.7**	**0.7**	**0.5**	**0.6**	**0.8**	**1.6**

pCO_2 NAC (Torr)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	45.8	39.5	41.1	38.4	37.6	35.5	35.0	32.1	32.4					
Subject 2	42.0	38.3	39.4	39.3	38.2	36.3	35.9	35.1	35.5	32.2	21.3	22.0	21.2	20.3
Subject 3	44.5	39.9	41.1	39.1	38.7	36.3	36.4	35.7	42.7	41.0	32.5	29.8	28.7	31.8
Subject 4		42.5	39.0	40.1	36.7	35.9	33.9	33.0	27.1	30.3				
Subject 5	42.4		40.1	41.4	38.4	37.5	35.6	34.0	32.6	31.7	28.4	29.2	29.0	33.2
Subject 6	41.6	40.1	41.8	41.7	40.5	39.1	38.6			34.2		28.6	30.0	33.8
Subject 7														
Subject 8	43.1	36.8	39.9	40.5	38.9	36.7	35.5	35.4	33.6	36.5	33.1	31.9	30.2	31.3
n	**6**	**6**	**7**	**7**	**7**	**7**	**7**	**6**	**6**	**6**	**4**	**5**	**5**	**5**
Mean	**43.2**	**39.5**	**40.3**	**40.1**	**38.4**	**36.8**	**35.8**	**34.2**	**34.0**	**34.3**	**28.8**	**28.3**	**27.8**	**30.1**
SD	**1.6**	**1.9**	**1.0**	**1.2**	**1.2**	**1.2**	**1.4**	**1.4**	**5.1**	**3.9**	**5.4**	**3.7**	**3.8**	**5.6**
SEM	**0.7**	**0.8**	**0.4**	**0.5**	**0.4**	**0.5**	**0.5**	**0.6**	**2.1**	**1.6**	**2.7**	**1.7**	**1.7**	**2.5**

pCO_2 CON (Torr)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	39.1	41.6	40.0	40.8	39.7	36.7	36.7	34.8	36.8	33.5	28.6	27.8	28.1	35.7
Subject 2	40.5	39.9	39.3	39.1	39.6	36.5	34.9	33.3	32.2	26.2	24.2	28.3	28.3	35.7
Subject 3	41.4	37.7	41.8	39.9	38.9	37.3	36.0	34.9	39.7	37.3	32.5	29.0	29.2	30.9
Subject 4														
Subject 5	41.7	39.3	40.0	40.6	40.6	38.2	38.1	34.7	35.4	34.4	30.8	29.1	29.2	
Subject 6														
Subject 7	41.7	39.2	41.1	38.6	36.9	34.0	32.9	29.7	30.2	31.2	27.3	28.4	31.0	34.9
Subject 8	43.4	40.6	40.9	42.0	38.6	39.5	36.8	35.9	34.0	35.6	32.3	32.9	33.3	36.7
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**5**
Mean	**41.3**	**39.7**	**40.5**	**40.2**	**39.1**	**37.0**	**35.9**	**33.9**	**34.7**	**33.0**	**29.3**	**29.3**	**29.9**	**34.8**
SD	**1.4**	**1.3**	**0.9**	**1.2**	**1.3**	**1.9**	**1.8**	**2.2**	**3.4**	**3.9**	**3.2**	**1.9**	**2.0**	**2.3**
SEM	**0.6**	**0.5**	**0.4**	**0.5**	**0.5**	**0.8**	**0.7**	**0.9**	**1.4**	**1.6**	**1.3**	**0.8**	**0.8**	**1.0**

Plasma Cl^- NAC (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	103	99	103	102	99	101	99	97	101	103	97	101	102	99
Subject 2	106	105		103	103	102	106	103	107	106	103	103	103	106
Subject 3	105	107	104	105	103	105	106	104	108	105	104	103	102	102
Subject 4	103	105	103	104	103	105	103	104	106	108	105	103	101	100
Subject 5	103	102	101	103	101	103	102	103	104	103	102	100	100	101
Subject 6	103	104	102	103	102	104	103	103	105	103		100	99	100
Subject 7	106	106	105	105	105	107	105	107	107	106	105	102	101	102
Subject 8	105	106	105	105	103	105	103	103	106	105	103	100	100	101
n	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
Mean	**104.0**	**103.9**	**103.5**	**103.5**	**102.1**	**103.6**	**103.1**	**102.9**	**105.1**	**104.4**	**102.4**	**101.1**	**102.1**	**101.1**
SD	**1.4**	**2.6**	**1.9**	**1.2**	**1.8**	**1.9**	**2.3**	**3.0**	**2.4**	**1.8**	**2.8**	**1.4**	**1.3**	**2.1**
SEM	**0.5**	**0.9**	**0.7**	**0.4**	**0.6**	**0.7**	**0.8**	**1.1**	**0.9**	**0.6**	**1.0**	**0.5**	**0.5**	**0.8**

Plasma Cl^- CON (mM)

Subjects	Rest	PE1	E1	PE2	E2	PE3	E3	PE4	FAT.	R+1	R+2	R+5	R+10	R+30
Subject 1	105	104	104	101	101	104	104	100	108	105	104	102	105	108
Subject 2	109	102		100	102	105	105	102	107	104	105	111	115	117
Subject 3	101	100	111	98	106	111	96	110	115	98	103	111	109	102
Subject 4	101	102	101	102	101	102	101	103	103	104	103	100	110	98
Subject 5	105	105	106	104	105	107	104	104	108	105	105	105	109	107
Subject 6	103	102	100	102	103	105	102	104	108	104	104	104	108	103
Subject 7	102	106	104	106	104	105	103	105	106	109	107	107	106	107
Subject 8	106	105	105	105	104	104	103	105	107	106	104	101	100	102
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**104.0**	**103.2**	**104.8**	**102.0**	**103.0**	**105.2**	**102.0**	**104.2**	**107.7**	**104.3**	**104.3**	**105.3**	**107.5**	**105.7**
SD	**3.2**	**2.2**	**3.3**	**3.0**	**2.0**	**3.1**	**3.2**	**3.4**	**4.0**	**3.6**	**1.5**	**5.0**	**5.1**	**6.7**
SEM	**1.3**	**0.9**	**1.4**	**1.2**	**0.8**	**1.2**	**1.3**	**1.4**	**1.6**	**1.5**	**0.6**	**2.0**	**2.1**	**2.7**

APPENDIX 9
RAW DATA STUDY 2

Attached a raw data spreadsheets for the following:

- General Information
- Time to fatigue
- Total plasma NAC
- Total blood NAC
- Reduced plasma NAC
- Reduced blood NAC
- Plasma K^+
- $\Delta[K^+]$ to work ratio
- Change in Plasma volume
- Haemoglobin (Hb)
- Haematocrit (Hct)
- Bicarbonate (HCO_3^-)
- Plasma Sodium (Na^+)
- Partial pressure oxygen (PO_2)
- Plasma Hydrogen (H^+)

General Information

Subjects	Age	Weight	Height	VO2	VO2	W/load 70%	W/load 90%
	(yr)	(kg)	(cm)	($l.min^{-1}$)	($kg.min^{-1}$)	(W)	(W)
Subject 1	24	91.15	188	5.36	59.05	228	327
Subject 2	24	76.50	187	3.54	47.28	129	199
Subject 3	20	70.69	180	4.50	64.35	164	263
Subject 4	24	87.10	179	4.20	49.84	153	208
Subject 5	20	66.50	174	4.05	59.62	160	238
Subject 6	18	73.90	177	3.65	50.23	146	220
Subject 7	18	91.20	180	3.71	40.71	154	231
Subject 8	23	65.40	173	3.06	47.59	110	154
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**21.38**	**77.81**	**179.75**	**4.01**	**52.33**	**156**	**230**
SD	**2.67**	**10.64**	**5.44**	**0.70**	**7.90**	**34**	**51**
SEM	**0.94**	**3.76**	**1.92**	**0.25**	**2.79**	**12**	**18**

Time to fatigue

Subjects	Var 1 (min)	Var 2 (min)	NAC (min)	CON (min)	Trial 1 (min)	Trial 2 (min)
Subject 1	11.61	12.61	11.45	5.45	11.45	5.45
Subject 2	15.20	14.18	11.61	16.26	16.26	11.61
Subject 3	24.63	25.63	19.30	11.05	19.30	11.05
Subject 4	13.70	12.43				
Subject 5	21.90	18.52	30.52	15.20	15.20	30.52
Subject 6	13.05	14.26	8.88	14.23	14.23	8.88
Subject 7	14.20	10.20	7.21	7.50	7.21	7.50
Subject 8	17.45	15.15	13.76	20.28	20.28	13.76
n	**8**	**8**	**7**	**7**	**7**	**7**
mean	**16.47**	**15.37**	**14.68**	**12.85**	**14.85**	**12.68**
SD	**4.58**	**4.79**	**7.98**	**5.18**	**4.50**	**8.33**
SEM	**1.62**	**1.69**	**3.02**	**1.96**	**1.70**	**3.15**

Total Plasma NAC ($mg.L^{-1}$)

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	198.03	177.46	169.32	166.16	162.31	158.7	144.23	141.06	128.18	98.338
Subject 2	127.18	108.7	109.79	106.52	96.379	81.162	89.495	105.8	80.437	75.002
Subject 3	167.55	154.31	135.95	185.38	137.02	171.26	146.19	108.76	121.82	77.86
Subject 4	204.59	193.96	178.23	194.75	188.85	166.82	163.87	121.77	98.163	68.065
Subject 5	137.93	135.75	123.43	110.63	109.91	113.53	93.239	87.925	76.331	65.219
Subject 6	93.308	93.573	95.338	101.52	84.304	79.007	128.73	60.028	60.028	45.904
Subject 7	178.03	228.03	198.69	198.69	180.39	166.82	135.34	115.28	103.67	75.54
n	7	7	7	7	7	7	7	7	7	7
mean	**158.09**	**155.97**	**144.39**	**151.95**	**137.02**	**133.9**	**128.73**	**105.8**	**95.518**	**72.275**
SD	**40.422**	**47.675**	**38.359**	**44.064**	**41.579**	**41.607**	**27.756**	**25.858**	**24.79**	**15.767**
SEM	**15.278**	**18.02**	**14.498**	**16.655**	**15.715**	**15.726**	**10.491**	**9.7733**	**9.3697**	**5.9593**

Total Blood NAC ($mg.L^{-1}$)

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	121.84	104.29	107.04	105.69	94.934	92.083	83.512	79.267	73.752	59.329
Subject 2	88.051	68.26	77.633	83.671	64.493	64.267	55.193	51.432	45.938	51.891
Subject 3	121.26	121.86	123.06	122.46	50.33	115.79	115.22	64.23	66.631	63.029
Subject 4	122.33	123.67	120.33	131	121	99.421	88.459	74.322	58.159	42.921
Subject 5	98.772	87.699	85.737	83.881	73.619	78.565	65.116	68.725	58.719	38.25
Subject 6	63.958	69.355	67.231	66.031	98.817	56.158	47.739	51.696	50.646	27.686
Subject 7	109.65	125.33	124.46	115.47	108.27	93.085	90.625	73.875	67.089	58.006
n	7	7	7	7	7	7	7	7	7	7
mean	**103.69**	**100.07**	**100.78**	**101.17**	**87.351**	**85.625**	**77.98**	**66.221**	**60.134**	**48.73**
SD	**21.865**	**25.153**	**23.685**	**23.831**	**25.299**	**20.705**	**23.403**	**11.063**	**9.7764**	**12.905**
SEM	**8.2643**	**9.5069**	**8.952**	**9.0074**	**9.562**	**7.8257**	**8.8453**	**4.1814**	**3.6951**	**4.8778**

Reduced Plasma NAC ($mg.L^{-1}$)

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	91.912	86.721	83.259	93.066	80.952	88.067	79.221	63.454	34.804	16.905
Subject 2	85.334	91.219	73.564	81.656	80.184	61.793	52.23	13.377	8.8276	17.206
Subject 3	105.18	57.373	57.818	62.265	79.307	58.262	61.629	54.704	54.482	31.355
Subject 4	89.53	76.324	76.518	86.034	91.278	86.228	76.712	60.981	40.201	8.5853
Subject 5	67.196	63.312	80.014	82.15	81.956	78.072	71.469	63.312	39.424	8.591
Subject 6	74.922	93.677	88.822	88.917	62.166	61.214	59.31	46.267	43.792	35.605
Subject 7	109	104.14	141.04	135.22	79.307	72.273	30.831	27.777	12.903	2.1972
n	7	7	7	7	7	7	7	7	7	7
mean	**89.011**	**81.824**	**85.863**	**89.901**	**79.307**	**72.273**	**61.629**	**47.125**	**33.491**	**17.206**
SD	**15.043**	**16.94**	**26.204**	**22.254**	**8.6373**	**12.29**	**16.714**	**19.545**	**16.637**	**12.333**
SEM	**5.6856**	**6.4026**	**9.904**	**8.411**	**3.2646**	**4.6451**	**6.3172**	**7.3874**	**6.2884**	**4.6614**

Reduced Blood NAC ($mg.L^{-1}$)

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	52.144	49.228	52.015	52.51	48.393	48.22	42.196	37.554	20.152	13.805
Subject 2	49.215	49.576	56.806	55.773	56.862	38.934	36.683	13.044	10.03	5.0096
Subject 3	82.851	43.932	41.015	49.583	49.583	53.228	37.734	35.729	29.052	24.038
Subject 4	69.098	68.439	72.734	73.699	66.258	63.133	45.041	48.495	14.835	5.6662
Subject 5	50.948	55.889	47.226	48.01	48.778	46.736	40.711	35.497	20.113	5.7557
Subject 6	60.432	56.067	61.716	59.037	47.566	42.974	40.125	27.936	26.216	21.683
Subject 7	76.672	74.53	78.124	89.813	52.907	48.871	16.434	15.477	9.4254	1.3168
n	7	7	7	7	7	7	7	7	7	7
mean	**63.051**	**56.809**	**58.519**	**61.204**	**52.907**	**48.871**	**36.989**	**30.533**	**18.546**	**11.039**
SD	**13.403**	**11.002**	**13.39**	**15.24**	**6.7194**	**7.7554**	**9.4761**	**12.668**	**7.5689**	**8.9216**
SEM	**5.0659**	**4.1584**	**5.061**	**5.7601**	**2.5397**	**2.9313**	**3.5816**	**4.788**	**2.8608**	**3.3721**

Plasma K+ NAC (mM)

	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	3.77	3.83	4.6	4.73	4.57	5.54	4.52	4.05	4.04	4.21	3.97
Subject 2	4.15	4.08	4.79	4.74	4.94	5.31	4.88	4.57	4.15	3.95	4.04
Subject 3	4.22	4.1	4.95	4.96	4.96	5.62	5.08	4.64	4.01	3.81	3.91
Subject 4	3.98	4.11	4.7	4.65	4.84	5.23	4.69	4.45	3.99	3.83	3.79
Subject 5	4	3.91	4.78	4.71	4.58	5.37	4.9	4.39	3.96	3.87	3.92
Subject 6	3.95	3.99	4.65	4.66	4.65	4.84	4.3	4.12	3.8	3.89	3.83
Subject 7	3.93	4.38	4.85	4.89	4.85	5.14	4.45	4.35	4.33	4.29	4.19
n	7	7	7	7	7	7	7	7	7	7	7
mean	**4**	**4.0571**	**4.76**	**4.7629**	**4.77**	**5.29286**	**4.6886**	**4.3671**	**4.04**	**3.9786**	**3.95**
SD	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**	**0.2305**
SEM	**0.0871**	**0.0871**	**0.0871**	**0.0871**	**0.0871**	**0.08712**	**0.0871**	**0.0871**	**0.0871**	**0.0871**	**0.0871**

Plasma K+ CON (mM)

	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	4.18	4.15	4.80	4.77	4.82	5.37	4.78	4.51	4.05	4.01	4.10
Subject 2	4.11	3.72	4.86	4.91	4.98	5.3	4.72	4.69	4.29	4.21	4.17
Subject 3	4.41	4.17	4.76	4.76	4.83	5.68	4.99		3.71	3.89	4.03
Subject 4	4.39	4.64	5.09	4.81	4.94	5.63	5.06	4.76	4.27	4.03	4.02
Subject 5	4.01	4.08	4.88	4.98	4.82	5.54	4.84	4.49	4.33	4.12	4.11
Subject 6	3.87	3.95	4.52	4.41	4.46	4.85	4.46	4.15	3.5	3.6	3.75
Subject 7	4.24	4.29	4.76	4.9	4.85	5.21	4.71	4.57	4.21	4.18	3.99
n	7	7	7	7	7	7	7	6	7	7	7
mean	**4.1717**	**4.1417**	**4.8117**	**4.795**	**4.8133**	**5.36833**	**4.7967**	**4.532**	**4.0517**	**4.005**	**4.0117**
SD	**0.2305**	**0.2947**	**0.1708**	**0.1884**	**0.1738**	**0.28795**	**0.1982**	**0.213**	**0.3238**	**0.2141**	**0.1338**
SEM	**0.0871**	**0.1114**	**0.0645**	**0.0712**	**0.0657**	**0.10883**	**0.0749**	**0.087**	**0.1224**	**0.0809**	**0.0506**

Delta [K+] to work ratio NAC ($nmol.l^{-1}.J^{-1}$)

