THE EFFECTS OF ACUTE NICOTINAMIDE RIBOSIDE SUPPLEMENTATION ON SUBSTRATE UTILISATION AND 5KM TIME-TRIAL PERFORMANCE

By

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A thesis submitted to The University of Birmingham for the degree of MASTERS BY RESEARCH

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August 2018

ABSTRACT

Nicotinamide Riboside (NR) administration has been shown to increase fat oxidation and improve endurance performance in rodents, whilst recent research has proven it is safe for human consumption. The present study aimed to investigate the influence of acute NR supplementation on substrate utilisation and exercise performance in humans. In this counter-balanced, crossover design study, eleven recreationally-active males performed a 60-minute bout of cycling at 55% VO_{2max}, followed by a 5km time-trial. Participants completed this twice during visits separated by at least one week, once following the consumption of 1000mg NR, and the other following placebo consumption. The contribution of fat oxidation to total substrate utilisation was not significantly different between the NR and placebo conditions during steady-state exercise (22.3±9.0% and 19.6±7.3%, respectively; $p < 0.05$). Similarly, there were no significant differences shown between 5km time-trial performance with NR (823.4±250.0 seconds) and placebo (819.5±239.8 seconds). To conclude, a single dose of NR does not influence substrate utilisation during moderate-intensity exercise, nor does it enhance exercise performance. Future research should investigate the effects of chronic NR supplementation, or the coupling of this with exercise training to further test the capacity of NR in humans during exercise.

ACKNOWLEDGEMENTS

Foremost, I would like to express my thanks to my thesis supervisor Dr Andrew Philp previously of the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham, who now however resides in Australia working for the Garvan Institute of Medical Research. Not only did Dr Philp encourage me to join his research team and enter postgraduate study, but also provided help and guidance whenever necessary.

I would also like to thank the remainder of the Philp lab for welcoming me into their team and for always answering any queries I may have had. A special thank you to Mr Ben Stocks and Ms Joanna Worthington for their assistance in the collection of the data presented in this thesis, and for their constant support throughout the duration of my studies.

Additionally, my fellow Masters students have made my time studying in Birmingham very enjoyable, alongside always being there to lend a hand or offer advice and guidance, and I would like to thank them for this.

Last but not least, I would like to thank my family and friends for their continued support throughout both my undergraduate and postgraduate studies.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
CHO	Carbohydrate
EDTA	Ethylenediamine tetraacetic acid
ERR	Estrogen-related receptor
FA	Fatty acid
GTE	Green tea extract
NA	Nicotinic acid
NAAD+	Nicotinic acid adenine dinucleotide
NAD	Nicotinamide adenine dinucleotide
NAMN	Nicotinic acid mononucleotide
NEFA	Non-esterified fatty acids
NMN	Nicotinamide mononucleotide
NR	Nicotinamide riboside
NRK	Nicotinamide riboside kinase
PARP	Poly-ADP-ribose polymerases
PBMC	Peripheral blood mononuclear cell
PGC-1α	PPAR-y coactivator-1α
PPAR	Peroxisome proliferator-activated receptor

RER	Respiratory exchange ratio
RPE	Ratings of perceived exertion
TF	Transcription factor
VE	Minute ventilation
WT	Wild type

INTRODUCTION

Regular physical activity is a preventative strategy for various chronic diseases such as type 2 diabetes, cardiovascular disease, hypertension and obesity (Warburton, Nicol & Bredin, 2006). Performing repeated bouts of exercise will, over time, induce various physiological adaptations that also facilitate an improvement in exercise performance (Jones & Carter, 2000). The type of training undertaken, such as endurance or resistance, determines the nature and magnitude of these changes (Jones & Carter, 2000).

Joyner & Coyle (2008) present a model of the multiple physiological factors that interact as determinants of performance (see Figure 1). Ultimately, they propose that exercise performance is determined by a combination of mechanical, cardiovascular, molecular, respiratory and anthropometric factors. The model suggests that morphological components such as stroke volume, haemoglobin content, aerobic enzyme activity and skeletal muscle fibre type together determine whole-body performance markers, such as maximal oxygen consumption (VO_{2max}), lactate threshold, mechanical efficiency and buffering capacity. These markers then interact at the whole-body level to determine the velocity and/or power of any given activity performed (Joyner & Coyle, 2008). Thus, changing any of these components has the potential to enhance these performance markers, and hence performance itself.

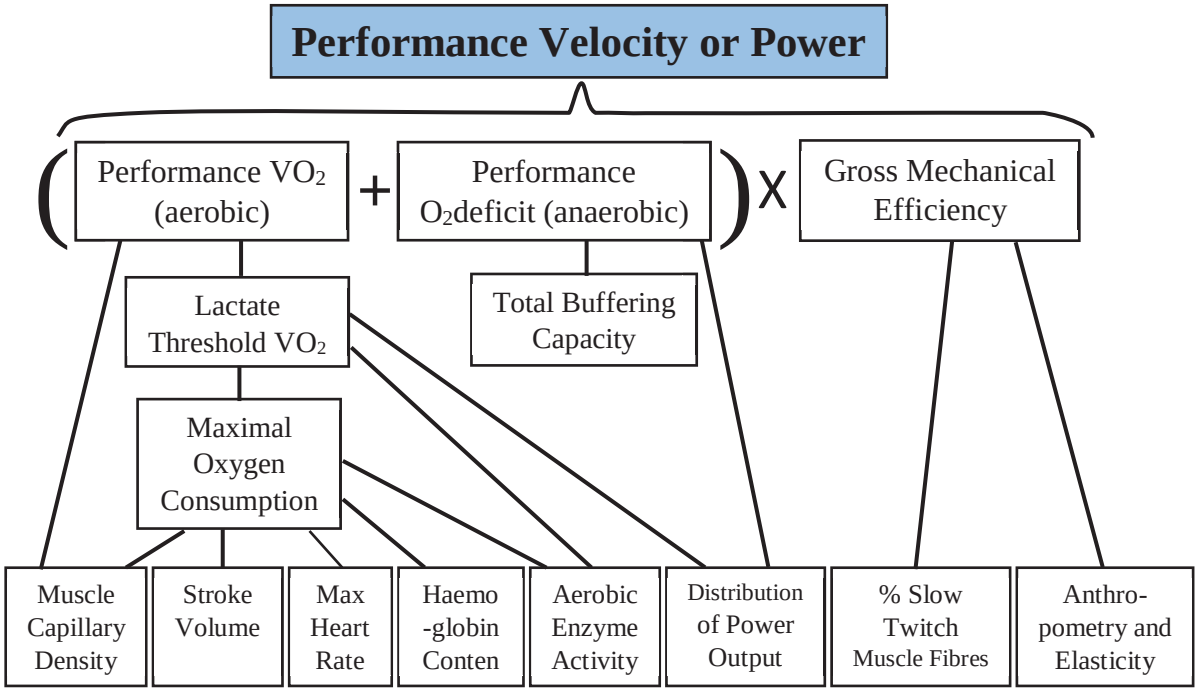


Figure 1. Schematic of the determinants of performance (reproduced from Joyner & Coyle, 2008)

SUBSTRATE UTLISATION

An additional factor that also influences performance is the contribution of fuels used during exercise. It is well known that carbohydrates (CHO) and fats are the dominant substrates oxidised in skeletal muscle during exercise in humans (Spriet, 2002), with a very minor contribution from protein. In comparison to CHO, fat is much more energy dense and is largely stored within the body – primarily in adipose tissue and as triglycerides within the muscle (Spriet, 2002). Following food consumption, macronutrients themselves do not directly provide energy – they require oxidation which releases adenosine triphosphate (ATP), and finally energy for work is released once ATP is hydrolysed (Melzer, 2011). The magnitude of energy expenditure, i.e. the intensity of exercise, is a determinant of whether this energy is generated anaerobically

or aerobically. During light exercise, aerobic respiration almost solely provides the energy required, however as this intensity increases, reliance on the anaerobic system becomes greater (Melzer, 2011). Although the oxidation of CHOs and fats occurs simultaneously, their contribution is largely dependent on various factors including exercise duration, exercise intensity, training status and nutrition (Venables, Achten & Jeukendrup, 2005). In an endurance trained state, fat oxidation at a given intensity is heightened, which corresponds with enhanced performance (Holloszy & Coyle, 1984).