	E15	E30	E45	Fatigue
Subject 1	6.6322	3.876	2.1246	3.5118
Subject 2	3.46	1.6082	1.397	1.4639
Subject 3	5.7588	2.9133	1.9422	2.0338
Subject 4	4.0972	1.875	1.6898	1.2906
Subject 5	6.621	3.0441	1.6996	2.8548
Subject 6	4.7619	2.417	1.5873	1.6481
Subject 7	4.7475	2.5758	1.5825	1.7919
n	7	7	7	7
mean	**5.1541**	**2.6156**	**1.7176**	**2.085**
SD	**1.2253**	**0.76**	**0.2433**	**0.8088**
sem	**0.4631**	**0.2872**	**0.092**	**0.3057**

Delta [K+] to work ratio CON ($nmol.l^{-1}.J^{-1}$)

	E15	E30	E45	Fatigue
Subject 1	4.6044	2.2756	1.5368	2.1707
Subject 2	5.5556	2.8996	2.0468	2.9127
Subject 3	3.9973	1.9986	1.4905	2.4467
Subject 4	3.125	0.5903	0.6944	1.5253
Subject 5	6.0883	3.4247	1.8772	2.5084
Subject 6	4.1126	1.6595	1.2266	1.7316
Subject 7	4.7475	3.0808	1.8855	1.8993
n	7	7	7	7
mean	**4.6044**	**2.2756**	**1.5368**	**2.1707**
SD	**1.0887**	**1.0635**	**0.511**	**0.5336**
sem	**0.4445**	**0.4342**	**0.2086**	**0.2179**

Change in Plasma Volume (%) NAC

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	1.5499	-9.918	-9.847	-10.59	-14.81	-12.32	-13.2		-5.399	3.338
Subject 2	-0.361	-10.75	-9.142	-8.108	-12.38	-11.31	-11.48	-10.75	-5.427	5.9346
Subject 3	-4.109	-16.71	-12.71	-15.11	-14.08	-13.93	-11.88	-9.03	-4.616	3.593
Subject 4	-1.572	-9.825	-8.718	-7.713	-16.28	-14.04	-14.04	-11.91	-7.426	-1.048
Subject 5	-1.087	-11.19	-10.54	-10.21	-12.42	-13.34	-12.58	-7.12	-4.421	0.6969
Subject 6	-2.11	-10.6	-12.91	-11.89	-14.49	-14.47	-13.45	-10.25	-5.614	3.156
Subject 7	-2.377	-8.074	-2.193	0.1465	-4.505	-2.959	-3.546	2.4357	4.6771	18.487
Subject 8	1.7941	-10.46	-9.181	-8.588	-17.61	-13.98	-7.108	-10.05	-5.609	3.7985
Subject 9	1.9942	2.5139	3.3232	4.4061	3.9766	3.8167	3.6689	4.8781	3.7101	5.9395
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**
mean	**-1.034**	**-10.94**	**-9.405**	**-9.007**	**-13.32**	**-12.04**	**-10.91**	**-8.096**	**-4.229**	**4.7445**
SD	**2.121**	**5.064**	**5.260**	**6.082**	**6.862**	**6.380**	**5.949**	**6.437**	**4.364**	**5.570**
SEM	**0.705**	**0.8888**	**1.1749**	**1.5578**	**1.4059**	**1.3494**	**1.2972**	**1.8438**	**1.3117**	**2.0999**

Change in Plasma Volume (%) CON

	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	-0.355	-6.721	-12.21	-10.18	-15.59	-15.06	-14.19	-10.28	-6.532	4.6033
Subject 2	-2.636	-9.746	-9.578	-8.987	-11.07	-10.75	-12.16	-7.879	-4.516	7.614
Subject 3	-2.757	-12.51	-12.36	-13.66	-16.93	-18.45	-17.53	-17.84	-15.62	-7.855
Subject 4	-3.763	-12.95	-11.29	-9.707	-17.09	-14.5		-12.14	-7.519	0.3571
Subject 5	-2.476	-9.767	-9.334	-6.528	-12.31	-12.24	-10.45	-5.712	-0.203	5.9192
Subject 6	-1.094	-10.92	-10.93	-10.02	-13.07	-12.89	-11.64	-7.417	-2.471	7.2931
Subject 7	-1.941	-8.361	-8.31	-8.31	-10.15	-9.762	-11.26	-7.957	1.5953	10.262
Subject 8	-0.182	-11.84	-10.47	-10.41	-13.62	-12.9	-12.73	-11.96	-6.29	-1.882
Subject 9	1.2574	2.1313	1.4221	2.0358	2.6011	2.7124	2.3795	3.8455	5.2906	5.9945
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**-1.901**	**-10.35**	**-10.56**	**-9.726**	**-13.73**	**-13.32**	**-12.85**	**-10.15**	**-5.194**	**3.2889**
SD	**1.578**	**4.614**	**4.210**	**4.358**	**5.962**	**5.916**	**5.818**	**5.891**	**6.059**	**5.679**
SEM	**0.4446**	**0.7535**	**0.5028**	**0.7198**	**0.9196**	**0.959**	**0.8994**	**1.3596**	**1.8705**	**2.1194**

Hb NAC (g/dl)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	14.9	14.9	15.7	15.6	15.6	15.9	15.8	15.8	15.7	15.3	14.7
Subject 3	14.1	14.5	15.9	15.2	15.6	15.3	15.5	15.0	14.8	14.5	13.8
Subject 4	14.2	14.3	15.0	14.9	14.9	15.5	15.3	15.3	15.1	14.8	14.3
Subject 5	13.4	13.5	14.3	14.3	14.3	14.5	14.6	14.5	14.0	13.8	13.4
Subject 6	15.0	15.1	15.8	16.0	16.0	16.2	16.1	16.1	15.8	15.4	14.7
Subject 7	16.3	16.5	17.0	16.6	16.5	16.7	16.5	16.7	16.1	16.0	15.0
Subject 8	14.6	14.5	15.5	15.4	15.3	16.1	15.7	15.2	15.4	15.1	14.4
n	7	7	7	7	7	7	7	7	6	7	7
mean	**14.7**	**14.7**	**15.6**	**15.5**	**15.5**	**15.8**	**15.7**	**15.6**	**15.3**	**15.0**	**14.4**
SD	**0.8**	**0.9**	**0.8**	**0.7**	**0.7**	**0.7**	**0.6**	**0.7**	**0.7**	**0.7**	**0.5**
SEM	**0.30**	**0.30**	**0.27**	**0.24**	**0.24**	**0.24**	**0.20**	**0.25**	**0.27**	**0.23**	**0.18**

Hb CON (g/dl)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	14.8	14.8	15.5	15.9	15.6	16.1	16.0	15.9	15.5	15.3	14.4
Subject 2	14.7	14.7	15.4	15.4	15.3	15.6	15.4	15.5	15.2	14.8	13.9
Subject 3	14.0	14.2	15.1	15.1	15.3	15.5	15.7	15.7	15.7	15.4	14.7
Subject 4	14.3	14.7	15.4	15.4	15.3	15.8	15.5		15.2	14.8	14.3
Subject 5	14.1	14.3	15.0	14.9	14.7	15.2	15.1	15.0	14.6	14.0	13.7
Subject 6	15.4	15.6	16.3	16.5	16.3	16.5	16.5	16.4	16.0	15.7	14.9
Subject 7	15.1	15.2	15.9	15.8	15.8	16.0	15.9	16.2	15.8	15.0	14.2
Subject 8	14.7	14.7	15.7	15.7	15.6	15.9	15.8	15.8	15.6	15.2	14.9
n	8	8	8	8	8	8	8	7	8	8	8
mean	**14.6**	**14.8**	**15.5**	**15.6**	**15.5**	**15.8**	**15.7**	**15.8**	**15.5**	**15.0**	**14.4**
SD	**0.5**	**0.5**	**0.4**	**0.5**	**0.5**	**0.4**	**0.4**	**0.5**	**0.4**	**0.5**	**0.4**
SEM	**0.17**	**0.16**	**0.15**	**0.18**	**0.16**	**0.14**	**0.15**	**0.17**	**0.16**	**0.18**	**0.16**

Hct NAC (%)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	44.6	44.8	47.9	47.3	46.7	48.2	47.9	48.0	47.9	46.2	42.1
Subject 3	42.4	43.2	45.9	45.8	45.9	46.3	45.5	46.0	45.0	43.5	41.6
Subject 4	43.1	43.6	45.8	45.5	44.9	48.0	47.3	47.3	46.7	45.1	43.3
Subject 5	42.6	42.8	45.6	45.2	45.0	45.6	45.8	45.7	44.3	43.5	42.2
Subject 6	45.1	45.9	48.3	49.0	48.4	49.3	49.6	49.0	48.1	46.8	44.5
Subject 7	49.1	49.7	51.2	49.3	48.4	50.2	50.0	49.7	48.5	47.7	44.5
Subject 8	45.3	44.7	48.0	47.6	47.6	50.3	49.4	47.1	48.1	46.6	44.0
n	7	7	7	7	7	7	7	7	6	7	7
mean	**44.5**	**44.8**	**47.4**	**47.0**	**46.7**	**48.2**	**47.9**	**47.5**	**46.9**	**45.6**	**43.2**
SD	**2.2**	**2.2**	**1.9**	**1.6**	**1.4**	**1.7**	**1.7**	**1.4**	**1.7**	**1.5**	**1.1**
SEM	**0.77**	**0.79**	**0.67**	**0.55**	**0.49**	**0.61**	**0.60**	**0.48**	**0.63**	**0.54**	**0.40**

Hct CON (%)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	43.7	43.9	45.0	46.9	46.7	48.3	48.3	48.1	47.1	45.6	42.7
Subject 2	43.1	44.6	46.2	46.1	46.1	46.3	46.8	47.3	45.8	45.3	42.1
Subject 3	41.5	42.3	44.8	44.7	44.8	46.2	46.5	45.9	46.1	45.7	43.4
Subject 4	44.0	44.6	47.5	46.5	45.9	48.7	48.1		47.7	46.4	43.8
Subject 5	45.1	45.7	47.3	47.4	46.5	45.1	48.4	47.7	46.4	45.6	43.5
Subject 6	47.5	47.4	50.5	49.9	50.0	51.1	51.0	50.6	49.5	47.8	45.5
Subject 7	45.8	46.5	47.7	48.0	48.0	48.4	48.5	48.4	47.8	45.3	43.8
Subject 8	45.2	45.3	48.4	47.6	47.9	48.8	48.7	48.6	48.8	46.9	45.5
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**44.5**	**45.0**	**47.2**	**47.1**	**47.0**	**47.9**	**48.3**	**48.1**	**47.4**	**46.1**	**43.8**
SD	**1.8**	**1.6**	**1.9**	**1.5**	**1.6**	**1.9**	**1.4**	**1.4**	**1.3**	**0.9**	**1.2**
SEM	**0.65**	**0.56**	**0.66**	**0.54**	**0.57**	**0.67**	**0.48**	**0.54**	**0.46**	**0.31**	**0.43**

HCO_3^- NAC (mM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	27.1	25.1	21.0	21.0	22.8	17.2	17.8	18.3	19.4	21.5	25.0
Subject 3	26.5	25.6	22.5	22.4	21.5	19.5	19.0	19.8	20.6	22.6	24.9
Subject 4	25.9	24.5	23.0	23.9	25.9	15.6	16.0	16.7	15.4	18.8	25.3
Subject 5	26.5	25.0	24.0	24.6	24.9	20.8	22.2	23.8	23.0	23.7	25.1
Subject 6	26.9	25.8	21.8	20.7	21.4	16.6	16.5	16.3	17.5	18.2	21.3
Subject 7	25.9	24.9	23.1	23.6	24.4	20.2	21.0	21.1	21.8	22.9	23.8
Subject 8	25.7	25.1	22.7	23.3	23.8	18.8	18.6	19.6	20.1	21.3	24.4
n	7	7	7	7	7	7	7	7	6	7	7
mean	**26.0**	**25.0**	**22.4**	**22.4**	**23.2**	**18.0**	**18.1**	**18.8**	**19.7**	**20.8**	**24.1**
SD	**1.0**	**0.5**	**1.1**	**1.8**	**1.8**	**2.2**	**2.8**	**3.0**	**2.6**	**2.3**	**1.4**
SEM	**0.36**	**0.19**	**0.39**	**0.62**	**0.64**	**0.77**	**0.98**	**1.05**	**0.97**	**0.83**	**0.48**

HCO_3^- CON (mM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	**26.4**	**25.4**	**23.1**	**23.0**	**23.9**	**18.0**	**19.1**	**19.1**	**19.5**	**20.3**	**23.1**
Subject 2	26.1	24.2	22.9	22.1	23.9	19.2	20.2	21.0	20.1	22.5	25.7
Subject 3	26.2	22.7	22.4	22.5	23.1	16.5	19.8	18.5	24.5	20.4	20.5
Subject 4	26.3	24.5	21.3	22.8	23.9	15.1	15.7		16.8	18.3	24.4
Subject 5	26.4	26.3	23.7	24.3	25.0	21.1	21.5	22.0	22.4	22.8	26.3
Subject 6	28.4	27.1	24.5	22.2	22.8	15.1	17.1	14.2	13.0	14.3	16.2
Subject 7	26.4	27.0	24.3	23.2	23.5	18.5	18.6	17.3	19.0	21.6	23.8
Subject 8	24.8	25.7	22.8	23.8	24.8	20.2	21.0	21.4	20.9	22.2	25.0
n	8	8	8	8	8	8	8	7	8	8	8
mean	**26.4**	**25.4**	**23.1**	**23.0**	**23.9**	**18.0**	**19.1**	**19.1**	**19.5**	**20.3**	**23.1**
SD	**1.2**	**1.6**	**1.1**	**0.8**	**0.8**	**2.4**	**2.1**	**3.0**	**3.8**	**3.1**	**3.6**
SEM	**0.47**	**0.61**	**0.43**	**0.31**	**0.31**	**0.92**	**0.80**	**1.22**	**1.43**	**1.16**	**1.36**

Plasma Na^+ NAC (mM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	136.1	140.5	139.3	142.8	140.1	141.8	139.8	138.9	139.3	139.1	136.7
Subject 3	138.8	139.1	142.8	143.1	143.2	144.3	142.2	142.6	140.8	141.3	139.1
Subject 4	137.0	138.0	139.7	138.7	140.6	143.8	140.8	140.5	138.8	140.0	140.3
Subject 5	139.0	138.4	142.9	139.0	141.4	139.2	138.9	138.1	138.0	138.0	136.7
Subject 6	137.9	139.3	138.8	139.0	139.9	143.5	140.8	136.8	142.4	134.9	138.7
Subject 7	143.2	142.8	144.5	141.9	141.8	143.4	141.7	141.9	141.1	140.2	138.4
Subject 8	141.8	142.0	144.1	144.4	143.9	146.0	143.3	143.5	143.5	142.8	141.8
n	7	7	7	7	7	7	7	7	6	7	7
mean	**139.1**	**139.9**	**141.8**	**141.4**	**141.6**	**143.4**	**141.1**	**140.4**	**140.6**	**139.4**	**138.9**
SD	**2.4**	**1.7**	**2.2**	**2.2**	**1.4**	**2.1**	**1.4**	**2.3**	**2.0**	**2.3**	**1.7**
SEM	**0.84**	**0.61**	**0.79**	**0.77**	**0.51**	**0.73**	**0.48**	**0.82**	**0.75**	**0.83**	**0.60**

Plasma Na^+ CON (mM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	**139.2**	**138.8**	**140.5**	**141.1**	**140.4**	**143.0**	**140.2**	**140.3**	**139.1**	**140.2**	**138.5**
Subject 2	136.7	136.7	137.0	138.6	139.2	139.6	137.7	138.3	135.6	137.0	135.9
Subject 3	133.7	136.3	140.1	139.6	139.5	142.5	138.9	140.4	136.4	140.0	137.6
Subject 4	137.6	137.5	140.1	142.1	142.9	145.0	141.9		141.3	141.6	140.7
Subject 5	142.1	143.4	144.6	139.6	140.6	144.7	140.4	141.8	140.5	141.0	137.1
Subject 6	139.8	138.0	138.4	141.2	137.7	141.4	139.2	138.6	138.2	137.7	137.5
Subject 7	142.6	141.4	144.2	144.0	143.3	144.6	142.3	139.8	140.3	143.1	140.5
Subject 8	141.7	138.0	139.3	142.5	139.3	143.1	141.2	142.7	141.7	141.1	140.1
n	8	8	8	8	8	8	8	7	8	8	8
mean	**139.2**	**138.8**	**140.5**	**141.1**	**140.4**	**143.0**	**140.2**	**140.3**	**139.1**	**140.2**	**138.5**
SD	**3.3**	**2.6**	**2.9**	**1.9**	**2.1**	**2.0**	**1.7**	**1.7**	**2.4**	**2.2**	**1.9**
SEM	**1.25**	**0.99**	**1.08**	**0.73**	**0.78**	**0.75**	**0.64**	**0.71**	**0.92**	**0.82**	**0.72**