FACTORS AFFECTING SUBSTRATE UTILISATION

The most thoroughly researched and seemingly important determinant of substrate utilisation is exercise intensity. In general, fat utilisation increases from low to moderate intensity exercise, and decreases from moderate to high intensities (Achten & Jeukendrup, 2003). Various studies have compared oxidation rates at different workload intensities, with results supporting this statement. In an early study by Romjin et al. (1993), five endurance-trained athletes completed three bouts of exercise on three consecutive days in a randomised order – one for 120 minutes at 25%VO_{2max}, another for 120 minutes at 65%VO_{2max}, and the third for just 30 minutes at 85%VO_{2max}. Exercise intensity and CHO oxidation rates displayed a significant positive relationship, however the reverse was not seen with fat oxidation. It was reported that after 30 minutes, fat oxidation rates were significantly higher at 65%VO_{2max} compared to both the lower and higher intensities. In fact, plasma free fatty acid (FFA) values were shown to persistently decrease at 85%, whereas a steady increase was observed with the moderate-intensity bout (Romjin et al., 1993).

Similarly in a later study, three consecutive 30-minute bouts of cycling were completed by eight participants, at 40%, 55% and finally 75%Watt max (Van Loon et al., 2001), with results indicating that whole-body fat oxidation was higher at 55%Wmax compared to 40%, with the lowest rates reported during exercise at 75%Wmax. However, total CHO oxidation was significantly higher with this increased intensity (Van Loon et al., 2001).

FAT_{MAX}

The exercise intensity at which the maximal fat oxidation rate occurs has been deemed 'Fat_{max}'. Various groups have attempted to determine this exact intensity, however this has proven difficult as it appears that the variation between individuals is large (Venables, Achten & Jeukendrup, 2005). Despite this, it has been reported that the majority of individuals' Fat_{max} occurs at a moderate-intensity exercise, ranging from 40%VO_{2max} up to 72%VO_{2max} (Bergman & Brooks, 1999; Achten, Gleeson & Jeukendrup, 2002a).

Achten, Gleeson & Jeukendrup (2002a) conducted a study in order to develop a test protocol to determine at which intensity Fat_{max} occurs. A set of incremental exercise tests with varying stage lengths and increment sizes were compared, with no significant differences in Fat_{max} shown between these. Furthermore, using intensities that corresponded to those in the incremental tests, various continuous prolonged exercise bouts at constant work rates were performed. In this study, the maximal rate of fat oxidation was calculated at, on average, 64±4 %VO_{2max}, with a Fat_{max} zone deemed between 55±3 % and 72±4 %VO_{2max} (Achten, Gleeson & Jeukendrup, 2002a).

The most time-efficient of the tested protocols from the study by Achten, Gleeson & Jeukendrup (2002a) was followed in a much larger study (Venables, Achten & Jeukendrup, 2005), in which 157 men and 143 women completed an incremental test to exhaustion on a treadmill, with intensity increasing every three minutes, then every one minute once RER reached a value of 1. The average intensity at which Fat_{max} occurred at in this study was lower in comparison, at 48.3%$VO_{2max.}$ However, the intensity at which Fat_{max} occurred in two more recent studies also following this same protocol differed from this, at 53±15%VO_{2max} in 281 student athletes (Randell et al., 2017) and at 60±16%VO_{2max} in 305 healthy volunteers (Fletcher et al., 2017), highlighting the large variability seen in this measure.

The intensity that Fat_{max} occurs at has also been associated with the onset of plasma lactate accumulation, with a significant correlation shown between the two when 26 male cyclists performed a graded test to exhaustion on a cycle ergometer (Achten, Gleeson & Jeukendrup, 2002b). Similar results were displayed in a later study by Achten & Jeukendrup (2004), in which it was concluded that there is a strong relationship between the onset of the decline in fat oxidation and the initial lactate increase above baseline during incremental exercise. Hence, at intensities below Fat_{max} (low or moderate-intensity exercise), lactate levels will likely remain at basal level.

CONTROL OF SUBSTRATE UTILISATION

Performing regular exercise is one way in which to alter an individuals' fat and CHO metabolism, with studies reporting that fat oxidation levels during exercise have increased by up to 44% in obese participants (Venables & Jeukendrup, 2008), and 29%

in healthy, non-obese participants (Schrauwen et al., 2002) after 4 and 12 weeks of training, respectively. In both studies, training sessions consisted of continuous exercise at a low-to-moderate intensity, and both also reported decreased CHO oxidation levels post-training.

As aforementioned, various studies have also demonstrated a change in the oxidation rates of fat and CHO during a single bout of exercise (Romjin et al., 1993; Van Loon et al., 2001), albeit largely dependent on exercise intensity and duration. The preference of substrate and the rate of substrate utilisation in skeletal muscle during these acute bouts is regulated by hormonal, metabolic and neural mechanisms (Esséen, 1977).

HORMONAL CONTROL OF SUBSTRATE UTILISATION

Collectively, hormones are involved in the regulation of many fundamental processes such as circulation, reproduction, growth and development, and metabolism (Galbo, 1986). Acute energy stress is a primary factor in hormone secretion, with marked changes observed in numerous hormones in response to stressors such as physical activity (Galbo, 1986). During times of stress, such as an acute exercise bout, the concentration of hormones that enhance lipolysis, glycolysis and gluconeogenesis rises (Bunt, 1986), emphasising their importance in regulating substrate metabolism.

Energy metabolism specifically is regulated primarily by the catecholamines, insulin, cortisol, glucagon and growth hormone (Bunt, 1986). The transition from rest to exercise causes the secretion of catecholamines, initiating the mobilisation of FFAs and

glucose. This response subsequently causes the release of secondary hormones, which then apply their actions to specific receptors on target cells (Bunt, 1986).

The catecholamines - adrenaline and noradrenaline - play an important role in controlling the metabolism of fats and CHOs, as well as stimulating the cardiac, respiratory and thermoregulatory systems (Zouhal et al., 2008). With exercise, catecholamine concentration rises proportionately with increased intensity and duration (Banister & Griffiths, 1972; Galbo, Holst & Christensen, 1975; Nilsson et al., 1975). Banister & Griffiths (1972) studied the effects of four different exercise intensities in five participants, and showed a progressive relationship between catecholamine concentrations and workload intensity/VO_2. Similar results were reported by Nilsson et al. (1975), where six healthy males completed a bout of exercise in which workload was increased every five minutes, for 15 minutes in total. A distinct increase in both noradrenaline and adrenaline concentrations were observed after 10 and 15 minutes, and these rises correlated with heart rate and mean blood pressure.

The increase in catecholamine secretion seen during exercise results in increases in glycogen utilisation and lipolytic rate. The infusion of adrenaline throughout a 40-minute cycle at 71%VO_{2peak} resulted in a rise in RER and net glycogen utilisation (Febbraio et al., 1998), whilst the inhibition of catecholamine secretion using a beta-adrenoceptor blockade was shown to reduce the exercise-induced rise in FFA concentrations during submaximal exercise (Hall et al., 1987), and glycerol during 30 minutes of cycling at ~66%Wmax (Arner et al., 1990).

On the contrary, plasma insulin concentration decreases during physical activity, and is inhibited by sympathetic nerves (Galbo, 1986). With an increase in both duration and

intensity, the inhibition of insulin secretion rises (Galbo, 1986). High levels of insulin during exercise would be counter-productive, as insulin works to enhance the synthesis of both fat and glycogen, and lower blood glucose levels (Bunt et al, 1986). Therefore, when insulin secretion is inhibited during exercise, the rate of lipolysis is accelerated, providing more glycerol and FFAs to be utilised (Wasserman et al., 1989).

MOLECULAR CONTROL OF SUBSTRATE UTILISATION

At the molecular level, this enhanced fat oxidation is the downstream result of the upregulation of various transcription factors (TF), co-activators and enzymes (Gerhart-Hines et al., 2007), as depicted in Figure 2. Both acute exercise bouts and long-term training can induce these changes (Pilegaard, Saltin & Neufer, 2003; Russell et al., 2003), and the coactivator peroxisome proliferator-activated receptor (PPAR)-y coactivator-1α (PGC-1α) appears to play arguably the most important role. Deemed the 'master regulator of mitochondrial biogenesis', PGC-1α has the ability to control the activity of several TFs, such as PPARs and estrogen-related receptors (ERRs) (Vega, Huss & Kelly, 2000; Schreiber et al., 2004), both regulators of the FA oxidation pathway (Finck & Kelly, 2006).

The overexpression of PGC-1α in mice was investigated during both voluntary and forced exercise, then compared to wild-type (WT) controls (Calvo et al., 2008). Not only did PGC-1α overexpression allow mice to run significantly further, faster, and for longer, they also elicited a 24% increase in VO_{2peak}. RER values observed were significantly lower at higher running speeds with overexpression, and the authors therefore propose that these mice were proficient at sparing CHO stores and hence were

utilising more FAs. Consistent with this result, analysis of muscle revealed elevated expression of FA transport/oxidation genes such as CD36, FA binding protein 3 (FABP3) and FA transport protein 1 (FATP1) in the PGC-1a overexpressed mice. It is plausible to propose that by upregulating PGC-1a via activation of its upstream targets during scenarios such as exercise or diet manipulation, that these same changes would occur.

PGC-1α itself is regulated by various pathways that are activated in response to different stimuli, namely energy stressors such as exercise and fasting (Fernandez-Marcos & Auwerx, 2011). Increased calcium levels, p38 mitogen-activated kinase (p38 MAPK) activation (Akimoto et al., 2005) and a heightened AMP/ATP ratio are all responses that occur due to a change in energy status following exercise, and they each initiate a number of mechanisms that induce the expression of PGC-1α. AMP-activated protein kinase (AMPK) is a fundamental energy sensor that is activated when there is a rise in the AMP/ATP ratio, and activates PGC-1α via phosphorylation (Fernandez-Marcos & Auwerx, 2011). When AMPK is activated acutely, glycogen and protein synthesis are suppressed, whilst glucose transport and fat metabolism are stimulated (Carling & Hardie, 1989; Bolster et al., 2002; Merrill et al., 1997; Winder & Hardie, 1996).

Another key energy sensor that when activated induces PGC-1α expression, is SIRT1. This well-researched member of the sirtuin family is also activated during times of energy stress, and upregulates PGC-1α via deacetylation (Fernandez-Marcos & Auwerx, 2011). Sirtuins are nicotinamide adenine dinucleotide (NAD)+-consumers, and require this coenzyme in order to be activated (Houtkooper et al., 2010).

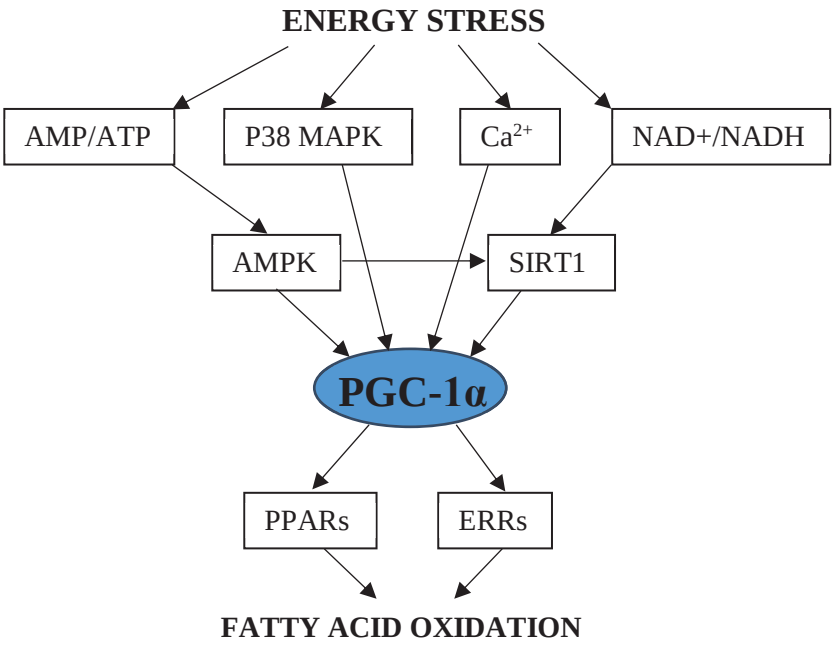


Figure 2. Molecular control of fat oxidation

NUTRITION

An important, additional factor influencing substrate metabolism and hence performance, is nutrition. Historically, research has focussed largely on the importance of CHO intake on performance. Krogh & Lindhard (1920) first established in human participants that exercising following the consumption of a high-CHO diet felt easier than following a high-fat diet. A few years later, it was shown that a decrease in blood glucose concentration was a hindrance to performance, yet that consuming CHOs prevented this decrease and therefore aided marathon running performance (Levine, Gordon & Derick, 1924). The relationship between CHO intake and muscle glycogen was first reported by Bergstrom et al. (1967), when it was shown that consuming a high-CHO diet increased muscle glycogen levels and hence enhanced one's capacity to complete prolonged bouts of exercise.

Following this discovery, studies investigating the effects of high CHO intake were in abundance, with new strategies emerging aiming to enhance glycogen stores prior to exercise. The classic approach to CHO loading for endurance events was studied first by Bergstrom et al. (1967), however a number of years later, Sherman et al. (1981) proposed a shorter, modified protocol involving a more gradual decrease in training coupled with an increase in CHO consumption. This protocol was later challenged by Bussau et al. (2002), who proved that just one day of high CHO intake resulted in maximal muscle glycogen content. Participants in this study were however endurance-trained athletes, so results may not be applicable to the general population.

Until more recently, research regarding the enhancement of fat oxidation via nutrition was limited. This is surprising, as the promotion of fat utilisation during endurance exercise should, in theory, slow the rate at which muscle glycogen is depleted and hence enhance exercise performance (Hawley, Brouns & Jeukendrup, 1998). Various nutritional strategies aiming to achieve heightened fat oxidation have been explored, including the ingestion of different supplements, the intake of fat before/during exercise, and the consumption of high-fat, low-CHO diets (Hawley, Brouns & Jeukendrup, 1998).

Despite efforts from several research groups, the ingestion of both medium- and long-chain triglycerides prior to and during exercise produced no significant effects on fat oxidation or exercise performance (Ivy et al., 1980; Satabin et al., 1987; Jeukendrup et al., 1995). Studies investigating the consumption of short-term (3 to 7 days) high-fat diets did however yield interesting results. When a five day high-fat diet was compared to a five day high-CHO diet, Jansson & Kaijser (1982) reported that following the high-

fat diet, fat oxidation was enhanced, however no evidence of glycogen sparing was distinguished during a 25-minute bout of cycling. On the contrary, long-term (>7 day) high-fat diets appear to increase fat oxidation levels without any detrimental effects on performance. Following 14 days of consuming a high-fat diet, trained cyclists performed significantly better in a moderate-intensity time to exhaustion test than after a high-CHO diet, despite beginning with lower muscle glycogen content (Lambert et al., 1994). This performance enhancement was accompanied by an increase in fat oxidation rates and a subsequent decrease in CHO oxidation rates.