PO_2 NAC (Torr)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	83.6	94.7	94.8	90.7	87.4	89.4	104.2	111.1	108.6	95.7	93.0
Subject 3	74.0	80.7	82.7	85.2	82.7	74.9	96.1	91.6	94.6	95.2	86.3
Subject 4	85.8	82.5	93.3	88.6	73.4	76.3	83.0	71.5	98.5	87.2	73.1
Subject 5	89.1	84.6	81.7	84.5	77.5	72.8	85.8	78.8	77.4	78.2	82.6
Subject 6	74.9	76.0	81.5	84.0	85.6	84.0	88.0	105.7	97.5	80.5	66.7
Subject 7	75.4	81.7	77.4	86.1	73.6	76.8	82.6	91.0	93.9	87.6	83.1
Subject 8	80.1	78.0	83.1	77.5	72.3	72.1	82.5	79.6	80.6	70.2	58.8
n	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**6**	**7**	**7**
mean	**79.1**	**85.8**	**85.8**	**85.9**	**81.2**	**79.6**	**90.3**	**91.1**	**93.0**	**85.6**	**78.0**
SD	**6.6**	**10.7**	**6.5**	**4.2**	**8.6**	**7.3**	**8.7**	**13.9**	**10.8**	**8.8**	**11.1**
SEM	**2.32**	**3.78**	**2.29**	**1.50**	**3.05**	**2.57**	**3.07**	**4.90**	**4.07**	**3.11**	**3.94**

PO_2 CON (Torr)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	70.2	108.4	91.5	90.2	97.0	90.2	100.4	99.2		90.4	80.5
Subject 2	89.3	96.6	89.7	85.0	81.3	85.1	100.4	102.1	98.3	92.5	90.4
Subject 3	79.1	84.9	85.4	79.4	76.9	78.6	96.3	101.4	75.7	95.6	97.4
Subject 4	82.0	78.4	83.8	80.6	88.6	86.7	90.2		97.4	85.2	82.7
Subject 5	74.9	83.8	88.5	84.2	82.7	74.8	77.5	90.6	89.1	86.0	86.1
Subject 6	64.6	53.5	86.8	78.0	74.1	80.3	87.6	95.0	90.2	67.9	60.8
Subject 7	65.7	75.7	77.9	81.1	87.9	74.7	78.8	90.7	91.4	85.2	93.5
Subject 8	68.5	71.5	87.9	81.5	81.8	84.0	95.3	96.1	95.9	78.3	68.1
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**
mean	**74.9**	**77.8**	**85.7**	**81.4**	**81.9**	**80.6**	**89.4**	**96.0**	**91.1**	**84.4**	**82.7**
SD	**10.1**	**13.4**	**4.0**	**2.7**	**5.8**	**4.9**	**8.8**	**5.0**	**7.7**	**9.2**	**14.8**
SEM	**4.11**	**5.05**	**1.50**	**1.11**	**2.36**	**1.84**	**3.31**	**2.04**	**2.91**	**3.47**	**6.05**

Plasma H^+ NAC (nM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1											
Subject 2	37.9	35.7	41.2	40.8	37.8	43.6	50.1	46.2	44.3	42.4	38.7
Subject 3	41.4	40.2	44.8	42.9	43.1	45.6	46.8	44.7	46.9	42.8	40.6
Subject 4	39.9	39.0	41.4	40.9	40.6	52.1	52.8	54.7	50.4	47.4	42.0
Subject 5	40.3	40.8	43.3	40.9	40.2	41.1	42.5	42.8	44.4	41.9	39.8
Subject 6	39.6	37.6	43.5	43.8	41.6	47.0	49.0	46.6	50.1	45.3	38.6
Subject 7	41.3	35.9	41.2	39.0	41.3	42.5	45.4	43.6	42.6	41.7	41.7
Subject 8	39.5	40.9	42.5	41.7	40.8	49.3	49.4	49.9	48.3	45.7	41.4
n	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**7**	**7**
mean	**39.1**	**37.8**	**42.4**	**41.5**	**40.7**	**46.0**	**48.2**	**47.2**	**46.7**	**43.7**	**40.0**
SD	**2.8**	**3.0**	**1.3**	**1.5**	**1.5**	**3.6**	**3.2**	**4.0**	**3.1**	**2.1**	**1.7**
SEM	**1.0**	**1.1**	**0.5**	**0.5**	**0.5**	**1.3**	**1.1**	**1.4**	**1.2**	**0.7**	**0.6**

Plasma H^+ CON (nM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	32.8	32.4	41.4	42.4	39.8	47.1	49.4	49.4		42.8	37.2
Subject 2	38.6	37.2	39.6	39.1	37.7	39.7	42.2	43.9	40.4	40.6	37.8
Subject 3	40.8	39.4	43.9	42.0	40.6	47.3	43.3	46.7	40.4	42.1	40.5
Subject 4	38.0	37.6	41.0	38.5	36.7	47.4	49.2		50.2	44.5	39.1
Subject 5	38.3	38.2	43.8	40.9	41.5	41.5	43.8	44.1	43.9	42.5	40.2
Subject 6	38.9	39.3	44.3	44.5	43.4	50.9	48.8	59.3	66.1	63.1	60.1
Subject 7	40.4	38.5	40.9	39.4	39.0	45.4	45.4	46.9	48.6	43.7	38.5
Subject 8	38.8	38.5	40.6	40.9	39.7	44.1	39.9	44.0	45.9	44.7	42.2
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**
mean	**39.1**	**38.4**	**42.0**	**40.8**	**39.8**	**45.2**	**44.6**	**47.5**	**47.9**	**45.9**	**42.6**
SD	**1.1**	**0.8**	**1.9**	**2.0**	**2.3**	**3.8**	**3.4**	**6.0**	**8.9**	**7.7**	**7.8**
SEM	**0.4**	**0.3**	**0.7**	**0.8**	**0.9**	**1.4**	**1.3**	**2.4**	**3.3**	**2.9**	**3.0**

APPENDIX 10
RAW DATA STUDY 3 – PART I

Attached a raw data spreadsheets for the following:

- General information
- Time to fatigue
- Coefficient of variance for variability trials
- 45 min biopsy sampling time
- Muscle NAC
- Muscle glutathione
- Muscle cysteine
- Muscle cystine
- Plasma reduced NAC
- Plasma total NAC
- Blood reduced NAC
- Blood total NAC
- Blood total glutathione (TGSH)
- Blood reduced glutathione (GSH)
- Blood calculated oxidised glutathione (GSSG)
- Plasma cysteine reduced
- Blood cysteine reduced
- Plasma cysteine total
- Plasma cysteine reduced
- Plasma total cystine
- Blood total cystine

GENERAL INFORMATION:

Subjects	Age	Weight	Height	VO2	VO2	NAC	CON
	(years)	(kg)	(cm)	($l.min^{-1}$)	($kg.min^{-1}$)	(min)	(min)
Subject 1	29	80.1	182	5.6	68.1	7.5	8.1
Subject 2	21	66.0	177	3.9	59.2	6.8	4.9
Subject 3	34	85.6	184	5.1	59.1	7.5	5.8
Subject 4	31	96.1	190	6.7	70.7	4.4	3.3
Subject 5	20	64.9	175	3.9	60.8	5.5	5.9
Subject 6	32	77.8	180	5.7	73.5	4.7	3.1
Subject 7	29	77.0	181	4.7	61.1	8.3	5.7
n	7	7	7	7	7	7	7
mean	**28.0**	**78.2**	**181.3**	**5.1**	**64.6**	**6.4**	**5.3**
SD	**5.4**	**10.8**	**4.9**	**1.0**	**6.0**	**1.5**	**1.7**
sem	**2.0**	**4.1**	**1.8**	**0.4**	**2.3**	**0.6**	**0.7**

TIME TO FATIGUE:

Subjects	Famil	Var 1	Var 2	Trial 1	Trial 2	NAC	CON
	(min)	(min)	(min)	(min)	(min)	(min)	(min)
Subject 1	7.0	5.3	5.9	8.1	7.5	7.5	8.1
Subject 2	3.1	4.9	5.4	6.8	4.9	6.8	4.9
Subject 3	5.0	6.1	5.6	5.8	7.5	7.5	5.8
Subject 4	3.9	5.1	4.7	4.4	3.3	4.4	3.3
Subject 5	4.6	3.3	3.6	5.5	5.9	5.5	5.9
Subject 6	3.5	4.2	4.6				
Subject 7	5.5	5.1	5.2	4.7	3.1	4.7	3.1
Subject 8	3.1	4.8	5.1	5.7	8.3	8.3	5.7
n	**8**	**8**	**8**	**7**	**7**	**7**	**7**
mean	**4.4**	**4.8**	**5.0**	**5.9**	**5.8**	**6.4**	**5.3**
SD	**1.3**	**0.8**	**0.7**	**1.3**	**2.1**	**1.5**	**1.7**
sem	**0.5**	**0.3**	**0.3**	**0.5**	**0.8**	**0.6**	**0.7**

COEFFECIENT OF VARIANCE:

Subjects	Var 1 (min)	Var 2 (min)	SD (min)	MEAN (min)	CV	CV (%)	VARIANCE
Subject 1	5.32	5.91	0.42	5.62	0.07	7.43	0.1741
Subject 2	4.94	5.40	0.33	5.17	0.06	6.29	0.1058
Subject 3	6.06	5.62	0.31	5.84	0.05	5.33	0.0968
Subject 4	5.05	4.67	0.27	4.86	0.06	5.53	0.0722
Subject 5	3.25	3.56	0.22	3.41	0.06	6.44	0.0480
Subject 6	4.15	4.58	0.30	4.37	0.07	6.97	0.0925
Subject 7	5.06	5.21	0.11	5.14	0.02	2.07	0.0112
Subject 8	4.79	5.13	0.24	4.96	0.05	4.85	0.0578
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**4.8**	**5.0**	**0.3**	**4.9**	**0.1**	**5.6**	**0.1**
SD	**0.8**	**0.7**	**0.1**	**0.8**	**0.0**	**1.7**	**0.0**
sem	**0.3**	**0.3**	**0.0**	**0.3**	**0.0**	**0.6**	**0.0**

BIOPSY TIME

Subjects	Trial 1 (s)	Trial 2 (s)	NAC (s)	CON (s)
Subject 1	39	33	33	39
Subject 2	38	42	38	42
Subject 3	56	47	56	47
Subject 4	71	80	71	80
Subject 5	96	89	89	96
Subject 6	47	54	47	52
Subject 7	43	41	43	41
Subject 8	48	47	47	48
n	**8**	**8**	**8**	**8**
mean	**54.8**	**54.1**	**53.0**	**55.6**
SD	**19.8**	**19.8**	**18.6**	**20.9**
sem	**7.0**	**7.0**	**6.6**	**7.4**

Muscle NAC (mg/kg wet wt)

NAC TOTAL	REST	45 MIN	FATIGUE
Subject 1	0	9.9	9.8
Subject 2	0	9.6	10.3
Subject 3	0	9.1	12.2
Subject 4	0	11.2	14.6
Subject 5	0	42.6	44.1
Subject 6	0	21.3	24.5
Subject 7	0	24.3	13.9
Subject 8	0	22.8	25.2
n	**8**	**8**	**8**
mean	**0**	**18.9**	**19.3**
SD	**0**	**11.6**	**11.6**
SEM	**0**	**4.1**	**4.1**

NAC REDUCED	REST	45 MIN	FATIGUE
Subject 1	0	5.8	7.2
Subject 2	0	8.5	8.1
Subject 3	0	7.2	10.6
Subject 4	0	11.2	8.5
Subject 5	0	20.0	16.0
Subject 6	0	9.8	12.9
Subject 7	0	22.6	10.6
Subject 8	0	16.1	22.1
n	**8**	**8**	**8**
mean	**0**	**12.7**	**12.0**
SD	**0**	**6.2**	**5.0**
SEM	**0**	**2.2**	**1.8**

Muscle Glutathione (nmol/g wet wt)

NAC TOTAL	REST	45 MIN	FAT
Subject 1	443	511	330
Subject 2	430	409	449
Subject 3	454	392	431
Subject 4	583	718	504
Subject 5	400	376	387
Subject 6	664	513	593
Subject 7	568	357	459
Subject 8	506	467	450
n	**8**	**8**	**8**
mean	**506.0**	**468.0**	**450.4**
SD	**98.7**	**126.8**	**83.8**
SEM	**37.3**	**47.9**	**31.7**

NAC REDUCED	REST	45 MIN	FAT
Subject 1	423	424	321
Subject 2	337	326	399
Subject 3	400	356	395
Subject 4	438	554	367
Subject 5	396	337	258
Subject 6	591	497	587
Subject 7	511	348	445
Subject 8	443	406	395
n	**8**	**8**	**8**
mean	**442.3**	**406.0**	**396.0**
SD	**83.9**	**89.0**	**103.6**
SEM	**31.7**	**33.6**	**39.2**

NAC OXIDISED	REST	45 MIN	FAT
Subject 1	20	87	9
Subject 2	93	83	50
Subject 3	54	36	36
Subject 4	145	164	137
Subject 5	4	39	129
Subject 6	73	16	6
Subject 7	57	9	14
Subject 8	62	63	55
n	**8**	**8**	**8**
mean	**63.7**	**62.0**	**54.4**
SD	**46.8**	**54.2**	**55.9**
SEM	**17.7**	**20.5**	**21.1**

CON TOTAL	REST	45 MIN	FAT
Subject 1	273	374	341
Subject 2	414	249	317
Subject 3	557	511	372
Subject 4	667	490	390
Subject 5	398	378	297
Subject 6	662	420	514
Subject 7	523	241	420
Subject 8	498	380	393
n	**8**	**8**	**8**
mean	**499.1**	**380.4**	**393.0**
SD	**145.6**	**106.0**	**80.9**
SEM	**55.0**	**40.1**	**30.6**

CON REDUCED	REST	45 MIN	FAT
Subject 1	265	357	325
Subject 2	340	204	235
Subject 3	478	430	368
Subject 4	491	377	336
Subject 5	343	344	240
Subject 6	587	386	501
Subject 7	445	227	353
Subject 8	421	333	336
n	**8**	**8**	**8**
mean	**421.3**	**332.1**	**336.9**
SD	**110.5**	**84.4**	**89.5**
SEM	**41.8**	**31.9**	**33.8**

CON OXIDISED	REST	45 MIN	FAT
Subject 1	8	17	16
Subject 2	74	45	82
Subject 3	79	81	104
Subject 4	176	113	54
Subject 5	55	34	57
Subject 6	75	34	13
Subject 7	78	14	67
Subject 8	78	49	56
n	**8**	**8**	**8**
mean	**77.9**	**48.3**	**56.1**
SD	**50.1**	**36.1**	**33.1**
SEM	**18.9**	**13.7**	**12.5**

Muscle Cysteine (nmol/g wet wt)

NAC TOTAL	REST	45 MIN	FAT
Subject 1	2.9	21.3	17.7
Subject 2	4	21.5	20.6
Subject 3	4.4	18.6	20.2
Subject 4	3.4	19.9	15.4
Subject 5	5	27.1	27.8
Subject 6	14.7	31.6	34
Subject 7	8.2	22.7	22.3
Subject 8	6.3	23.8	22.9
n	**8**	**8**	**8**
mean	**6.1**	**23.2**	**22.6**
SD	**4.2**	**4.6**	**6.4**
SEM	**1.6**	**1.7**	**2.4**

NAC REDUCED	REST	45 MIN	FAT
Subject 1	2.6	18	14.7
Subject 2	3.5	17.2	19.3
Subject 3	2.8	16	16.7
Subject 4	2.6	16	12.1
Subject 5	4.6	22.6	20.1
Subject 6	7.3	22	25.3
Subject 7	5.9	18.1	18.1
Subject 8	4.3	18.7	17.9
n	**8**	**8**	**8**
mean	**4.2**	**18.6**	**18.0**
SD	**1.8**	**2.7**	**4.2**
SEM	**0.7**	**1.0**	**1.6**

NAC OXIDISED	REST	45 MIN	FAT
Subject 1	0.3	3.3	3
Subject 2	0.5	4.3	1.3
Subject 3	1.6	2.6	3.5
Subject 4	0.8	3.9	3.3
Subject 5	0.4	4.5	7.7
Subject 6	7.4	9.6	8.7
Subject 7	2.3	4.6	4.2
Subject 8	2.0	5.1	5.0
n	**8**	**8**	**8**
mean	**1.9**	**4.7**	**4.5**
SD	**2.5**	**2.3**	**2.7**
SEM	**1.0**	**0.9**	**1.0**

CON TOTAL	REST	45 MIN	FAT
Subject 1	3.8	4.3	4.3
Subject 2	4.3	4	5
Subject 3	4.8	4.1	3.7
Subject 4	3	3.4	4.3
Subject 5	3.4	4.3	2.8
Subject 6	7.8	7.2	8.3
Subject 7	5	7.7	5.3
Subject 8	4.7	4.9	4.9
n	**8**	**8**	**8**
mean	**4.6**	**5.0**	**4.8**
SD	**1.6**	**1.7**	**1.7**
SEM	**0.6**	**0.6**	**0.7**

CON RED	REST	45 MIN	FAT
Subject 1	3	3.8	3.1
Subject 2	3.4	3	3.4
Subject 3	3.9	3.1	2.6
Subject 4	2.4	2.8	2.9
Subject 5	2.7	4	2.3
Subject 6	5.1	4.4	5.4
Subject 7	4.1	6.3	4
Subject 8	3.4	3.8	3.5
n	**8**	**8**	**8**
mean	**3.5**	**3.9**	**3.4**
SD	**0.9**	**1.2**	**1.0**
SEM	**0.4**	**0.5**	**0.4**

CON RED	REST	45 MIN	FAT
Subject 1	0.8	0.5	1.2
Subject 2	0.9	1	1.6
Subject 3	0.9	1	1.1
Subject 4	0.6	0.6	1.4
Subject 5	0.7	0.3	0.5
Subject 6	2.7	2.8	2.9
Subject 7	0.9	1.4	1.3
Subject 8	1.3	1.1	1.4
n	**8**	**8**	**8**
mean	**1.1**	**1.1**	**1.4**
SD	**0.7**	**0.8**	**0.7**
SEM	**0.3**	**0.3**	**0.3**

Plasma Reduced NAC ($mg.L^{-1}$)

	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	1.06	45.51	99.58	163.64	241.56	155.84	136.21	142.13	111.58	110.03	127.64	145.71	97.03	86.87	67.25	44.10
Subject 2	9.54	56.79	90.65	153.26	278.66	171.78	164.73	150.79	133.51	150.62	97.88	106.88	95.59	93.12	76.19	53.96
Subject 3	3.85	7.93	84.01	181.76	183.11	144.59	153.60	140.09	117.79	119.37	123.92	112.24	110.45	104.46	97.12	72.07
Subject 4	1.38	21.83	52.35	103.29	133.02	84.51	90.77	78.25	96.24	78.01	82.94	85.46	76.76	48.75	46.56	36.38
Subject 5	9.71	49.84	116.67	202.27	216.83	87.54	115.70	147.41	105.83	104.76	95.31	95.95	102.69	83.07	86.08	56.80
Subject 6	4.12	35.32	57.97	80.38	118.99	81.65	164.06	143.28	128.24	199.16	170.15	138.63	152.60	146.87	80.14	65.91
Subject 7	4.69	96.71	126.76	165.26	170.89	164.32	147.42	128.64	106.10	148.36	128.64	131.46		117.37	99.53	42.63
Subject 8	3.25	30.83	108.11	171.17	123.12	117.12	103.10	73.87	79.68	109.11	110.11	116.31	140.14	97.36	88.29	59.96
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
Mean	**4.70**	**43.09**	**92.01**	**152.63**	**183.27**	**125.92**	**134.45**	**125.56**	**109.87**	**127.43**	**117.07**	**116.62**	**110.80**	**97.22**	**80.14**	**53.98**
SD	**3.56**	**26.76**	**26.54**	**40.69**	**58.62**	**37.89**	**28.22**	**31.24**	**17.28**	**37.38**	**27.16**	**22.59**	**29.17**	**30.54**	**18.60**	**13.22**
SEM	**1.34**	**9.46**	**9.38**	**14.39**	**20.72**	**13.39**	**9.98**	**11.05**	**6.11**	**13.22**	**9.60**	**8.54**	**11.91**	**11.54**	7.03	**5.00**

Plasma Total NAC ($mg.L^{-1}$)

	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	1.87	63.80	166.36	247.75	390.53	276.16	258.36	239.62	198.20	162.70	237.58	250.91	189.38	159.38	158.02	92.87
Subject 2	15.76	85.87	119.79	212.91	375.70	273.43	250.96	228.18	185.81	216.21	219.45	222.36	212.53	126.55	125.45	91.08
Subject 3	7.31	12.62	123.89	287.53	369.60	246.15	268.24	275.36	218.50	241.25	254.31	268.87	227.62	218.91	194.17	134.10
Subject 4	2.43	37.50	95.99	172.33	197.92	148.86	115.82	123.99	133.45	143.01	135.33	145.43	127.99	74.63	80.37	51.26
Subject 5	17.32	85.62	225.27	415.11	473.94	156.60	222.89	205.39	213.39	223.62	229.24	182.94	274.18	220.61	152.77	118.82
Subject 6	7.39	58.25	212.00	353.91	220.25	191.04	202.15	212.22	273.95	273.68	221.45	207.44	253.46	187.18	144.60	74.15
Subject 7	7.95	60.89	86.80	133.96	193.66	168.46	216.34	206.14	214.67	246.75	272.05	229.03	222.10	240.91	215.99	65.95
Subject 8	7.39	58.25	212.00	353.91	220.25	191.04	182.41	155.72	154.38		200.14		261.77	246.95	154.61	112.18
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**8.43**	**57.85**	**155.26**	**272.18**	**305.23**	**206.47**	**214.65**	**205.85**	**199.04**	**215.32**	**221.44**	**214.11**	**222.40**	**184.39**	**157.22**	**92.50**
SD	**5.55**	**24.09**	**55.94**	**97.99**	**109.06**	**51.61**	**49.48**	**56.08**	**42.95**	**46.79**	**48.64**	**45.41**	**55.19**	**60.52**	**42.65**	**30.43**
SEM	**1.96**	**8.52**	**19.78**	**34.64**	**38.56**	**18.25**	**17.49**	**22.89**	**15.19**	**17.68**	**19.86**	**18.54**	**22.53**	**21.40**	**16.12**	**11.50**

Blood Reduced NAC ($mg.L^{-1}$)

	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	1.91	23.96	67.32	136.89	97.06	148.11	89.03	85.38	72.94	72.11	77.37	83.59	62.84	54.06	42.57	29.79
Subject 2	8.44	33.40	37.59	119.37	223.49	102.86	108.57	95.08	84.57	92.30	64.40	69.38	62.48	61.24	51.54	38.29
Subject 3	5.35	8.84	48.16	154.05	116.76	125.68	93.78	83.46	69.92	71.87	72.81	66.35	71.35	61.73	58.73	42.65
Subject 4	6.14	12.87	27.18	66.48	111.27	40.85	60.85	47.46	59.62	49.01	51.55	52.62	47.86	33.51	32.46	25.55
Subject 5	5.30	29.30	94.95	89.71	99.61	48.17	73.36	74.32	67.15	68.17	59.71	59.07	60.16	48.30	67.57	34.54
Subject 6	5.09	83.82	137.91	168.72	252.18	154.39	102.75	90.92	83.89	117.38	99.30	85.82	66.14	87.72	59.30	39.27
Subject 7	23.83	53.75	74.37	148.56	128.28	74.70	95.11	79.52	68.45	86.52	74.62	75.66	87.89	72.34	54.21	28.62
Subject 8	7.68	20.43	60.18	136.58	149.55	81.44	68.47	41.44	49.37	63.06	62.70	65.12	69.55	54.41	71.71	55.14
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**7.97**	**33.30**	**68.46**	**127.54**	**147.27**	**97.03**	**86.49**	**74.75**	**69.49**	**77.55**	**70.31**	**70.36**	**66.02**	**59.16**	**54.84**	**36.73**
SD	**6.70**	**24.65**	**35.25**	**34.30**	**58.80**	**43.16**	**17.07**	**21.37**	**11.68**	**20.90**	**14.50**	**12.26**	**12.29**	**16.15**	**13.81**	**9.46**
SEM	**2.37**	**8.72**	**12.46**	**12.13**	**20.79**	**15.26**	**6.04**	**8.08**	**4.13**	**7.39**	**5.13**	**4.63**	**4.64**	**5.71**	**5.22**	**3.34**

Blood Total NAC ($mg.L^{-1}$)

	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	4.03	36.73	123.65	219.78	170.19	256.28	155.84	148.01	125.82	111.21	143.76	149.93	95.85	100.48	100.40	52.80
Subject 2	14.87	59.63	77.41	166.09	381.74	185.21	162.08	145.90	121.57	139.49	140.23	75.94	82.46	80.36	94.15	62.37
Subject 3	11.30	13.98	81.03	272.01	245.58	252.79	163.30	161.06	129.47	139.92	146.17	159.42	135.54	125.69	114.41	81.31
Subject 4	11.23	22.38	46.92	111.73	197.98	74.61	81.62	79.65	84.19	90.11	90.36	88.67	78.50	53.40	52.90	32.78
Subject 5	9.95	61.71	205.85	153.72	169.80	79.34	143.06	103.26	141.75	142.67	138.89	105.06	172.15	128.41	97.50	73.41
Subject 6	8.84	146.94	269.62	370.43	385.55	263.89	145.08	130.38	155.42	165.22	166.52	136.34	111.32	112.95	87.24	43.09
Subject 7	41.06	93.16	131.35	232.81	233.51	143.68	129.34	88.11	122.73	140.17	155.90	138.39	137.44	139.69	126.46	47.88
Subject 8	8.65	14.22	89.04	238.28	213.21	202.53	176.84	148.78	149.56	138.86	139.14	135.98	117.57	129.45	113.77	71.81
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**13.74**	**56.09**	**128.11**	**220.61**	**249.70**	**182.29**	**144.64**	**125.64**	**128.81**	**132.68**	**140.11**	**123.72**	**113.68**	**108.81**	**98.35**	**58.18**
SD	**11.45**	**45.84**	**74.64**	**80.05**	**86.92**	**76.72**	**29.33**	**31.05**	**22.03**	**24.45**	**24.06**	**30.11**	**35.90**	**29.31**	**22.35**	**16.81**
SEM	**4.05**	**16.21**	**26.39**	**28.30**	**30.73**	**27.13**	**10.37**	**10.98**	**7.79**	**9.24**	**9.09**	**10.64**	**14.65**	**10.36**	**7.90**	**5.94**

Blood TGSH NAC (nmol.l^{-1})

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	823.74	779.52	716.24	747.42	795.04	647.54	574.37	590.12	647.86	572.47	700.13	783.15	652.26	606.17	635.22	425.62	404.17
Subject 2	855.57	831.19	806.16	836.94	926.18	881.55	914.51	858.18	523.45	529.62	931.08	900.56	902.03	861.6	880.34	893.13	856.09
Subject 3	790.64	890.67	855.39	898.15	820.56	785.58	737.8	863.92	661.34	844.93	750.75	698.78	623.43	803.77	803.15	842.7	818.3
Subject 4	743.87	756.88	786.7	709.23	784.46	716.98	725.91	926.59	1100	824.51	888.5	1072.3	855.87	868.05	833.93	860.12	893.37
Subject 5	792.22	754.85	850.84	869.74	664.64	846.9	852.66	898.99	875.78	1204.5	797.7	791.86	805.3	1030.5	1059.3	857.2	914.65
Subject 6	834.98	898.65	870.49	801.17	1072.2	1010.4	1088.6	967.26	1191.5	968.75	927.06	1037.1	978	948.13	790.31	1055.4	1051.5
Subject 7	783.92	789.26	843.18	865.24	553.26	693.45	622.54	520.79	652.79	679.08	647.05	518.4	809.5	509.66	521.14	555.89	642.68
Subject 8	791.26	754.85	662.32	681.45	743.99	881.55	879.93	945.52	847.83	782.93	739.34	599.46	849.64	711.71	880.82	759.73	715.26
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**802.03**	**806.98**	**798.92**	**801.17**	**795.04**	**807.99**	**799.54**	**821.42**	**812.56**	**800.84**	**797.70**	**800.20**	**809.50**	**792.45**	**800.53**	**781.23**	**787.00**
SD	**34.80**	**59.77**	**74.32**	**80.33**	**157.13**	**120.08**	**167.89**	**169.36**	**236.20**	**218.59**	**107.30**	**196.61**	**119.57**	**174.32**	**163.19**	**200.42**	**198.51**
SEM	**12.30**	**21.13**	**26.28**	**28.40**	**55.55**	**42.46**	**59.36**	**59.88**	**83.51**	**77.28**	**37.94**	**69.51**	**42.27**	**61.63**	**57.70**	**70.86**	**70.18**

Blood TGSH CON (nmol.l^{-1})

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	887.32	810.78	774.53	747.11	849.44	829.3	851.23	825.89	962.52	983.57	907.9	886.32	951.01	1017.7	1160.8	1025.9	986.29
Subject 2	832.93	735.07	797.39	756.82	703.82	1007.1	913.87	1197.8	1087.6	841.39	739.17	809.65	721.72	579.68	973.16	902.07	839.43
Subject 3	733.68	794.22	742.41	743.6	719.58	757.74	781.52	700.78	798.68	722.04	642.02	603.05	642.78	749.64	571.06	674.01	728.49
Subject 4	775.53	800.64	770.77	704.26	710.34	766.57	706.52	740.48	772.85	461.56	348.15	531.46	639.62	554.15	486.38	557.31	557.65
Subject 5	729.97	788.99	884.4	958.04	1045	774.7	871.49	885.66	1013.4	955.6	874.34	780.29	776.89	948.68	749.56	819.91	791.43
Subject 6	954.59	991.99	880.12	862.82	751.19	901.53	760.93	768.34	639.72	676.94	694.86	786.56	800.48	937.59	792.5	858.76	878.77
Subject 7	761.06	799.28	742.61	743.22	774.58	687.64	780.69	772.34	657.79	851.03	928.39	809.65	797.95	807.7	786.76	710.51	692.02
Subject 8	714.35	728.05	845.43	855.83	790.66	680.59	772.59	634.2	659.82	1016.3	1449.5	1270.2	1115.4	799.31	819.85	764.2	838.64
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**798.68**	**806.13**	**804.71**	**796.46**	**793.08**	**800.65**	**804.86**	**815.69**	**824.05**	**813.55**	**823.04**	**809.65**	**805.73**	**799.31**	**792.50**	**789.09**	**789.09**
SD	**85.65**	**81.26**	**58.00**	**86.33**	**112.73**	**109.86**	**67.87**	**171.93**	**175.81**	**186.43**	**315.12**	**220.22**	**159.96**	**169.04**	**211.67**	**145.61**	**130.16**
SEM	**30.28**	**28.73**	**20.51**	**30.52**	**39.86**	**38.84**	**24.00**	**60.79**	**62.16**	**65.91**	**111.41**	**77.86**	**56.55**	**59.76**	**74.84**	**51.48**	**46.02**

Blood GSH NAC ($nmol.l^{-1}$)

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	727.31	703.83	602.34	667.02	668.68	602.27	543.46	482.64	437.08	454.19	385.23	554.77	496.18	448.48	419.45	330.27	373.82
Subject 2	745.31	732.1	701.23	690.36	812.1	818.87	674.99	751.28	338.11	474.05	739.28	454.44	750.15	728.41	668.62	733.85	636
Subject 3	718.15	783.98	763.31	769.52	699.58	684.13	675.37	707.39	609.99	539.75	552.76	446.76	534.55	598.28	526.75	565.77	705.29
Subject 4	632.29	689.3	646.72	609.5	640.85	683.93	557.54	622.09	747.66	552.81	811.17	759.2	675.49	652.4	637.96	611.98	635.08
Subject 5	748.5	639.53	743.58	671.33	612.5	719.92	666.92	715.5	790.58	877.33	753.67	612.67	543.25	689	795	587.42	755.25
Subject 6	722.76	781.17	695.52	793.17	744.34	776.21	935.72	891.86	888.21	782.21	782.21	862.62	749.31	694.48	731.03	800.48	804.14
Subject 7	713.52	672.96	749.63	794.01	518.58	654.75	598.02	512.22	536.54	464.57	446.79	407.1	614.7	448.21	436.54	444.94	490.74
Subject 8	680.23	673.11	594.07	592.88	652.77	699.77	747.85	649.77	568.93	574.92	518.02	502.77	553.95	527.01	583.9	619.83	658.76
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**711.01**	**709.5**	**687.05**	**698.47**	**668.68**	**704.98**	**674.98**	**666.59**	**614.64**	**589.98**	**623.64**	**575.04**	**614.7**	**598.28**	**599.91**	**586.82**	**632.39**
SD	**38.149**	**52.359**	**66.168**	**79.283**	**87.8**	**67.871**	**125.57**	**131.97**	**185.05**	**156.42**	**166.86**	**161.63**	**99.591**	**111.73**	**134.32**	**148.99**	**140.18**
SEM	**13.488**	**18.512**	**23.394**	**28.031**	**31.042**	**23.996**	**44.395**	**46.657**	**65.424**	**55.301**	**58.993**	**57.145**	**35.211**	**39.503**	**47.488**	**52.675**	**49.56**

Blood GSH CON ($nmol.l^{-1}$)

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	821.43	753.28	632.54	696.18	785.88	808.46	780.7	748.1	819.2	783.94	829.57	715.5	705.13	891.78	699.95	736.24	767.35
Subject 2	778.57	675.08	674.05	660.94	576.21	858.87	861.03	986.05	886.05	587.08	619.69	550.64	538.7	449.01	782.77	663.18	592.51
Subject 3	696.5	697.34	683.98	654.06	649.01	648.21	666.27	637.8	600.88	575.52	543.66	464.32	540.26	555.36	479.28	506.59	513.74
Subject 4	695.69	662.45	665.05	628.68	658.58	630	607.89	572.84	605.34	324.75	309.17	432.55	491.03	431.27	452.06	515.69	483.19
Subject 5	660.92	599.08	696.25	829.42	889	682.3	781.75	750.83	726.54	649.25	772.92	569.75	658.08	772.92	613.92	702.25	618.33
Subject 6	750.62	876.21	732.34	728.69	653.1	732.34	762.9	727.38	551.93	593.17	646.97	683.52	616.23	763.93	614.07	726.34	785.86
Subject 7	685.68	749.63	695.86	664.57	628.58	610.37	559.44	626.73	555.8	598.7	673.95	696.85	643.09	644.05	530	467.84	522.04
Subject 8	610.79	553.33	677.29	630	656.95	488.08	515.03	513.22	458.14	784.52	820.45	906.67	736.61	644.05	482.09	635.99	676.72
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**712.53**	**695.8**	**682.17**	**686.57**	**687.16**	**682.33**	**691.88**	**695.37**	**650.49**	**612.12**	**652.05**	**627.48**	**616.14**	**644.05**	**581.77**	**619.27**	**619.97**
SD	**67.557**	**99.961**	**28.665**	**66.571**	**100.41**	**117.35**	**123.14**	**145.21**	**146.34**	**144.28**	**171.28**	**154.69**	**86.458**	**161.92**	**117.35**	**107.3**	**115.14**
SEM	**23.885**	**35.342**	**10.135**	**23.536**	**35.499**	**41.488**	**43.537**	**51.338**	**51.74**	**51.009**	**60.555**	**54.691**	**30.567**	**57.248**	**41.488**	**37.937**	**40.708**