NUTRITIONAL SUPPLEMENTS

The use of nutritional supplements to enhance fat utilisation is a thriving area of research, with this group of supplements often referred to as 'fat burners' (Jeukendrup & Randell, 2011). One substance that has received a great deal of attention is caffeine. Despite the vast number of studies that have investigated the effects of this supplement, results still appear to be inconclusive regarding its effects on fat oxidation. In an early study by Costill et al. (1978), trained cyclists were administered with caffeinated coffee 60 minutes prior to a bout of exercise to exhaustion at 80%VO_{2max}. Compared to a decaffeinated control, participants exhibited significantly increased fat oxidation during exercise, and this was supported by increased measurements of plasma FFAs and glycerol, and a decrease in RER. These results prompted the proposal of the theory that caffeine could enhance exercise capacity by increasing and decreasing fat and CHO oxidation, respectively, hence promoting glycogen sparing leading to enhanced endurance (Graham et al. 2008). However, it has more recently been proposed that

central mechanisms are more likely the cause of this, with evidence that caffeine increases the activity of the sympathetic nervous system, hence increasing adrenaline levels which can increase FA availability (Jeukendrup & Randell, 2011). In contrast however, numerous studies have also shown that caffeine has no significant effect on fat and CHO metabolism during exercise compared to a placebo, with measures such as RER and glycogenolysis remaining unchanged (Graham et al., 2000; Jackman et al., 1996). Additionally, one study has shown a tendency towards increased activity of muscle glycogen phosphorylase following caffeine ingestion (Chelsey et al., 1998), which should promote glycogenolysis and hence enhance CHO metabolism rather than reduce it.

Another supplement of interest in this area is green tea extract (GTE). The first study to explore how GTE effects fat utilisation at rest was conducted by Dulloo et al. (1999), who showed a significant increase in 24-hour fat oxidation following GTE consumption in comparison to caffeine and a placebo. However, a later study by Berube-Parent et al. (2005) reported no differences in fat oxidation between GTE and a placebo, despite finding an 8% increase in 24-hour energy expenditure. Supplementing GTE prior to exercise has produced similar ambiguous results. One study investigated the effects of GTE ingestion prior to a 30-minute cycle at 50%Wmax, finding positive results (Venables et al., 2008). Whole-body fat oxidation increased significantly with GTE, and the contribution of fat was 17% higher compared to placebo supplementation. However, a later study saw lower fat oxidation during a 60-minute cycle at 60%VO_{2max} following six days of supplementation, compared to caffeine and a placebo (Dean, Braakhuis & Paton, 2009), indicating that further research into this supplement may be necessary to confirm its effects on metabolism.

Very recently, an additional supplement with the potential to influence substrate metabolism has been discovered. Nicotinamide Riboside (NR) is a naturally occurring substance found in cow's milk (Bieganowski & Brenner, 2004), although it can also be obtained from unprocessed foods such as meat, eggs and green vegetables. NR is a form of vitamin B3, and works as an NAD+ precursor, meaning its consumption has the potential to increase NAD+ levels in various tissues.

NAD+

NAD+ is a cellular coenzyme, whose keystone function, along with its reduced form, NADH, was initially identified as inducing hydrogen transfer in metabolic oxidation-reduction reactions (Berger, Ramirez-Hernandez & Ziegler, 2004). More recent studies have revealed that NAD+ is also associated with additional reactions, acting as a co-substrate for various enzymes such as poly-ADP-ribose polymerases (PARPs) (Bai & Canto, 2012) and sirtuins (Houtkooper et al., 2010).

Oxidation-reduction reactions are essential to numerous metabolic pathways such as glycolysis, the Kreb's cycle, oxidative phosphorylation and anaerobic metabolism (Berg, Tymoczko & Stryer, 2002). NAD+ is an electron carrier, and when reduced to NADH, this electron can be transferred to other transported molecules – ultimately to deliver the electron to oxygen - via the above pathways (Berg, Tymoczko & Stryer, 2002). NAD+ and NADH together hence play a major role in the synthesis of ATP from various dietary sources (White & Schenk, 2012).

Alterations in NAD+ levels have the potential to change the activity of various enzymes involved in important metabolic pathways (Imai, 2009). As the amount of NAD+ is limited in a cell, it must be regenerated via the re-oxidation of NADH for these pathways to ensue (Berg, Tymoczko & Stryer, 2002). The balance of the oxidation-reduction state of NAD+ and NADH was previously assumed to determine the NAD+/NADH ratio, however the discovery of the biosynthetic and salvage pathways of NAD+ and the actions of NAD+-consumers has enhanced the complexity of this ratio greatly (White & Schenk, 2012).

NAD+ CONCENTRATIONS

NAD+ is primarily located within three cellular compartments – the cytosol, the nucleus, and the mitochondria (White & Schenk, 2012). The total NAD+ and NADH concentrations in skeletal muscle have been estimated at ~1.5-1.9 and ~0.08-0.2mmol/kg dry muscle, respectively (Graham et al. 1978). However, the way in which this is distributed between each compartment varies greatly; in the cytosol, values have been reported at around 0.15 and 0.00028mmol/kg wet muscle, and in the mitochondria, values are much higher at 3.15 and 0.5mmol/kg wet muscle (Cabrera et al., 1998). In general, little is known about nuclear NAD+ levels, although it is thought that the cytosolic and nuclear compartments are in equilibrium, and hence this coenzyme can pass freely through these membranes (Easlon et al., 2008).

To enable the movement of NAD+/NADH into the mitochondria however, shuttles are required due to the impermeability of the mitochondrial membrane (Palmieri, 2004). In

skeletal muscle, the glycerol 3-phosphate and malate-aspartate shuttles allow for this movement (White & Schenk, 2012).

NAD+ AND EXERCISE

Intracellular NAD+ levels increase in response to energy stressors, such as low glucose levels and exercise (Gerhart-Hines et al., 2007). In early animal studies, it was concluded that in mammalian muscle, this rise predominantly occurred within the mitochondria (Jobsis & Stainsby 1968). In human studies however, results are conflicting regarding NAD+/NADH action at various exercise intensities. Graham et al. (1978) measured muscle NAD+ at rest and following a bout of cycling at both ~75% and ~100% VO_{2max}. Following work at both of these intensities, muscle NAD+ content significantly decreased. Conversely, in a later study, both NAD+ and the cytosolic NAD+/NADH ratio increased above resting levels at these same intensities (Sahlin et al., 1987).

NAD+ CONSUMERS

Both the synthesis and breakdown of NAD+ influences its abundance within a cell. NAD+ acts as a co-substrate for three types of enzymes – sirtuins, poly-ADP-ribose polymerases (PARPs) and cADP-ribose synthases – these each consume NAD+ and release nicotinamide (Nam) and an ADP-ribosyl product (Belenky, Bogan & Brenner, 2007).

Sirtuins act as both transcriptional activity effectors and energy sensors, through their ability to control the acetylation state of transcriptional regulators, such as histones (Houtkooper et al., 2012). Sirtuins are NAD+-dependent, and when activated they trigger pathways that enhance mitochondrial oxidative metabolism and metabolic efficiency (Imai & Guarente, 2014). Although the sirtuin family consists of seven different proteins (SIRT1-SIRT7), SIRT1 and SIRT3 are the most widely studied and seemingly important of these (North & Verdin, 2004).