Blood GSSG NAC ($nmol.l^{-1}$)

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	65.89	57.5	141.98	50.93	63.56	20.85	70.53	77.8	143.32	199.63	78.33	170.82	245.88	125.94	174.44	289.68	218.95
Subject 2	54.36	59.99	123.34	95.88	127.61	148.24	52.84	211.75	201.59	254.31	119.48	197.34	183.02	130.68	190.39	238.89	246.92
Subject 3	37.17	96.88	58.44	89.54	70.57	109.54	115.25	62.98	197.79	146.51	98.37	138.73	102.53	194.28	91.79	167.43	214.75
Subject 4	79.84	138.19	105.73	75.58	51.76	136.57	98.63	167.64	167.5	136.81	38.99	197.34	148.59	122.88	34.32	41.62	74.47
Subject 5	69.05	189.91	188.15	128.62	156.01	122.02	89.74	134.83	286.86	306.35	101.42	210.54	118.81	175.76	135.64	117.66	173.09
Subject 6	203.97	115.78	147.78	134.13	98.09	169.18	129.4	40.96	87.79	83.77	47.89	103.04	190.35	173.66	174.44	132.41	92.91
Subject 7	75.38	49.65	46.75	78.65	146	77.27	221.24	145.61	101.98	252.32	254.44	197.34	154.86	153.87	256.76	242.67	169.98
Subject 8	103.56	174.72	168.14	225.83	133.71	192.52	257.56	120.98	201.68	231.76	629.01	363.57	378.76	153.87	337.76	128.21	161.92
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**86.153**	**110.33**	**122.54**	**109.9**	**105.91**	**122.02**	**129.4**	**120.32**	**173.56**	**201.43**	**170.99**	**197.34**	**190.35**	**153.87**	**174.44**	**169.82**	**169.12**
SD	**51.343**	**54.081**	**50.05**	**54.275**	**40.35**	**54.099**	**72.611**	**57.125**	**63.731**	**74.021**	**196.64**	**76.38**	**88.212**	**26.137**	**93.865**	**81.711**	**60.3**
SEM	**18.153**	**19.12**	**17.695**	**19.189**	**14.266**	**19.127**	**25.672**	**20.197**	**22.532**	**26.17**	**69.523**	**27.004**	**31.188**	**9.2409**	**33.186**	**28.889**	**21.319**

Blood GSSG CON ($nmol.l^{-1}$)

	Basal	1	2	5	10	15	25	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	96.43	75.69	113.9	80.4	126.36	45.27	30.91	107.49	210.78	118.28	314.9	228.38	156.07	157.69	215.77	95.35	30.34
Subject 2	110.26	99.09	104.93	146.59	114.08	62.68	108.13	106.9	185.34	55.57	191.8	446.12	151.87	133.19	211.73	159.28	220.09
Subject 3	72.49	106.69	92.08	128.63	120.98	101.44	62.43	156.54	51.35	305.18	197.99	252.02	88.88	192.55	276.4	276.93	113.02
Subject 4	111.59	67.58	139.98	99.73	143.61	33.05	168.37	304.5	352.3	271.71	77.34	313.11	180.38	215.66	195.97	248.14	258.29
Subject 5	43.72	115.32	107.26	198.41	52.14	126.98	185.74	183.49	85.19	327.13	192.64	179.2	262.05	341.49	264.34	269.79	159.4
Subject 6	112.22	117.48	174.97	116.22	327.81	234.16	152.87	75.4	303.26	186.54	144.85	174.46	228.69	253.65	59.28	254.96	247.33
Subject 7	70.4	116.3	93.55	71.23	34.68	38.7	24.52	8.56	116.24	214.51	200.26	111.3	194.8	61.45	84.6	110.96	151.94
Subject 8	111.03	81.74	68.25	88.57	91.22	181.77	132.08	295.75	278.9	208.01	221.31	96.69	295.69	184.71	296.92	139.9	56.51
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	**91.018**	**97.486**	**111.87**	**116.22**	**126.36**	**103.01**	**108.13**	**154.83**	**197.92**	**210.87**	**192.64**	**225.16**	**194.8**	**192.55**	**200.63**	**194.41**	**154.62**
SD	**25.852**	**19.916**	**32.679**	**41.705**	**89.609**	**73.651**	**62.415**	**103.73**	**108.68**	**92.131**	**67.013**	**114.29**	**66.138**	**83.185**	**86.923**	**75.619**	**84.904**
SEM	**9.14**	**7.0413**	**11.554**	**14.745**	**31.681**	**26.039**	**22.067**	**36.676**	**38.423**	**32.573**	**23.693**	**40.409**	**23.383**	**29.41**	**30.732**	**26.735**	**30.018**

Plasma Cysteine Reduced CON ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	5.0192	7.2692	8.8962	8.1346	9.1385	7.2	10.973	9.9692	9.6577	10.835	6.7154
Subject 2	6.3596	10.658	10	13.965	9.0789	11.798	17.544	14.737	13.816	12.675	10.053
Subject 3	6.8983	9.0508	16.373	20.644	20.237	16.271	13.525		9.3559	8.8983	7.5424
Subject 4	8.8144	10.567	13.093	15.387	8.1443	13.789	14.639	14.665	16.701	14.82	12.912
Subject 5	4.2683	6.3821	8.6585	8.9431	9.7439	12.642	11.626	10.041	12.561	10.081	8.1707
Subject 6	6.4578	8.634	12.088	13.376	11.881	13.608	13.428	12.242	11.546	13.789	9.6314
Subject 7	4.8983	8.0339	8.7288	8.661	6.9661	7.4068		6.7797	6.7458	5.9492	7.3051
Subject 8	9.8897	11.262	9.6324	12.377	9.1054	11.605	8.7377	8.7377	8.7868	11.679	14.583
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**	**8**
mean	**6.5926**	**8.9822**	**10.934**	**12.686**	**10.537**	**11.79**	**12.925**	**11.024**	**11.146**	**11.091**	**9.6117**
SD	**2.1096**	**1.7418**	**2.7342**	**4.2047**	**4.16**	**3.1265**	**2.826**	**2.9957**	**3.1735**	**2.8377**	**3.0516**
sem	**0.7974**	**0.6158**	**0.9667**	**1.4866**	**1.4708**	**1.1054**	**1.0681**	**1.1323**	**1.122**	**1.0033**	**1.1534**

Blood Cysteine Reduced CON ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	3.9254	4.7977	6.6046	6.6669	6.1062	8.5362	7.9754	7.7262	7.5454	7.4146	5.9162
Subject 2	9.1579	9.1579	7.7632	9.7263	8.0263	8.8421	10.289	11.132	8.3684	8.8421	7.3421
Subject 3	6.322	7.678	11.695	13.429	13.13	11.384	10.158	8.565	7.9718	7.2768	6.2034
Subject 4	14.639	11.572	7.1134	10.979	8.7113	8.5052	13.093	8.5567	9.4845	11.392	10.639
Subject 5	7.8293	13.098	12.878	12.878	15.951	13.244	13.829	13.756	15.732	14.195	16.902
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
mean	**8.3748**	**9.2607**	**9.2108**	**10.736**	**10.385**	**10.102**	**11.069**	**9.9471**	**9.8204**	**9.8241**	**9.4007**
SD	**4.0051**	**3.2595**	**2.8682**	**2.7147**	**4.037**	**2.1275**	**2.3834**	**2.4843**	**3.3822**	**2.9505**	**4.5938**
sem	**1.7912**	**1.4577**	**1.2827**	**1.2141**	**1.8054**	**0.9514**	**1.0659**	**1.111**	**1.5126**	**1.3195**	**2.0544**

Plasma Total Cysteine CON ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	47.106	32.133	34.113	37.823	38.797	36.306	56.057	48.25	38.445	40.706	30.526
Subject 2	48.272	51.53	48.525	62.51	48.117	58.738	52.942	56.31	56.963	61.525	54.528
Subject 3	47.677	50.115	59.667	59.305	58.875	59.142	53.24	51.698	53.777	56.598	54.085
Subject 4	44.247	56.118	81.321	51.678	40.144	61.532	61.007	67.135	67.87	64.699	63.407
Subject 5	54.606	57.149	50.986	53.738	50.082	60.667	46.603	47.291	49.452	54.742	45.02
Subject 6	52.321	49.36	53.311	46.776	65.402	64.871	59.574	53.375	50.92	66.652	63.453
Subject 7	52.163	49.36	56.584	53.362	49.361	59.45	52.63	46.102	41.088	39.909	40.004
Subject 8	65.203	51.515	56.647	60.925	45.392	73.763	39.619			69.869	76.048
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**
mean	**51.325**	**49.406**	**55.144**	**53.251**	**49.544**	**59.313**	**52.634**	**52.88**	**51.216**	**56.837**	**53.384**
SD	**7.0069**	**10.103**	**13.184**	**8.7934**	**9.678**	**12.459**	**8.1957**	**7.2485**	**9.8811**	**11.346**	**14.584**
sem	**2.6484**	**4.5184**	**4.6613**	**3.3236**	**3.6579**	**5.0862**	**3.3459**	**2.7397**	**3.7347**	**4.0115**	**5.1561**

Blood Total Cysteine CON ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	31.444	24.019	23.12	24.644	26.57	32.573	33.215	29.056	23.53	25.682	23.968
Subject 2	37.259	34.267	37.968	43.523	38.199	44.617	40.958	42.106	41.208	42.187	41.041
Subject 3	39.598	39.689	48.108	46.721	43.471	43.333	32.425	37.386	38.668	38.832	38.702
Subject 4	34.331	38.477	49.642	39.349	34.078	43.897	44.955	46.366	43.273	42.279	42.749
Subject 5	44.594	46.613	48.066	36.5334	46.206	44.334	39.57	43.466	49.104	33.61	33.003
n	**5**	**5**	**5**	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
mean	**37.445**	**36.613**	**41.381**	**38.559**	**37.705**	**41.751**	**38.224**	**39.676**	**39.157**	**36.518**	**35.893**
SD	**5.0372**	**8.3224**	**11.217**	**9.7557**	**7.7925**	**5.1535**	**5.3223**	**6.765**	**9.5463**	**7.01**	**7.615**
sem	**2.2527**	**3.7219**	**5.0164**	**4.8779**	**3.4849**	**2.3047**	**2.3802**	**3.0254**	**4.2692**	**3.135**	**3.4055**

Plasma Reduced Cysteine NAC (µmol.l^{-1})

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	4.8462	82.927	76.016	56.911	87.805	55.691	65.854	88.618	72.358	68.699	60.569
Subject 2	4.4737	123.25	86.842	94.737	112.28	69.737	82.895	71.053	76.754	69.737	51.21
Subject 3	7.2316	76.949	73.446	67.797	69.492	75.141	90.395		96.045	97.74	90.96
Subject 4	10.954	67.526	63.866	61.907	64.371	66.186	75.773	56.443	58.247	30.412	18.428
Subject 5	4.5122	70.615	56.077	48.115	58.154	64.731	65.769	57.115	55.385	61.615	44.308
Subject 6	7.368	63.144	38.918	42.784	71.907	50.773	73.34	100		73.969	69.072
Subject 7	10.554	58.125	42.589	64.196	64.375	69.554	64.732		48.75	55.714	35.893
Subject 8	11.569	58.039	54.167	57.108	70.098	53.431	67.157		65.809	64.951	39.461
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**5**	**7**	**8**	**8**
mean	**7.7342**	**75.071**	**61.49**	**61.694**	**74.81**	**63.155**	**73.225**	**74.646**	**67.621**	**65.355**	**51.242**
SD	**3.2306**	**21.322**	**16.699**	**15.681**	**17.432**	**8.8088**	**10.114**	**19.298**	**15.868**	**18.828**	**24.089**
sem	**1.2211**	**7.5384**	**5.904**	**5.5441**	**6.1631**	**3.1144**	**3.8225**	**8.6302**	**5.9976**	**6.6567**	**9.1048**

Blood Reduced Cysteine NAC (µmol.l^{-1})

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	4.9223	48.566	45.565	42.576	51.015	33.6	38.517	48.454	41.171	42.283	36.366
Subject 2	7.3421	74.916	52.453	58.158	69.174	44.247	49.774	42.916	48.079	42.6	26.163
Subject 3	7.5424	46.749	41.858	37.871	41.431	41.298	49.461	46.841	54.081	57.092	54.244
Subject 4	8.1701	38.747	36.473	36.903	38.216	39.407	40.368	30.006	32.084	28.9	13.381
Subject 5	7.6098	47.715	51.204	47.715	46.753	49.32	45.796	48.064	40.475	35.74	27.279
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
mean	**7.1173**	**51.339**	**45.497**	**44.645**	**49.318**	**41.574**	**44.783**	**43.256**	**43.178**	**41.323**	**31.487**
SD	**1.2649**	**13.75**	**7.6514**	**8.6909**	**12.136**	**5.8201**	**5.1617**	**7.7243**	**8.3265**	**10.449**	**15.129**
sem	**0.5657**	**6.1494**	**3.8257**	**3.8867**	**5.4275**	**2.6028**	**2.3084**	**3.4544**	**3.7237**	**4.6728**	**6.7659**

Plasma Total Cysteine NAC ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	19.531	222.86	233.02	222.99	217.18	237.94	232.01	194.72	166.65	152.03	116.784
Subject 2	21.436	306.51	295.59	289.97	298.87	175.42			187.24		126.4
Subject 3	51.382	286.15	297.46	283.52	297.29	289	282.12	199.1	181.3	148.6	101.23
Subject 4	64.069	162.02	168.07	165.23	161.08	180.18	185.46	162.27	153.61	136.9	125.78
Subject 5	44.63	153.5	153.45	153.49	158.24	150.31	157.95	151.27	129.54	124.82	117.54
Subject 6	44.242	145.6	78.988	101.62	128.563	129.24		131.44	256.03	171.68	176.28
Subject 7	61	120.86	97.648	94.063	114.75	106.79	92.634		102.97	107.99	70.202
Subject 8	52.4	241.94	245.53	144.94	147.42	131.55	157.76		163.28	148.26	94.698
n	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**5**	**8**	**7**	**8**
mean	**44.97**	**204.93**	**195.13**	**181.98**	**199.26**	**175**	**184.66**	**167.76**	**167.58**	**141.47**	**116.02**
SD	**19.591**	**69.365**	**95.627**	**75.881**	**73.962**	**66.118**	**65.798**	**28.855**	**45.094**	**20.573**	**33.189**
sem	**7.9979**	**24.524**	**39.04**	**26.828**	**27.955**	**24.99**	**26.862**	**12.904**	**15.943**	**7.776**	**12.544**

Blood Total Cysteine NAC ($\mu mol.l^{-1}$)

	Basal	R	E15	E30	E45	Fat	F+1	F+2	F+5	F+10	F+30
Subject 1	17.267	131.24	136.54	130.8	133.67	142.05	127.01	105.35	94.704	89.324	89.024
Subject 2	28.711	192.47	184.1	182.46	186.43	236.8	220.36	151.03	109.25	73.706	76.454
Subject 3	34.154	172.2	171.76	167.48	172.74	165.52	157.74	116.07	111.34	83.902	60.764
Subject 4	38.692	93.479	94.036	93.851	99.183	98.186	99.511	89.119	88.735	82.211	74.638
Subject 5	30.173	101.02	109.75	100.05	100.75	97.499	92.749	85.363	80.221	79.017	69.541
n	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
mean	**29.799**	**138.08**	**139.24**	**134.93**	**138.55**	**148.01**	**139.47**	**109.39**	**96.85**	**81.632**	**74.084**
SD	**8.0067**	**43.395**	**38.697**	**39.496**	**40.197**	**57.575**	**51.99**	**26.376**	**13.329**	**5.7947**	**10.334**
sem	**3.5807**	**19.407**	**17.306**	**17.663**	**17.976**	**25.749**	**23.251**	**11.796**	**5.961**	**2.5915**	**4.6216**

APPENDIX 11
RAW DATA STUDY 3 – PART II

Attached a raw data spreadsheets for the following:

- Maximal 3-*O*-MFPase activity ($nmol.min^{-1}.(g\ wt\ weight)^{-1}$)
- Percentage change from pre-infusion in maximal *in vitro* 3-*O*-MFPase activity
- Maximal 3-*O*-MFPase ($nmol.min^{-1}.(g\ protein)^{-1}$)
- Plasma K^+
- $\Delta[K^+]$
- $\Delta[K^+]$ to work ratio
- Change in Plasma volume
- Plasma Hydrogen (H^+)
- Bicarbonate (HCO_3^-)
- Partial pressure oxygen (PO_2)
- Partial pressure carbon dioxide (PCO_2)
- Plasma Sodium (Na^+)
- Haemoglobin (Hb)
- Haematocrit (Hct)
- Plasma Calcium (Ca^{2+})
- Plasma Chloride (Cl^-)

Maximal 3-*O*-MFPase (nmol.min^{-1}.(g wt weight)$^{-1}$)

CON					NAC			
	Pre-Inf	**45 min**	**Fatigue**			**Pre-Inf**	**45 min**	**Fatigue**
Subject 1	210.50	148.00	147.00		Subject 1	201.50	189.90	169.00
Subject 2	240.00	209.75	192.00		Subject 2	204.50	170.33	156.67
Subject 3	225.00	157.33	154.25		Subject 3	202.75	202.75	144.33
Subject 4	184.50	142.67	130.33		Subject 4	199.50	179.67	136.67
Subject 5	195.50	137.50	147.67		Subject 5	207.00	157.33	184.48
Subject 6	177.50	146.75	151.67		Subject 6	186.67	165.25	139.33
Subject 7	192.50	173.20	161.50		Subject 7	190.33	170.67	163.43
Subject 8	213.25	169.10	158.33		Subject 8	214.00	173.50	156.25
n	**8**	**8**	**8**		**n**	**8**	**8**	**8**
mean	**204.84**	**160.54**	**155.34**		**mean**	**200.78**	**176.18**	**156.27**
SD	**21.20**	**23.49**	**17.56**		**SD**	**8.80**	**14.41**	**16.14**
sem	**7.50**	**8.31**	**6.21**		**sem**	**3.11**	**5.09**	**5.70**

Percentage change from pre-infusion in maximal *in vitro* 3-*O*-MFPase activity (%)

CON				NAC		
	45 min	**Fatigue**			**45 min**	**Fatigue**
Subject 1	-29.7	-30.17		Subject 1	-5.8	-16.13
Subject 2	-12.6	-20.00		Subject 2	-16.7	-23.39
Subject 3	-30.1	-31.44		Subject 3	0.0	-28.81
Subject 4	-22.7	-29.36		Subject 4	-9.9	-31.50
Subject 5	-29.7	-24.47		Subject 5	-24.0	-10.88
Subject 6	-17.3	-14.55		Subject 6	-11.5	-25.36
Subject 7	-10.0	-16.10		Subject 7	-10.3	-14.14
Subject 8	-20.7	-25.75		Subject 8	-18.9	-26.99
n	**8**	**8**		**n**	**8**	**8**
mean	**-21.60**	**-23.98**		**mean**	**-12.14**	**-22.15**
SD	**7.91**	**6.47**		**SD**	**7.60**	**7.51**
sem	**2.80**	**2.29**		**sem**	**2.69**	**2.65**

Maximal 3-*O*-MFPase (nmol.min^{-1}.(g protein)$^{-1}$)

CON					**NAC**			
	Pre-Inf	**45 min**	**Fatigue**			**Pre-Inf**	**45 min**	**Fatigue**
Subject 1	1173.65	826.10	747.09		Subject 1	1083.12	1051.11	1452.04
Subject 2	1194.92	1320.58	1187.91		Subject 2	1493.06	1053.64	1392.29
Subject 3	1655.20	1144.27	1343.35		Subject 3	1343.99	1083.16	930.65
Subject 4	1072.89	1016.36	700.88		Subject 4	1413.32	1114.81	753.47
Subject 5	1431.35	754.27	664.09		Subject 5	1110.25	1256.56	1122.01
Subject 6	926.16	997.95	756.79		Subject 6	1009.01	855.80	852.39
Subject 7	1064.17	1316.64	998.64		Subject 7	1145.75	1145.86	889.29
Subject 8	1244.64	847.49	821.38		Subject 8	1247.99	1058.07	721.65
n	**8**	**8**	**8**		**n**	**8**	**8**	**8**
mean	**1220.37**	**1027.96**	**902.52**		**mean**	**1230.81**	**1077.38**	**1014.22**
SD	**229.82**	**217.70**	**249.14**		**SD**	**172.36**	**112.73**	**279.95**
sem	**81.25**	**76.97**	**88.08**		**sem**	**60.94**	**39.86**	**98.98**

Plasma K^+ NAC (mM)

Subjects	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	4.62	5.10	5.19	5.36	6.31	5.13		4.02	4.42	4.79
Subject 2	4.14	4.89	4.91	4.70	5.23	3.59	4.09	3.33	3.65	4.04
Subject 3	4.66	5.08	5.16	5.23	6.56		4.27	4.01	4.31	4.47
Subject 4	4.44	5.37	5.27	5.4	5.99	4.68	4.25	4.08	4.00	4.15
Subject 5	4.15	5.19	5.25	5.27	5.97	4.53	4.05	3.96	3.96	4.03
Subject 6	4.20	5.17	5.06	5.37	6.14	4.70	4.16	3.96	4.15	4.23
Subject 7	4.18	5.33	5.42	5.48	6.24	4.48	4.01	4.80	3.92	4.10
Subject 8	3.96	5.24	5.20	5.27	5.70	5.26	3.80	3.63	3.89	3.91
n	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**	**8**
mean	**4.29**	**5.17**	**5.18**	**5.26**	**6.02**	**4.62**	**4.09**	**3.97**	**4.04**	**4.22**
SD	**0.25**	**0.16**	**0.15**	**0.24**	**0.41**	**0.54**	**0.18**	**0.42**	**0.25**	**0.29**
sem	**0.09**	**0.06**	**0.05**	**0.09**	**0.14**	**0.21**	**0.07**	**0.15**	**0.09**	**0.10**

Plasma K^+ CON (mM)

Subjects	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	4.62	5.10	5.16	5.41	6.31	5.13		4.01	4.42	4.67
Subject 2	4.18	4.88	4.89	4.89	5.90	4.04	3.74	3.49	3.75	3.89
Subject 3	4.48	4.96	5.32	5.32	6.44	4.85	4.48	4.11	4.30	4.56
Subject 4	4.42	5.26	5.23	5.27	6.26	4.86	4.13	3.89	3.77	3.85
Subject 5	4.06	5.16	5.25	5.37	6.21	4.52	4.05	3.75	3.74	3.93
Subject 6	4.27	5.20	5.32	5.55	6.25	4.98	4.13	3.76	3.85	4.07
Subject 7	4.31	5.49	5.47	5.62	6.39	4.68	4.07	3.85	3.93	4.11
Subject 8	3.96	5.20	5.23	5.25	6.45	4.63	3.76	3.68	3.85	3.95
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**4.29**	**5.16**	**5.23**	**5.34**	**6.28**	**4.71**	**4.05**	**3.82**	**3.95**	**4.13**
SD	**0.22**	**0.19**	**0.17**	**0.22**	**0.18**	**0.33**	**0.25**	**0.19**	**0.26**	**0.31**
sem	**0.08**	**0.07**	**0.06**	**0.08**	**0.06**	**0.12**	**0.09**	**0.07**	**0.09**	**0.11**

Delta K^+ NAC (mM)

Subjects	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	0.48	0.57	0.74	1.69	-1.18		-2.29	-1.89	-1.52
Subject 2	0.75	0.77	0.56	1.09	-1.64		-1.9	-1.58	-1.19
Subject 3	0.42	0.5	0.57	1.9		-2.29	-2.55	-2.25	-2.09
Subject 4	0.93	0.83	0.96	1.55	-1.31	-1.74	-1.91	-1.99	-1.84
Subject 5	1.04	1.1	1.12	1.82	-1.44	-1.92	-2.01	-2.01	-1.94
Subject 6	0.86	0.86	1.17	1.94	-1.44	-1.98	-2.18	-1.99	-1.91
Subject 7	1.15	1.24	1.3	2.06	-1.76	-2.23	-1.44	-2.32	-2.14
Subject 8	1.28	1.24	1.31	1.74	-0.44	-1.9	-2.07	-1.81	-1.79
n	**7**	**8**	**8**	**8**	**7**	**6**	**8**	**8**	**8**
mean	**0.86**	**0.89**	**0.97**	**1.72**	**-1.32**	**-2.01**	**-2.04**	**-1.98**	**-1.80**
SD	**0.33**	**0.28**	**0.31**	**0.30**	**0.43**	**0.21**	**0.33**	**0.24**	**0.31**
sem	**0.12**	**0.10**	**0.11**	**0.11**	**0.16**	**0.09**	**0.11**	**0.08**	**0.11**

Delta K^+ CON (mM)

Subjects	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	0.48	0.54	0.79	1.69	-1.18		-2.3	-1.89	-1.64
Subject 2	0.7	0.71	0.71	1.72	-1.86	-2.16	-2.41	-2.15	-2.01
Subject 3	0.48	0.84	0.84	1.96	-1.59	-1.96	-2.33	-2.14	-1.88
Subject 4	0.84	0.81	0.85	1.84	-1.4	-2.13	-2.37	-2.49	-2.41
Subject 5	1.1	1.19	1.31	2.15	-1.69	-2.16	-2.46	-2.47	-2.28
Subject 6	0.93	1.05	1.28	1.98	-1.27	-2.12	-2.49	-2.4	-2.18
Subject 7	1.18	1.16	1.31	2.08	-1.71	-2.32	-2.54	-2.46	-2.28
Subject 8	1.24	1.27	1.29	2.49	-1.82	-2.69	-2.77	-2.6	-2.5
n	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**0.87**	**0.95**	**1.05**	**1.99**	**-1.57**	**-2.22**	**-2.46**	**-2.33**	**-2.15**
SD	**0.30**	**0.26**	**0.27**	**0.26**	**0.25**	**0.23**	**0.15**	**0.24**	**0.29**
sem	**0.11**	**0.09**	**0.10**	**0.09**	**0.09**	**0.09**	**0.05**	**0.08**	**0.10**

Delta [K^+] to work ratio NAC ($nmol.^{-1}.J^{-1}$)

Subjects	E15	E30	E45	FAT.
Subject 1	1.94	1.15	1.00	1.84
Subject 2	4.66	2.39	1.16	1.86
Subject 3	2.37	1.41	1.07	2.84
Subject 4	2.95	1.32	1.02	1.45
Subject 5	6.11	3.23	2.19	3.03
Subject 6	5.01	2.70	1.89	2.61
Subject 7	6.61	3.20	2.26	2.38
n	7	7	7	7
mean	**4.24**	**2.20**	**1.51**	**2.29**
SD	**1.84**	**0.90**	**0.58**	**0.59**
sem	**0.70**	**0.34**	**0.22**	**0.22**

Delta [K+] to work ratio CON ($nmol.^{-1}.J^{-1}$)

Subjects	E15	E30	E45	FAT.
Subject 1	1.94	1.09	1.06	1.81
Subject 2	4.35	2.20	1.47	3.08
Subject 3	2.71	2.37	1.58	3.07
Subject 4	2.67	1.29	0.90	1.77
Subject 5	6.47	3.50	2.57	3.54
Subject 6	5.14	2.53	1.90	2.76
Subject 7	6.41	3.28	2.22	3.64
n	7	7	7	7
mean	**4.24**	**2.32**	**1.67**	**2.81**
SD	**1.85**	**0.91**	**0.60**	**0.76**
sem	**0.70**	**0.34**	**0.23**	**0.29**

Change in Plasma Volume (%) NAC

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	0	-0.06	-6.84	-11.24	-8.50	-16.82	-12.48		-7.55	-5.85	0.58
Subject 2	0	0.45	-14.40	-10.57	-9.32	-17.93	-12.16		-7.86	-3.34	3.67
Subject 4	0		-16.67	-18.05	-16.65	-22.13	-20.27	-17.23	-17.03		-9.73
Subject 5	0	-2.61	-12.76	-14.48	-13.98	-15.09	-13.83	-11.18	-8.72	-6.03	-0.39
Subject 6	0	-1.41	-9.16	-9.62	-8.90	-14.40	-10.50	-8.06	-5.89	-1.59	3.85
Subject 7	0	-1.73	-10.99	-8.21	-8.39	-14.51	-11.50	-9.43	-3.12	6.40	10.33
Subject 8	0	-0.79	-10.11	-9.58	-7.82	-11.46	-13.79		-5.62	-2.19	5.20
n	**8**	**6**	**7**	**7**	**7**	**7**	**7**	**4**	**7**	**6**	**7**
mean	**0**	**-1.03**	**-11.56**	**-11.68**	**-10.51**	**-16.05**	**-13.51**	**-11.47**	**-7.97**	**-2.10**	**1.93**
SD	**0**	**1.12**	**3.32**	**3.43**	**3.40**	**3.37**	**3.21**	**4.04**	**4.40**	**4.55**	**6.21**
sem	**0**	**0.46**	**1.25**	**1.30**	**1.29**	**1.27**	**1.21**	**2.02**	**1.66**	**1.86**	**2.35**

Change in Plasma Volume (%) CON

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	0	-6.08	-8.69	-8.90	-8.16	-14.10		-7.98	-7.61	-3.96	3.31
Subject 2	0	-2.62	-11.35	-12.22	-11.00	-15.78	-12.22	-11.70	-7.92	-1.58	4.45
Subject 4	0	-3.81	-10.15	-12.96	-11.13	-15.30	-13.60	-10.81	-8.83	-6.29	
Subject 5	0	0.00	-12.30	-14.98	-13.56	-17.74	-14.43	-12.53	-9.82	-7.29	1.21
Subject 6	0	-2.46	-9.88	-9.10	-9.47	-13.93	-13.24	-13.09	-8.79	-2.79	6.07
Subject 7	0	-0.90	-11.23	-11.22	-10.20	-13.58	-8.62	-9.84	-5.17	-1.97	5.04
Subject 8	0	1.58	-6.45	-3.19	-2.98	1.46	-6.42	1.22	0.66	5.04	14.54
n	**7**	**7**	**7**	**7**	**7**	**7**	**6**	**7**	**7**	**7**	**6**
mean	**0**	**-2.04**	**-10.01**	**-10.37**	**-9.50**	**-12.71**	**-11.42**	**-9.25**	**-6.78**	**-2.69**	**5.77**
SD	**0**	**2.53**	**1.96**	**3.82**	**3.32**	**6.41**	**3.18**	**4.92**	**3.59**	**4.03**	**4.61**
sem	**0**	**0.96**	**0.74**	**1.44**	**1.26**	**2.42**	**1.30**	**1.86**	**1.36**	**1.52**	**1.88**

Plasma H^+ NAC (nM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	40.27	40.64	45.08	43.55	47.10	57.81	64.12		63.53	55.98	48.64
Subject 2	39.17	35.32	43.95	42.56	39.36	56.62	57.41		59.02	53.83	38.46
Subject 3	41.69	41.69	41.78	42.27	42.27	54.20		62.81	62.95	52.97	41.02
Subject 4	36.90	37.58	39.45	36.81	35.65	48.64	51.64	52.72	49.77	45.71	38.28
Subject 5	38.99	39.54	44.46	43.05	43.45	44.67	49.09	50.47	48.64	46.24	41.59
Subject 6	40.36	38.37	45.29	43.65	43.65	56.10	60.81	60.67	56.91	51.17	43.25
Subject 7	37.07	37.58	46.03	44.77	43.55	54.45	58.61	58.34	57.02	51.52	40.55
Subject 8	38.82	38.99	43.45	41.78	39.99	52.12	59.29	61.66	61.52	52.36	37.33
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**6**	**8**	**8**	**8**
mean	**39.16**	**38.71**	**43.69**	**42.31**	**41.88**	**53.08**	**57.28**	**57.78**	**57.42**	**51.22**	**41.14**
SD	**1.64**	**1.98**	**2.15**	**2.41**	**3.48**	**4.45**	**5.23**	**5.06**	**5.65**	**3.57**	**3.61**
sem	**0.58**	**0.70**	**0.76**	**0.85**	**1.23**	**1.57**	**1.98**	**2.07**	**2.00**	**1.26**	**1.28**

Plasma H^+ CON (nM)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	40.74	40.64	45.08	42.95	44.57	63.39	64.12		63.68	55.21	46.67
Subject 2	38.28	38.19	46.24	44.77	43.95	50.35	59.16	61.09	61.09	57.15	41.11
Subject 3	40.93	40.46	44.16	40.83	39.72	48.98	54.45	53.46	54.83	47.21	40.09
Subject 4	35.81	36.48	37.84	36.90	37.33	41.40	50.47	52.72	48.53	35.81	38.37
Subject 5	39.99	38.11	45.39	46.67	45.29	50.35	57.28	59.98	58.75	54.58	44.06
Subject 6	40.09	40.93	42.46	41.78	40.18	57.68	62.52	65.46	59.29	53.70	40.64
Subject 7	38.46	38.02	42.76	43.35	42.85	46.24	51.88	52.24	50.70	45.92	36.98
Subject 8	38.73	38.90	44.26	43.55	42.17	49.55	58.48	60.81	59.84	52.84	39.90
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**39.13**	**38.97**	**43.52**	**42.60**	**42.01**	**50.99**	**57.29**	**57.97**	**57.09**	**50.30**	**40.98**
SD	**1.68**	**1.57**	**2.62**	**2.91**	**2.73**	**6.76**	**4.82**	**5.14**	**5.26**	**7.03**	**3.09**
sem	**0.60**	**0.56**	**0.93**	**1.03**	**0.97**	**2.39**	**1.70**	**1.94**	**1.86**	**2.49**	**1.09**