When PARPs consume NAD+, this is either to cause an ADP-ribosyl protein modification or to create the ADP-ribose polymer, PAR (Bai & Canto, 2012). PARP activity is upregulated by breaks in DNA strands (Malanga & Althaus, 2005), and plays a role in transcriptional regulation, cell division and energy metabolism (Schreiber et al., 2006).

NAD+ BIOSYNTHESIS

NAD+ is synthesised from smaller units, either via the *de novo* or salvage pathways (see Figure 3). *De novo* synthesis is an eight-step process and begins with the amino acid tryptophan, which is, in short, converted into quinolinic acid, then into nicotinic acid mononucleotide (NAMN). NAMN is then adenylated to form nicotinic acid adenine dinucleotide (NAAD+), which is finally converted to NAD+ by the enzyme NAD+ synthase (Bogan & Brenner, 2008). NAD+ can also be generated from the salvageable precursor vitamins nicotinic acid (Na), nicotinamide (Nam) and NR. As detailed below in Figure 3, fewer steps are required to rebuild NAD+ from these precursors. Alongside tryptophan, each of the precursors is available through one's diet, and hence dietary

manipulation can impact NAD+ levels. However, as tryptophan plays a role in various other biosynthetic processes, its efficiency is limited in comparison to the salvage pathways (Canto, Menzies & Auwerx, 2015).

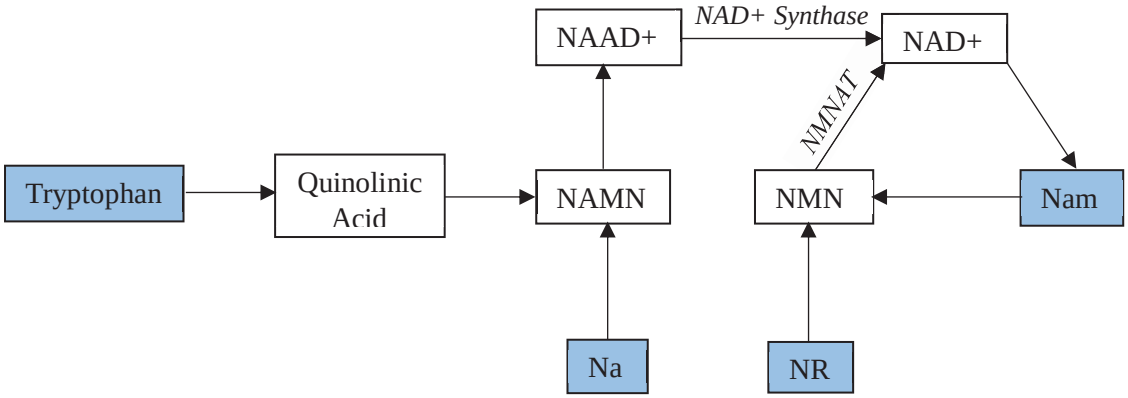


Figure 3. NAD+ biosynthesis

DEPLETED NAD+ LEVELS

Nam and Na are collectively termed niacin, which is essentially the vitamin form of NAD+ (vitamin B3). Dietary intake of niacin, NR and/or tryptophan prevent pellagra, a disease carrying symptoms such as dermatitis, diarrhoea and dementia (Bogan & Brenner, 2008). A lack of niacin was determined as the cause of this disease, hence why eating foods containing high levels of this vitamin (e.g. milk, eggs and meat) acted as a cure (Bogan & Brenner, 2008). Another condition associated with depleted NAD+ levels is dyslipidemia, which involves the elevation of triglycerides in the blood (Ratajczak et al., 2016). As Nam can inhibit sirtuin activity and is hence less efficient at lowering these lipid levels, high doses of Na are often prescribed to prevent and treat

dyslipidemia (Ratajczak et al., 2016). Although successful, these doses cause painful flushing as a side effect, as a result of the activation of the GPR109A receptor (Wise et al., 2003). It is believed however, that the effectiveness of Na is likely due to its ability to increase NAD+ levels, which can also be achieved by another vitamin precursor that does not activate GPR109A and hence gives rise to no adverse side effects – NR (Canto et al., 2012).

NICOTINAMIDE RIBOSIDE

Prior to 2004, it was believed that niacin was the only dietary NAD+-precursor available. However, Bieganowski & Brenner (2004) discovered that in yeast, NR is also an effective precursor that enables NAD+ biosynthesis. Unlike niacin, NR is unique as it directly enhances the synthesis of NAD+ via the nicotinamide riboside kinase (NRK) pathway in skeletal muscle (Bieganowski & Brenner, 2004). Once NR enters the cell it is first phosphorylated to NMN by these NRKs, and NMNAT then converts this into NAD+ (Belenky, Bogan & Brenner, 2007).

Following the initial discovery of NR and its pathways, Belenky et al. (2007) went on to investigate the effects that exogenous NR had on WT yeast cells, rather than modified cells with gene deletions as had been examined previously (Bieganowski & Brenner, 2004). It was discovered that intracellular NAD+ levels in yeast cells were doubled with the addition of 10μM NR, however only when grown in Na-free media. This supplementation also significantly prolonged lifespan in both WT and mutant strains, in comparison to those strains that were NR-deficient and showed no extension in lifespan.

Based on the mechanisms by which NR works, much research has investigated whether the use of this vitamin would be beneficial in various clinical populations. Mice with Alzheimer's (Gong et al. 2013), noise-induced hearing loss (Brown et al. 2014), mitochondrial myopathy (Khan et al. 2014), muscular dystrophy (Ryu et al. 2016) and type 2 diabetes (Yoshino et al. 2011) have all shown improvements following NR administration, albeit some more pronounced than others.

The first study to provide evidence for the effects NR has on skeletal muscle was conducted by Canto et al. (2012), where mammalian cells and mouse tissues were supplemented with NR, and the effects of this on oxidative metabolism and high-fat diet-induced obesity were assessed. This study was designed on the premise that increased NAD+ levels leads to enhanced sirtuin (specifically SIRT1 and SIRT3) activity, which in turn may lead to protection against metabolic diseases and may also heighten fat oxidation (Pfluger et al., 2008). Firstly, mice and human cells were treated with NR, with reports that NAD+ content was significantly enhanced in muscle and liver cells, and importantly, in the mitochondria. In response to this increase in NAD+, enhanced deacetylation of both SIRT1 and SIRT3 targets were seen in skeletal muscle, liver and brown adipose tissue cells in vivo, and hence, increased acetylation of PGC-1a was also observed. The effect of NR on mice consuming a normal chow diet and mice consuming a high-fat diet were compared following this, producing positive results. Although NR had no effect on body weight in mice on the chow diet, those on a high-fat diet showed a decrease in weight gain due to reduced fat mass. It was confirmed that this decrease was due to enhanced energy expenditure, which was linked to heightened oxygen consumption rates with NR administration. These results are promising for

future research looking into NR and exercise, as an increase in oxygen consumption should correlate with improvements in performance (Joyner & Coyle, 2008).

However, as shown in a recent study by Kourtzidis et al. (2016), it appears that NR supplementation has the opposite effect on exercise performance. Following 21 days of NR supplementation (300mg/kg BW/day), nine Wistar rats completed an incremental swimming performance test, in which they swam until exhaustion under an incremental load in warm water. Although results were not significant, the NR group tended towards a poorer physical performance by 35% in comparison to control rats. This protocol however initiated a survival response mechanism, as the rats were swimming to avoid drowning. It is possible that use of an alternative exercise mode would produce opposing results, as was seen with enhanced treadmill running performance in mice administered with NR (Canto et al., 2012). More research into the relationship between NR and exercise performance would provide a clearer insight into whether this supplement has the potential to be performance enhancing.

NR SUPPLEMENTATION IN HUMANS

Until recently, NR administration had only been investigated in yeast, mammalian cells and rodents. However, Trammell et al. (2016) conducted the first human trial in the form of a pilot study in one healthy, male participant, in which the participant supplemented with 1,000mg of NR daily, for seven days. Frequent blood samples were taken in the hours following ingestion of the first dose, and analysis showed that NAD+ levels peaked 7.7 hours post-ingestion (2.7-fold increase from baseline), with no changes seen in the first 2.7 hours (see Figure 4 for the PBMC NAD+ blood response).

Consistent with previous NR literature, no adverse side-effects were observed throughout or following the supplementation period.

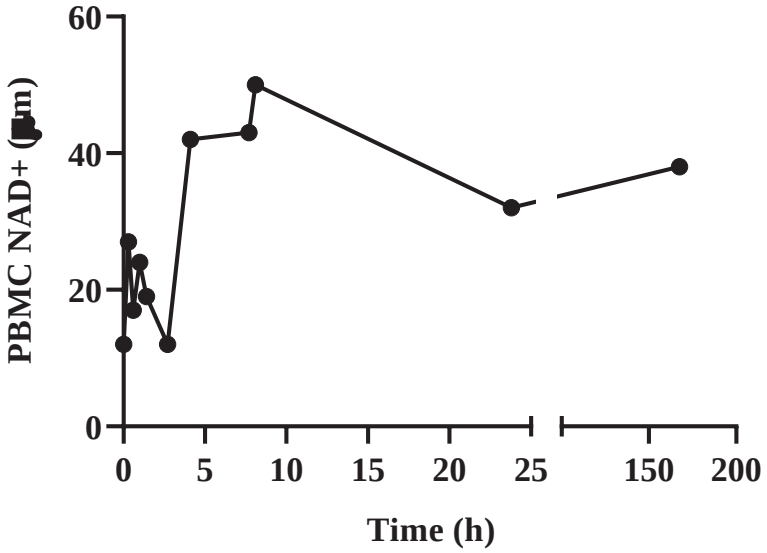


Figure 4. PBMC NAD+ concentration with respect to whole blood volumes following consumption of 1,000mg NR daily for seven days in one healthy, male participant (data from Trammell et al. 2016).

This pilot study provided confirmation that NR is effective, and safe for human consumption, leading to the conduction of the first clinical trial in humans (Trammell et al., 2016). Twelve healthy participants (six male and six female) were recruited to establish whether NR works in a dose-dependent manner, and hence each orally consumed a single dose of 100mg, 300mg and 1,000mg NR in a randomised order, with a seven day washout period between each. After analyses of various NAD+ metabolites,

it was concluded that NR works dose-dependently, with the greatest rise in NAD+ levels observed following the consumption of 1,000mg NR (see Figure 5).

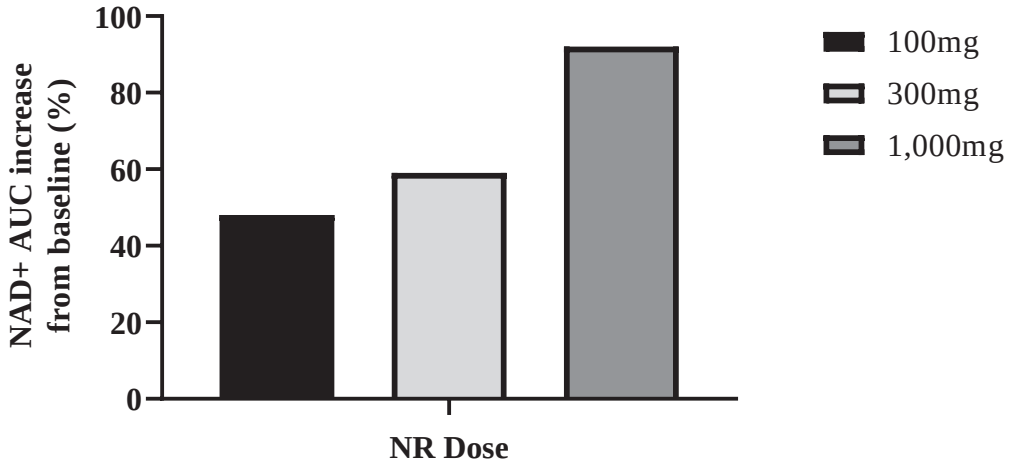


Figure 5. NAD+ metabolite AUC increase from baseline (%) in response to 100mg, 300mg and 1,000mg doses of NR in 12 participants (data from Trammell et al. 2016).

NR DOSING STRATEGIES

The optimal dosing strategy of NR is currently unknown, with rodent studies having only studied NR in a chronic fashion, and in clinical populations such as in models with obesity (Canto et al., 2012), diabetes (Yoshino et al., 2011) and mitochondrial myopathy (Khan et al., 2014).

However, a recent study investigated the effects of a range of NR concentrations in mice consuming a mildly obesogenic diet, with metabolic flexibility as the main outcome measure (Shi et al., 2017). This study was designed not only because the authors believed the NR doses used in previous studies appeared “strikingly high

compared to most commercial supplements", but also because the benefits of vitamin B3 on metabolic health are currently unidentified (Shi et al., 2017). NR doses ranging from to 5 to 900mg/kg of diet (5NR, 15NR, 30NR, 180NR and 900NR) were administered in nine-week old mice for a 15 week supplementation period. Despite the 900NR dose being significantly higher than all other administered doses, no differences were reported in serum or liver levels of NR, tryptophan or Nam when compared to the 15, 30 or 180NR doses. The gene expression of NR pathway enzymes (NRK1/2, NAMPT and NMNAT) in skeletal muscle, liver and white adipose tissue also displayed no variation with changes in dose. Results showed that with 30NR, fasting metabolic flexibility was significantly enhanced in comparison to 5NR, and refeeding metabolic flexibility was also significantly greater compared to 5NR, 15NR and 900NR respectively. This dose also exhibited the highest expressions of PGC-1a and PPARγ, both key mitochondrial genes that play important roles in fat metabolism.

Although lacking in depth, the research looking at NR consumption in humans appears to promote the daily dose of 1,000mg NR for the greatest effects on the NAD+ metabolome (Trammell et al., 2016). However, the length of the supplementation period required to optimally induce these changes needs further investigation.

Whilst the evidence surrounding NR administration in cells and rodents is fairly extensive, with many studies reporting the clear benefits of this supplement, research in human participants is lacking. This opens the door to an exciting realm of study into how NR consumption may affect various human clinical populations, or even if it has the capacity to work as a performance-enhancing supplement.

An increase in NAD+ levels heightens the NAD+/NADH ratio, which has the potential to enhance the enzymatic activity of SIRT3 (Hirschey et al., 2010). Upregulating SIRT3 expression results in increased deacetylation of various metabolic enzymes including those involved in beta-oxidation, and can subsequently enhance fat oxidation (Hirschey et al., 2010). With evidence in mice that NR supplementation enhances NAD+ levels, boosts sirtuin activity and hence improves oxidative capacity and mitochondrial function (Canto et al., 2012), together with the confirmation from Trammell et al., (2016) that consumption of a single dose of NR significantly increases NAD+ levels in human participants, it is viable to suggest that NR administration has the potential to promote fat oxidation in humans. Acute bouts of moderate-intensity exercise also have the ability to increase fat oxidation, via various hormonal, metabolic and neural pathways. Hence, the present study aims to investigate whether the consumption of NR prior to an acute bout of steady-state exercise will have an added effect on whole-body fat oxidation, in comparison to a placebo.

An increase in fat oxidation during a bout of steady-state exercise should also, in theory, enhance one's performance in a higher intensity time-trial immediately post-exercise, via muscle glycogen sparing. Elevated levels of glycogen spared during the steady-state exercise should provide more glycogen for the latter stages of the performance, perhaps allowing one to produce a higher power output for longer and complete the time-trial more rapidly. Hence, following the steady-state exercise bout, the present study will also include a 5km time-trial performance test in order to compare exercise performance following both NR and placebo consumption.