HCO_3^- NAC (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	24.3	20.9	20.5	20.6	12.6		11.9	11.7	13.7	19.3
Subject 2	26.5	18.6	19.2	20.8	11.6	11.2		10.9	12.9	20.4
Subject 3	28.0	19.3	19.2	19.6	12.8	12.7	13.4	13.2	15.2	21.0
Subject 4	31.2	20.6	21.2	21.7	15.5	14.3	15.0	15.4	17.7	18.3
Subject 5	27.0	17.5	17.6	16.7	14.2	13.9	15.4	16.0	16.2	21.0
Subject 6	29.5	20.1	19.2	18.8	11.5	12.5	13.9	13.5	16.5	21.9
Subject 7	28.6	17.7	16.8	18.5	12.1	12.4	11.9	12.2	14.1	21.4
Subject 8	28.9	19.5	19.9	20.2	12.4	11.6	12.3	13.0	15.5	24.8
n	**8**	**8**	**8**	**8**	**8**	**7**	**7**	**8**	**8**	**8**
mean	**28.0**	**19.3**	**19.2**	**19.6**	**12.8**	**12.7**	**13.4**	**13.2**	**15.2**	**21.0**
SD	**2.1**	**1.3**	**1.4**	**1.6**	**1.4**	**1.1**	**1.4**	**1.7**	**1.6**	**1.9**
sem	**0.7**	**0.4**	**0.5**	**0.6**	**0.5**	**0.4**	**0.5**	**0.6**	**0.6**	**0.7**

HCO_3^- CON (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	24.4	21.2	19.5	20.6	11.3	11.9		11.9	13.8	19.3
Subject 2	27.2	18.1	18.2	18.6	13.0	11.2	11.0	10.0	11.3	20.3
Subject 3	25.0	22.1	24.0	24.5	15.2	13.4	12.9	14.5	16.3	24.2
Subject 4	28.0	25.6	24.0	22.4	17.9	15.0	15.2	16.3	18.4	25.0
Subject 5	27.3	20.8	17.0	17.6	13.4	12.1	12.3	13.3	14.6	21.7
Subject 6	29.9	21.7	21.3	22.4	12.7	11.5	12.3	13.4	15.8	24.1
Subject 7	27.3	19.0	18.2	19.2	15.0	14.4	14.3	14.8	16.6	23.4
Subject 8	29.3	17.9	18.1	19.8	13.2	13.4	13.1	13.1	14.8	23.7
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**
mean	**27.3**	**20.8**	**20.0**	**20.6**	**14.0**	**12.9**	**13.0**	**13.4**	**15.2**	**22.7**
SD	**1.9**	**2.5**	**2.8**	**2.3**	**2.0**	**1.4**	**1.4**	**1.9**	**2.1**	**2.0**
sem	**0.7**	**0.9**	**1.0**	**0.8**	**0.7**	**0.5**	**0.5**	**0.7**	**0.7**	**0.7**

PO_2 NAC (Torr)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	70.9	71.4	79.1	66.5	74.0	89.2		96.1	85.4	61.7
Subject 2	76.0	84.8	85.7	82.0	83.3	109.4		104.0	98.4	77.9
Subject 3	71.7	84.3	85.5	78.0	63.0		96.0	96.5	91.3	76.8
Subject 4	71.2	64.9	64.6	63.4	70.9	88.6	88.1	89.4	74.5	75.0
Subject 5	64.8	78.0	76.3	75.6	76.7	97.3	101.5	98.3	90.9	74.7
Subject 6	61.6	71.8	71.2	78.0	87.1	101.1	107.2	104.6	100.2	88.5
Subject 7	50.2	81.9	72.0	81.1	86.1	108.3	111.4	102.1	99.6	67.0
Subject 8	51.8	86.9	75.8	80.2	72.7	87.4	104.6	95.2	87.2	75.8
n	**8**	**8**	**8**	**8**	**8**	**7**	**6**	**8**	**8**	**8**
mean	**64.8**	**78.0**	**76.3**	**75.6**	**76.7**	**97.3**	**101.5**	**98.3**	**90.9**	**74.7**
SD	**9.6**	**7.9**	**7.2**	**6.9**	**8.3**	**9.3**	**8.4**	**5.1**	**8.7**	**7.9**
sem	**3.4**	**2.8**	**2.5**	**2.4**	**2.9**	**3.5**	**3.4**	**1.8**	**3.1**	**2.8**

PO_2 CON (Torr)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	69.6	76.3	71.4	69.4	80.1		100.1	97.9	77.8	62.6
Subject 2	62.3	86.9	85.2	78.3	76.2	104.7	107.3	107.0	108.3	68.3
Subject 3	88.1	85.9	80.6	84.3	75.4	87.0	89.4	93.6	90.8	84.2
Subject 4	66.7	53.8	55.9	51.9	62.0	91.2	94.5	87.9	76.3	59.6
Subject 5	66.7	77.9	76.2	71.8	74.7	94.6	101.1	99.8	92.5	75.4
Subject 6	57.2	74.7	80.2	70.3	74.7		102.6	106.3	104.7	94.5
Subject 7		81.0	80.9	78.0	78.6	98.7	111.0	102.4	86.6	
Subject 8	56.5	87.0	79.4	70.3	76.0	91.5	103.0	103.6	102.9	83.4
n	**7**	**8**	**8**	**8**	**8**	**6**	**8**	**8**	**8**	**7**
mean	**66.7**	**77.9**	**76.2**	**71.8**	**74.7**	**94.6**	**101.1**	**99.8**	**92.5**	**75.4**
SD	**10.6**	**10.9**	**9.1**	**9.6**	**5.5**	**6.3**	**6.8**	**6.5**	**12.1**	**12.7**
sem	**4.0**	**3.9**	**3.2**	**3.4**	**1.9**	**2.6**	**2.4**	**2.3**	**4.3**	**4.8**

PCO_2 NAC (Torr)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	41.0	40.1	36.4	41.1	30.6	31.9		31.1	31.5	36.5
Subject 2	42.7	34.0	33.9	33.9	27.2	26.7		26.7	28.7	32.5
Subject 3	45.5	37.3	34.4	34.9	28.7	29.8	31.7	31.1	32.0	36.7
Subject 4	49.3	34.6	33.3	34.1	31.2	30.7	32.8	31.8	34.1	40.3
Subject 5	44.9	43.7	33.6	32.7	29.9	28.7	33.0	32.3	32.8	36.4
Subject 6	46.8	38.6	35.3	34.7	26.7	31.3	31.9	32.8	32.4	35.3
Subject 7	45.5	34.9	33.4	34.0	27.7	30.5	29.1	29.4	30.6	36.2
Subject 8	48.3	35.0	35.0	33.7	27.3	28.7	31.8	33.4	33.6	39.4
n	**8**	**8**	**8**	**8**	**8**	**8**	**6**	**8**	**8**	**8**
mean	**45.5**	**37.3**	**34.4**	**34.9**	**28.7**	**29.8**	**31.7**	**31.1**	**32.0**	**36.7**
SD	**2.7**	**3.4**	**1.1**	**2.6**	**1.7**	**1.7**	**1.4**	**2.1**	**1.7**	**2.4**
sem	**1.0**	**1.2**	**0.4**	**0.9**	**0.6**	**0.6**	**0.6**	**0.8**	**0.6**	**0.8**

PCO_2 CON (Torr)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	44.4	34.5	36.1	35.5	28.4		30.2	30.1	31.5	35.5
Subject 2	42.2	34.4	33.8	33.9	27.1	27.5	27.9	26.0	27.3	34.7
Subject 3	42.2	40.5	41.2	40.7	31.2	30.5	28.8	33.2	32.1	40.2
Subject 4	42.5	40.9	38.0	35.5	30.7	31.5	33.2	32.5	34.5	41.0
Subject 5	43.4	36.5	36.0	36.1	30.7	30.5	31.0	31.2	32.2	38.3
Subject 6	49.7	38.2	36.9	37.3	30.3	30.3	33.3	32.9	35.2	40.5
Subject 7	44.5	33.6	33.3	34.8	39.4	31.1	31.1	31.3	31.9	36.2
Subject 8	38.2	33.1	32.4	35.1	27.5	32.3	32.4	32.1	32.7	39.7
n	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**8**
mean	**43.4**	**36.5**	**36.0**	**36.1**	**30.7**	**30.5**	**31.0**	**31.2**	**32.2**	**38.3**
SD	**3.2**	**3.1**	**2.9**	**2.1**	**3.9**	**1.5**	**2.0**	**2.3**	**2.4**	**2.5**
sem	**1.1**	**1.1**	**1.0**	**0.7**	**1.4**	**0.6**	**0.7**	**0.8**	**0.8**	**0.9**

Plasma Na^+ NAC (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	140.6	139.0	144.0	146.5	150.2	144.3		143.3	143.7	140.0
Subject 2	140.3	142.1	142.2	142.2	147.0	143.1		142.0	139.8	142.3
Subject 3	139.0	140.6	140.0	140.8	149.2		141.7	141.2	137.4	137.7
Subject 4	136.7	141.0	137.6	140.9	145.7	141.6	141.5	143.3	142.0	139.1
Subject 5	136.5	139.4	139.9	139.1	141.5	140.5	138.1	137.2	138.0	136.3
Subject 6	142.4	141.5	142.1	141.7	144.5	141.6	141.4	140.8	139.9	138.5
Subject 7	140.2	142.8	142.0	141.5	145.9	140.7	140.9	139.3	139.6	138.3
Subject 8	138.8	143.6	142.1	141.9	145.7	143.5	142.6	141.3	140.7	139.4
n	**8**	**8**	**8**	**8**	**8**	**7**	**6**	**8**	**8**	**8**
mean	**139.3**	**141.3**	**141.2**	**141.8**	**146.2**	**142.2**	**141.0**	**141.1**	**140.1**	**139.0**
SD	**2.0**	**1.6**	**2.0**	**2.1**	**2.7**	**1.5**	**1.5**	**2.0**	**2.0**	**1.8**
sem	**0.7**	**0.6**	**0.7**	**0.7**	**1.0**	**0.6**	**0.6**	**0.7**	**0.7**	**0.6**

Plasma Na^+ CON (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	145.2	139.3	141.7	138.5	147.6		141.2	136.4	140.5	138.5
Subject 2	139.1	143.3	141.9	143.5	145.4	144.2	143.8	142.3	139.7	138.1
Subject 3	138.1	140.0	140.1	138.6	143.2	140.3	139.7	137.1	137.1	135.7
Subject 4	139.9	141.1	140.5	142.3	145.7	143.7	142.3	142.4	141.0	139.4
Subject 5	139.5	144.3	142.5	140.9	145.0	140.6	141.7	140.2	139.3	138.8
Subject 6	141.2	142.7	143.7	141.9	144.9	141.3	140.0	139.0	138.7	138.1
Subject 7	139.4	144.5	141.9	142.0	142.4	139.5	139.5	138.7	137.9	135.9
Subject 8	140.9	141.1	145.1	141.9	145.9	141.0	140.5	141.6	139.3	138.4
n	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**8**
mean	**140.4**	**142.0**	**142.2**	**141.2**	**145.0**	**141.5**	**141.1**	**139.7**	**139.2**	**137.9**
SD	**2.2**	**2.0**	**1.6**	**1.8**	**1.6**	**1.8**	**1.5**	**2.3**	**1.3**	**1.3**
sem	**0.8**	**0.7**	**0.6**	**0.6**	**0.6**	**0.7**	**0.5**	**0.8**	**0.5**	**0.5**

Hb (g/dl)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	15.7	15.8	16.4	16.5	16.5	17.1	16.7		16.2	16.1	15.7
Subject 2	15.8	15.7	17.1	16.8	16.7	17.4	16.8		16.5	16.1	15.5
Subject 3	15.1	15.3	15.6	15.6	15.2	16.2		16.1	15.5	15.1	15.0
Subject 4	14.6	15.7	16.7	16.3	16.3	16.6	16.5	16.2	16.1		15.5
Subject 5	14.5	14.5	15.4	15.6	15.4	15.6	15.4	15.1	14.9	14.6	14.2
Subject 6	15.9	15.9	16.7	16.6	16.5	17.1	16.7	16.5	16.3	15.9	15.5
Subject 7	16.6	16.8	17.4	17.2	17.3	17.8	17.5	17.3	16.7	15.8	15.4
Subject 8	16.1	16.2	17.0	16.9	16.7	17.1	17.3		16.4	16.2	15.6
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**5**	**8**	**7**	**8**
mean	**15.5**	**15.7**	**16.5**	**16.4**	**16.3**	**16.9**	**16.7**	**16.2**	**16.1**	**15.7**	**15.3**
SD	**0.7**	**0.7**	**0.7**	**0.6**	**0.7**	**0.7**	**0.7**	**0.8**	**0.6**	**0.6**	**0.5**
sem	**0.3**	**0.2**	**0.3**	**0.2**	**0.2**	**0.2**	**0.3**	**0.4**	**0.2**	**0.2**	**0.2**

Hb CON (g/dl)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	16.0	16.5	16.8	16.7	16.6	17.2		16.5	16.4	16.3	15.7
Subject 2	15.3	16.1	16.8	16.8	16.7	17.2	16.8	16.8	16.3	15.9	15.4
Subject 3	15.1	15.5	15.6	15.7	15.9	16.8	16.4	16.3	15.8	15.8	15.2
Subject 4	14.8	15.2	15.7	16.0	15.7	16.2	16.0	15.7	15.5	15.3	
Subject 5	14.4	14.4	15.5	15.7	15.5	15.9	15.6	15.4	15.1	14.9	14.3
Subject 6	15.0	15.2	15.9	15.9	15.8	16.3	16.2	16.2	15.9	15.2	14.4
Subject 7	16.2	16.2	17.2	17.1	17.1	17.4	16.9	17.0	16.5	16.2	15.7
Subject 8	16.7	16.5	17.3	17.0	16.9	16.4	17.1	16.2	16.5	16.1	15.4
n	**8**	**8**	**8**	**8**	**8**	**8**	**7**	**8**	**8**	**8**	**7**
mean	**15.4**	**15.7**	**16.4**	**16.4**	**16.3**	**16.7**	**16.4**	**16.3**	**16.0**	**15.7**	**15.2**
SD	**0.8**	**0.7**	**0.8**	**0.6**	**0.6**	**0.6**	**0.5**	**0.5**	**0.5**	**0.5**	**0.6**
sem	**0.3**	**0.3**	**0.3**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**

Hct NAC (%)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	47.9	47.6	49.3	51.4	49.9	52.8	51.5		50.3	49.7	47.6
Subject 2	47.0	47.1	50.9	49.6	49.2	52.1	50.5		49.0	47.8	46.1
Subject 3	43.9	44.3	47.0	47.4	47.0	48.8	48.1	46.1	46.5	45.3	43.8
Subject 4	42.4	43.1	45.1	47.3	46.4	49.0	48.1	47.1	47.3		44.8
Subject 5	38.7	40.3	43.2	43.6	44.0	44.0	43.9	43.3	42.5	42.0	40.2
Subject 6	43.3	44.1	45.9	46.5	46.4	47.8	46.7	45.9	45.3	44.2	42.6
Subject 7	44.8	45.1	48.5	47.5	47.3	49.4	48.5	47.9	46.2	44.1	43.5
Subject 8	43.0	43.1	45.9	45.9	45.5	46.4	47.2		45.2	43.9	41.9
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**5**	**8**	**7**	**8**
mean	**43.9**	**44.3**	**47.0**	**47.4**	**47.0**	**48.8**	**48.1**	**46.1**	**46.5**	**45.3**	**43.8**
SD	**2.9**	**2.3**	**2.5**	**2.3**	**1.9**	**2.9**	**2.3**	**1.7**	**2.4**	**2.6**	**2.4**
sem	**1.0**	**0.8**	**0.9**	**0.8**	**0.7**	**1.0**	**0.8**	**0.8**	**0.9**	**1.0**	**0.8**

Hct CON (%)

Subjects	BASAL	REST	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	49.1	50.7	51.2	51.6	51.5	53.0		51.7	51.8	50.2	48.4
Subject 2	47.4	46.1	48.8	49.3	48.9	50.2	49.3	49.0	48.4	46.2	44.7
Subject 3	44.1	44.4	46.6	46.8	46.7	47.6	46.4	46.8	46.1	44.9	43.1
Subject 4	42.4	43.1	45.1	45.8	45.7	46.6	46.2	45.5	45.0	44.2	44.1
Subject 5	41.1	41.1	44.4	45.4	45.2	46.5	45.4	44.9	44.3	43.5	40.8
Subject 6	39.7	40.4	42.4	41.9	42.5	43.6	43.5	43.4	41.7	40.6	38.6
Subject 7	44.3	44.8	47.5	47.8	47.2	48.3	46.9	47.3	46.2	45.4	43.3
Subject 8	44.9	44.7	46.6	45.7	45.9	45.1	47.2	45.9	45.2	44.2	41.8
n	**8**	**8**	**8**	**8**	**8**	**8**	7	**8**	**8**	**8**	**8**
mean	**44.1**	**44.4**	**46.6**	**46.8**	**46.7**	**47.6**	**46.4**	**46.8**	**46.1**	**44.9**	**43.1**
SD	**3.1**	**3.2**	**2.7**	**2.9**	**2.7**	**2.9**	**1.8**	**2.6**	**3.0**	**2.7**	**2.9**
sem	**1.1**	**1.1**	**1.0**	**1.0**	**0.9**	**1.0**	**0.7**	**0.9**	**1.1**	**1.0**	**1.0**