It should however be noted that muscle glycogen sparing is not certain. Despite the hypothesis put forward by numerous researchers that CHO consumption during moderate-intensity exercise will reduce the rate of muscle glycogenolysis and hence improve performance, the literature surrounding this mechanism indicates that utilisation of muscle glycogen is not consistently reduced with CHO consumption (Karelis et al., 2010).

HYPOTHESES

1. Nicotinamide riboside supplementation will promote enhanced fat oxidation during a bout of steady-state exercise compared to a placebo trial.
2. Nicotinamide riboside supplementation will improve time-trial performance compared to a placebo trial.

METHODOLOGY

PARTICIPANTS

Eleven healthy, young males volunteered to participate in this study (see Table 1 for participant characteristics). All participants were recreationally active, achieving the UK government physical activity guidelines for adults (150 minutes of moderate intensity, or 75 minutes of vigorous intensity activity per week), however were excluded if they were specifically endurance-trained athletes. Inclusion criteria required participants to be aged between 18 and 35, have a body mass index (BMI) between 18 and 30kg/m^2, and a VO_{2max} below 60mL/kg/min. This latter value was determined during the participant's first visit to the laboratory following the completion of an incremental exercise test on a cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). In addition to not fulfilling the inclusion criteria, participants were also excluded from participating in the study if they were: currently taking part in another study; consuming a high-fat, low-carbohydrate diet; answered 'yes' to any questions on the health screening form (e.g. history of elevated blood pressure, heart condition, chest pain, elevated blood cholesterol, asthma, diabetes, hepatitis, bleeding disorders); had a past history of substance abuse, smoking or was taking medicine/supplements that may influence normal metabolic processes; or finally, had donated blood within twelve weeks of the study commencing.

Ethical approval was granted by the University of Birmingham's School of Sport, Exercise and Rehabilitation Sciences Ethics Health and Safety Committee before the study began.

Table 1. *Participant characteristics*

Variable	n=11
Age (years)	24.91±2.34
Weight (kg)	77.86±5.89
Height (cm)	181.36±8.51
VO_{2max} (ml/kg/min)	43.8±6.50

Values are means ± S.D.

STUDY DESIGN

This was a single-centre study with a randomised, counter-balanced, crossover, double-blind design. Participants were required to visit the Human Performance Laboratory in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham on five separate occasions, over a period of approximately four weeks. Visit 1 consisted of the completion of a health and activity questionnaire, the determination of anthropometric measures (height and weight), and the signing of informed consent. If eligible thus far based on the inclusion/exclusion criteria, participants then performed an incremental test on a cycle ergometer to determine VO_{2max}. Visits 2 and 3 acted as familiarisation trials for the 5 kilometre time-trial (TT) that participants were to complete as part of the experimental trials, and hence the results of these visits were not used in final analysis. Visits 4 and 5 were both experimental trials, identical other than the supplement that was consumed prior to the trial. Participants completed one bout of steady-state exercise followed by a 5km TT per

visit, once having supplemented with NR, and the other having supplemented with a placebo (cellulose).

DETAILS OF LABORATORY VISITS

VISIT 1.

Participants had the opportunity to discuss the study with the investigators in more depth and ask any questions they may have had. If they decided to participate, they then signed informed consent, had anthropometric measures recorded, and completed the screening and health/activity questionnaire. If the individual was deemed eligible to partake in the study based on the inclusion/exclusion criteria, they then completed an exercise test on a cycle ergometer.

Prior to the onset of the exercise test, participants completed a 5-minute warm-up period of cycling at 50 Watts (W). Participants then undertook an incremental exercise test, and began cycling at a resistance of 75W for 3 minutes. Every 3 minutes, investigators increased the resistance of the bike by 25W. Ratings of perceived exertion (RPE) were determined using a 6-20 Borg scale (Borg, 1973), and recorded during every 3-minute increment. However once participants reached 15 (‘Hard’) or above on this scale, workload was increased by 25W every 1 minute. Participants were instructed to continue cycling until they could not maintain a cadence above 60 revolutions per minute (RPM) or requested that the test was stopped (volitional fatigue). Thereafter, they completed a 5-minute cool down period of unloaded cycling.

Using a breath-by-breath metabolic cart (Vyntus CPX, Jaeger, CareFusion, Germany), respiratory variables were measured allowing the calculation of maximal oxygen consumption (VO_{2max}, mL/min/kg), minute ventilation (VE) (L/min), VO_2 (mL/min), VCO_2 (mL/min) and the respiratory exchange ratio (RER). Prior to use, the metabolic cart was calibrated for volume using a 3 litre gas syringe, and was gas calibrated with commercial gases of known concentration.

VO_{2max} was calculated using 30-second averages from the metabolic cart output, with the maximum average value determined as the participants' VO_{2max} score. This output was also used to calculate the power at which each individual would be required to cycle at during the steady-state exercise in the experimental trials; a scatter graph of VO_2 values at each power output during the incremental test was created, and from this the power in Watts corresponding to 55%VO_{2max} was calculated.

VISITS 2 AND 3.

In a study by Sewell & McGregor (2008), the use of one familiarisation trial (20km TT) was reported to lower the variance shown between the subsequent three TTs completed by seven recreationally active adults (mean coefficient of variance = 3.4%). In order to ensure reduced levels of variance between experimental trials in the present study, it was decided participants would complete two familiarisation sessions prior to these. Results of these visits were however not used during analysis.

Following a 5-minute warm-up of cycling at 50W, the bike was programmed to the linear mode, and participants were instructed to complete a 5km self-paced TT, which typically lasted between 10-15 minutes.

For each participant, the resistance added during the trial was determined using an alpha value that was calculated so that power output would require ~86%VO_{2max}, whilst cycling at 80RPM (as used in Sewell & McGregor, 2008);

$$Alpha = (0.86*Wmax) / 80^2$$

Participants were provided with verbal encouragement, and feedback in regard to distance cycled every 1km, then 500m and 250m from the end. They were however blinded from time, power output (Watts) and RPM throughout the trial.

VISITS 4 AND 5.

Following their evening meal the night before each experimental trial (~8pm), participants were instructed to consume 1,000mg NR or placebo in the form of four 250mg capsules. This dosing strategy was chosen based on the results of the first clinical NR trial in humans, in which it was concluded that NR works in a dose-dependent fashion, with the greatest rise in NAD+ levels observed following the consumption of 1,000mg NR (Trammell et al., 2016). Additionally, in the n=1 study from Trammell et al. (2016), it was shown that following the consumption of a single 1,000mg dose of NR, NAD+ levels in peripheral blood mononuclear cells (PBMC) rose significantly, remaining elevated for up to 24 hours post-consumption (Trammell et al., 2016). Participants were provided with these capsules after the completion of visits 3

and 4, and were sent text message reminders to consume the supplements the evening before both trials.

Upon arrival on the day of the trial, participants had a cannula (BD Venflon™, India) placed in a forearm vein to allow for a baseline and then repeated blood sampling during the experimental session. Participants then completed a 60-minute steady-state cycle at 55%VO_{2max} (as calculated from Visit 1). The intensity of 55%VO_{2max} was chosen based on evidence that moderate-intensity exercise is most likely to promote enhanced fat utilisation. Although variable between individuals, 'Fat_{max}' has been deemed to occur at exercise intensities ranging between 40% and 72%VO_{2max} (Bergman & Brooks, 1999; Achten, Gleeson & Jeukendrup, 2002a).

For five minute periods every 15 minutes during the trial (at 15, 30, 45 and 60 minutes), participants wore a mask connected to the metabolic cart to enable the collection of breath-by-breath data for analysis. During each of these five minute periods, RPE and heart rate (HR) (Polar RCX5, New York) were also recorded. After a 2-minute break following completion of the steady-state exercise, participants then completed a 5km self-paced time-trial, as they did in visits 2 and 3. These experimental visits lasted approximately two hours.