Plasma Ca^{2+} NAC (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	1.22	1.20	1.19	1.21	1.35	1.32	1.32	1.19	1.25	1.20
Subject 2	1.23	1.22	1.27	1.26	1.34	1.34	1.30	1.23	1.21	1.18
Subject 3	1.20	1.25	1.20	1.25	1.30	1.38	1.35	1.26	1.22	1.19
Subject 4	1.26	1.27	1.26	1.29	1.34	1.36	1.32	1.27	1.25	1.23
Subject 5	1.23	1.26	1.23	1.25	1.26	1.23	1.25	1.19	1.18	1.22
Subject 6	1.33	1.28	1.32	1.25	1.28	1.28	1.23	1.24	1.25	1.25
Subject 7	1.28	1.29	1.25	1.25	1.31	1.28	1.22	1.20	1.21	1.23
Subject 8	1.19	1.22	1.20	1.20	1.22	1.25	1.22	1.21	1.16	1.14
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**1.24**	**1.25**	**1.24**	**1.25**	**1.30**	**1.31**	**1.28**	**1.22**	**1.22**	**1.21**
SD	**0.05**	**0.03**	**0.04**	**0.03**	**0.05**	**0.05**	**0.05**	**0.03**	**0.03**	**0.04**
sem	**0.02**	**0.01**	**0.02**	**0.01**	**0.02**	**0.02**	**0.02**	**0.01**	**0.01**	**0.01**

Plasma Ca^{2+} CON (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	1.18	1.21	1.26	1.26	1.20	1.27	1.25	1.32	1.27	1.26
Subject 2	1.18	1.28	1.30	1.34	1.28	1.27	1.38	1.31	1.24	1.22
Subject 3	1.24	1.15	1.34	1.31	1.32	1.14	1.24	1.52	1.40	1.16
Subject 4	1.24	1.26	1.25	1.28	1.29	1.25	1.32	1.37	1.35	1.33
Subject 5	1.31	1.31	1.34	1.30	1.32	1.31	1.28	1.27	1.25	1.26
Subject 6	1.27	1.22	1.23	1.23	1.28	1.25	1.22	1.20	1.18	1.19
Subject 7	1.26	1.23	1.23	1.25	1.25	1.24	1.19	1.21	1.18	1.20
Subject 8	1.25	1.26	1.26	1.21	1.28	1.29	1.25	1.18	1.20	1.21
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**1.24**	**1.24**	**1.28**	**1.27**	**1.28**	**1.25**	**1.27**	**1.30**	**1.26**	**1.23**
SD	**0.04**	**0.05**	**0.05**	**0.04**	**0.04**	**0.05**	**0.06**	**0.11**	**0.08**	**0.05**
sem	**0.02**	**0.02**	**0.02**	**0.02**	**0.01**	**0.02**	**0.02**	**0.04**	**0.03**	**0.02**

Plasma Cl^- NAC (mM)

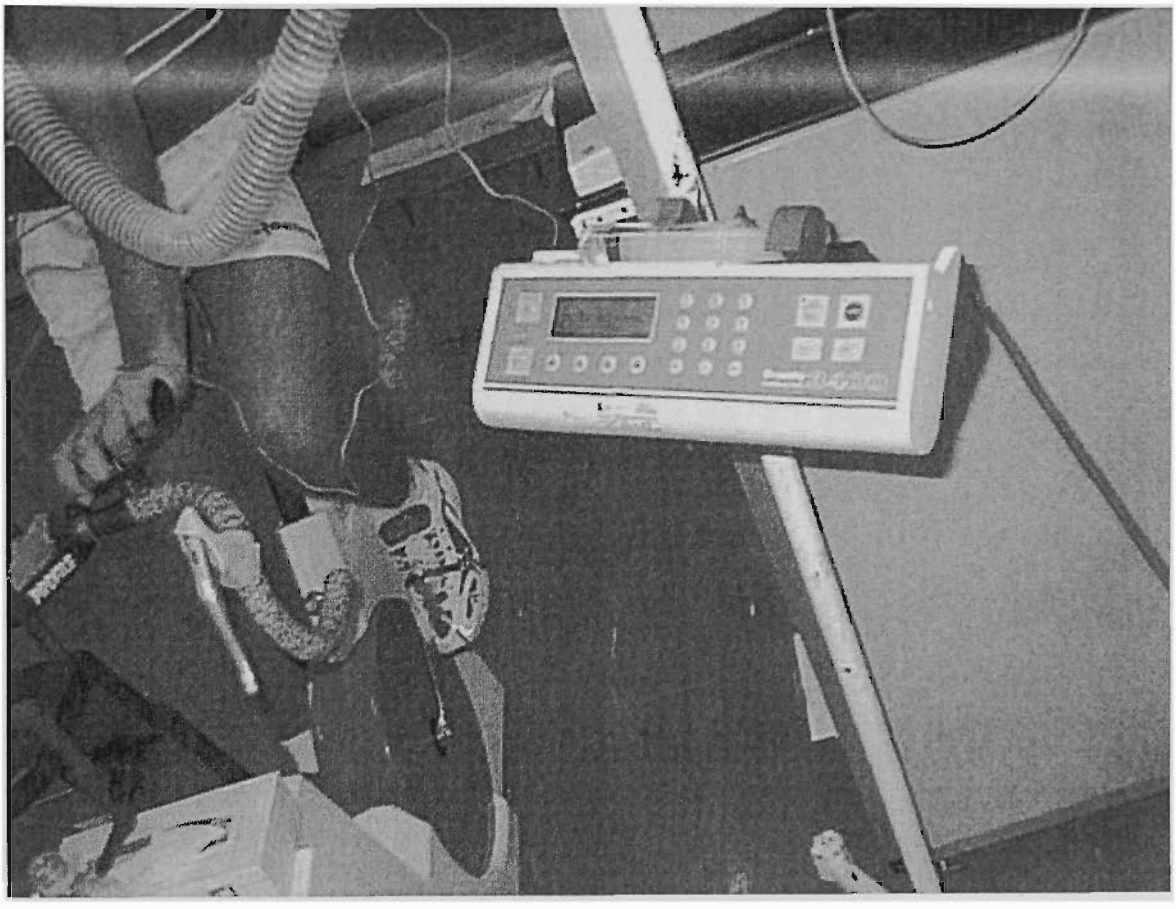
Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	103	103	102	99	99	97	101	103	97	101
Subject 2	106	107	103	103	106	103	107	106	103	103
Subject 3	105	104	105	103	106	104	108	105	104	103
Subject 4	103	103	104	103	103	104	106	108	105	103
Subject 5	106	106	105	103	103	100	99	103	102	103
Subject 6	105	108	105	106	106	103	102	102	103	104
Subject 7	108	104	104	105	105	102	101	101	101	102
Subject 8	106	106	105	106	107	103	101	104	103	107
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**105**	**105**	**104**	**104**	**104**	**102**	**103**	**104**	**102**	**103**
SD	**2**	**2**	**1**	**2**	**3**	**2**	**3**	**2**	**2**	**2**
sem	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Plasma Cl^- CON (mM)

Subjects	BASAL	E15	E30	E45	FAT.	R1	R2	R5	R10	R30
Subject 1	105	104	101	101	104	100	108	105	104	102
Subject 2	109	104	100	102	105	102	107	104	105	111
Subject 3	101	111	98	106	96	110	115	98	103	111
Subject 4	101	101	102	101	101	103	103	104	103	100
Subject 5	106	106	103	106	107	103	102	102	103	103
Subject 6	107	104	107	107	111	106	104	104	104	107
Subject 7	104	107	106	105	107	103	101	102	102	102
Subject 8	105	105	101	105	107	102	101	99	101	103
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**105**	**105**	**102**	**104**	**105**	**104**	**105**	**102**	**103**	**105**
SD	**3**	**3**	**3**	**2**	**5**	**3**	**5**	**3**	**1**	**4**
sem	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**1**	**0**	**2**

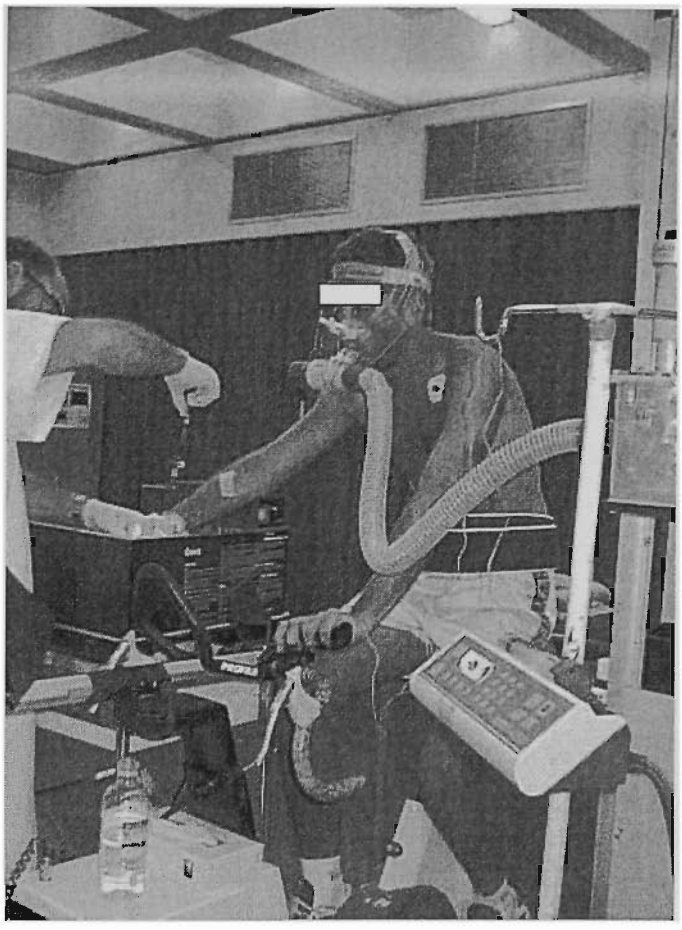
APPENDIX 12
PHOTOS FROM EXPERIMENTAL DAYS

Infusion pump

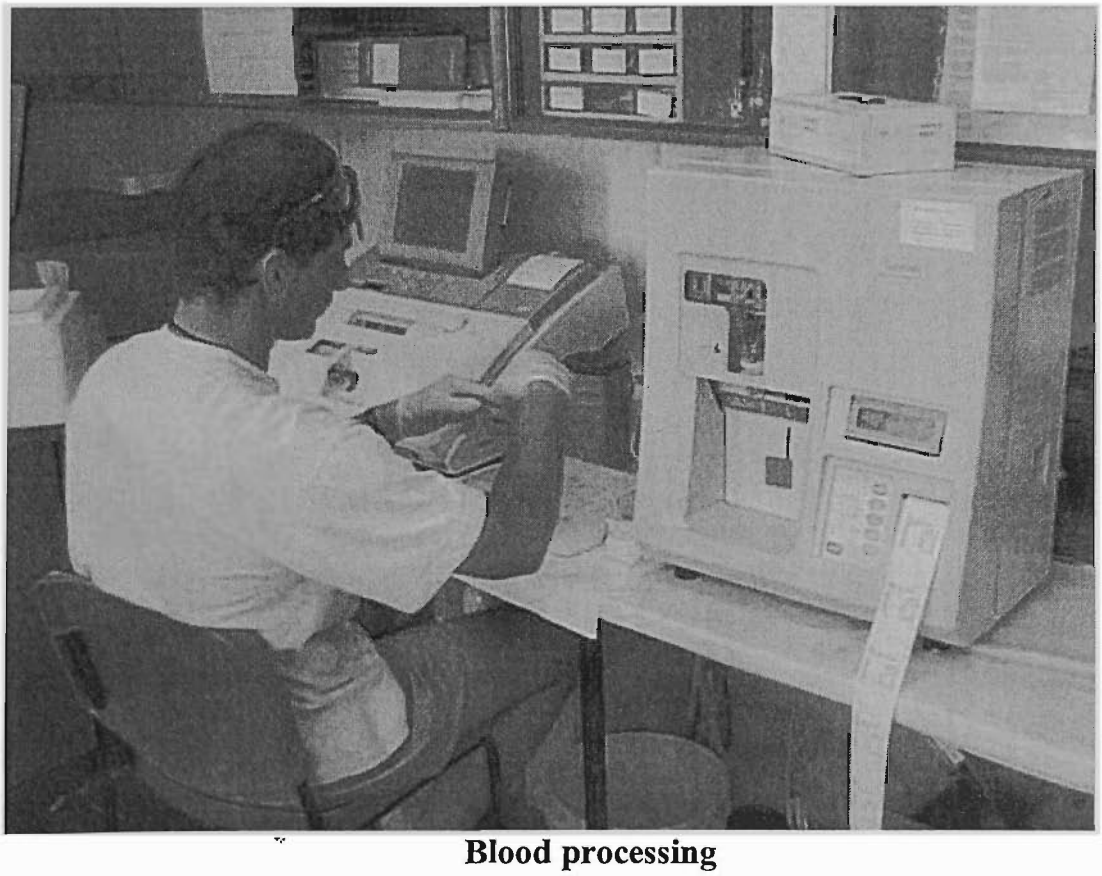


Data collection

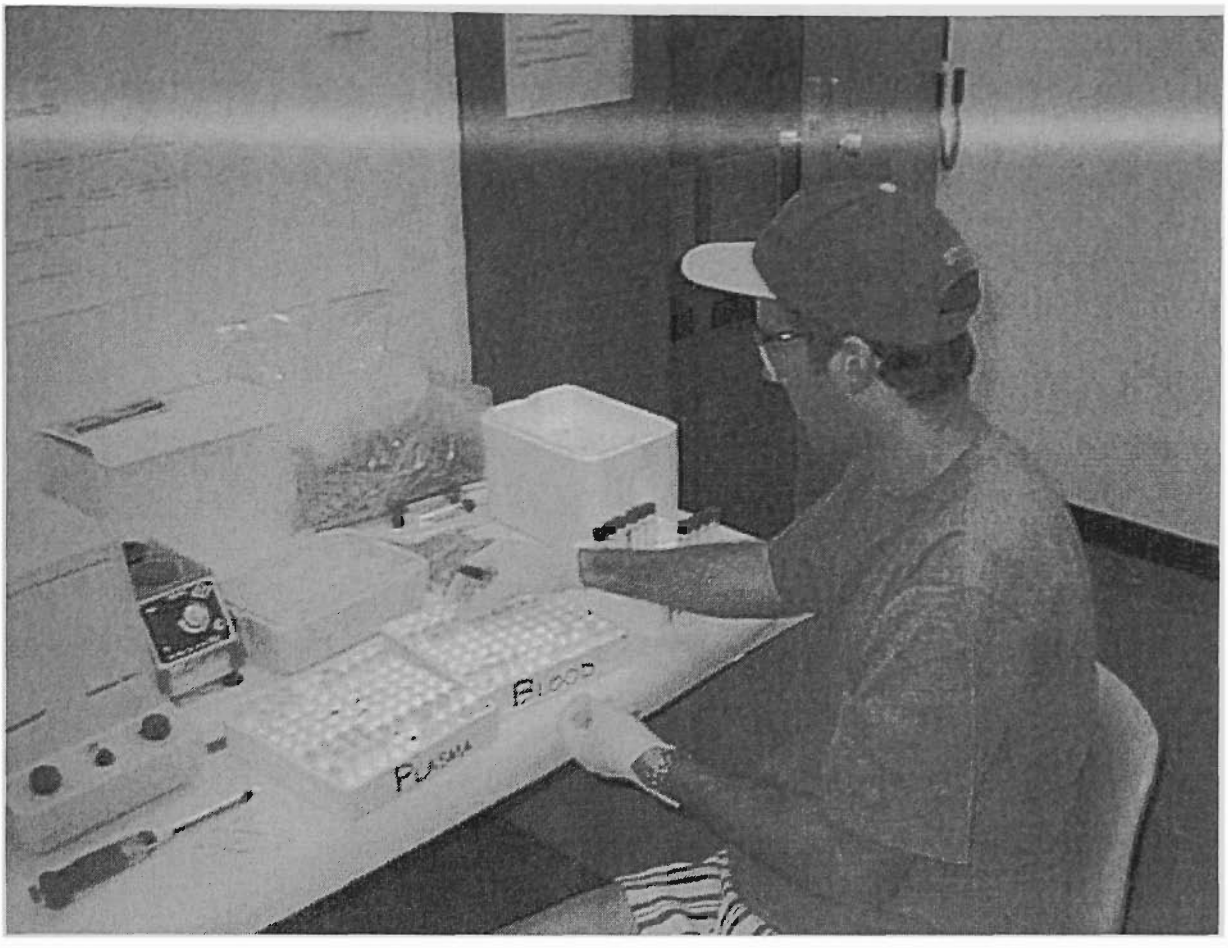
Arterialised venous blood sampling and expired gases collection



Blood gas, haematocrit and haemoglobin analysis



Blood processing



All smiles at the end