Visit 5 was identical to visit 4, however participants consumed the alternative supplement prior to this visit. Both participants and investigators were blinded to supplement order, which was randomised. Visits 4 and 5 were separated by at least one week, to allow for wash-out of the supplement.

Following each experimental trial, breath-by-breath data was extracted from the metabolic cart. From each of the five minute periods recorded, the first minute of data

was disregarded, and four minute averages were calculated for VE (minute ventilation), VO_2, VCO_2 and RER at each time point.

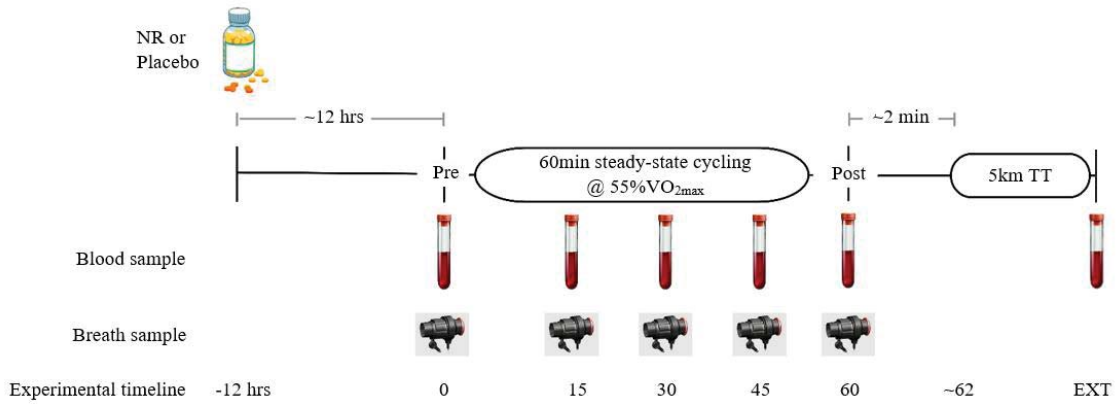


Figure 6. Experimental trial schematic

RANDOMISATION AND BLINDING

Participants were enrolled by the study investigator, however participant trial order (NR or placebo supplement order) was randomly assigned by another researcher, who was not involved in participant recruitment, data collection or analyses. Randomisation was achieved using the free online research randomiser software (www.randomizer.org), which generated a random trial order for all (n=11) participants. The designated laboratory member labelled the supplements 'A' and 'B', and until all data collection was complete, only this laboratory member had access to the randomiser output and the record of which supplement carried each label. All relevant information was disclosed to the study investigator and participants following completion of all analyses.

SUPPLEMENTATION

Following their third visit to the laboratory, participants were provided with either 1,000mg NR or cellulose (placebo), dependent on their randomised trial order. They were then provided with 1,000mg of the remaining supplement following the completion of visit 4, and were instructed to consume this prior to visit 5, after a washout period of approximately seven days. This washout period was chosen based on the methodology of the first clinical NR trial in humans, in which twelve participants consumed a single dose of 100mg, 300mg and 1,000mg NR in a randomised order with a seven day washout period between data collection (Trammell et al., 2016). Supplements were given in the form of 250mg capsules, and participants were instructed to orally consume four the night before each trial (~12 hours prior); a total dose of 1,000mg per visit. The timing and size of the NR dose was based on the results of the first human NR trial, in which one male participant consumed 1,000mg NR for seven days (Trammell et al., 2016). Blood analysis showed that following just one 1,000mg dose of NR, PBMC NAD+ levels were heightened significantly, peaking after 7.7 hours. To allow participants a normal night of sleep, supplements were consumed ~12 hours prior to exercise rather than 8 hours prior, however as NAD+ levels remained elevated up to 24 hours post-consumption in the pilot study (Trammell et al., 2016), this was deemed adequate.

Both the experimental and placebo supplements were commercially available:

- NR: NIAGEN® capsules, ChromaDex. For more information please see https://www.chromadex.com/niagen/

- Cellulose: Microcrystalline Cellulose, Blackburn Distributions. For more information, please see http://www.blackburndistributions.com/microcrystalline-cellulose-mcc-powder.html

NUTRITION AND PHYSICAL ACTIVITY CONTROLS

Participants were instructed to arrive fasted to visits 1, 4 and 5, having not consumed any food after 8pm the previous night. They were however permitted to drink ~300mL of water prior to these visits, to ensure they were sufficiently hydrated. As visits 2 and 3 were familiarisations and completed purely for the benefit of the participant, these nutrition/activity controls were not applied. However, exhaustive activity and the consumption of alcohol, caffeine or any other supplement was prohibited in the 24 hours leading up to all visits.

One day prior to the first experimental visit (visit 4), participants were required to complete a food diary. They were then asked to replicate this diet as closely as possible on the day before their second experimental visit (visit 5). Participants were also required to provide the experimenters with a copy of this diary at visit 4.

CANNULATION

The cannula was inserted into a vein in the forearm upon arrival to the laboratory on visits 4 and 5. To allow for plasma, red blood cell (RBC) and PBMC analysis, 26mL (2 x 10mL, 1 x 6mL) samples were taken at the onset of steady-state exercise, following completion of steady-state exercise, and upon completion of the 5km time-trial. At 15,

30 and 45 minutes into exercise, 6mL samples were taken for plasma and RBC analysis. In total, 6 blood samples (96mL in total) were taken throughout each visit.

BLOOD PREPARATION

PLASMA AND RBC:

For all time points (baseline, 15, 30, 45, 60 and post-TT), 6mL blood was drawn into ethylenediamine tetraacetic acid (EDTA) collection tubes (BD, Plymouth, UK), inverted, and left to stand on ice until the experimental trial was complete. Upon completion of the trial, these tubes were centrifuged at 1,600xG at 4°C for 8 minutes (Thermo Scientific, Massachusetts, USA). Plasma was then extracted and transferred into 1.5mL tubes (Eppendorf, Germany). From the baseline, 60 and post-TT samples, 100µL RBC was aliquoted into chilled 2mL tubes (Eppendorf, Germany), each containing 1mL 0.5M Perchloric Acid. All plasma and RBC samples were flash frozen in liquid nitrogen, then stored at -80°C until further analysis.

PBMC:

The 20mL (2 x 10mL) blood samples taken at baseline, 60 and post-TT time points were drawn into heparin collection tubes (BD, Plymouth, UK), inverted, and left to stand in ice until the experimental trial was complete. Samples were transferred into 50mL conical centrifuge tubes (Falcon, Mexico), and then diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco™, Paisley, Scotland), and mixed via inversion. 5mL Ficoll-Pacque Plus (GE Healthcare, Uppsala, Sweden) was pipetted into 15mL conical tubes (Falcon, Mexico) and 10mL of blood/RPMI was pipetted

carefully on top of the Ficoll-Paque. Tubes were then centrifuged at 400xG for 30 minutes at room temperature (22°C). 15mL concical tubes were then prepared with a small amount (approximately 2mL) of autoMACS Running Buffer (Miltenyi Biotec, Germany). The PBMC layer from the previously centrifuged 15mL tubes was carefully pipetted off, and decanted into the tubes containing autoMACS Running Buffer. The tubes were then filled with autoMACS Running Buffer, and centrifuged at 300xG for 10 minutes at 4°C. Tubes were removed from the centrifuge, and the supernatant was poured away. Each remaining pellet was then re-suspended in 1mL freezing medium (90% fetal bovine serum, 10% dimethyl sulfoxide), and pipetted into 2mL tubes (Eppendorf, Germany) to be frozen. The tubes were transferred into a Mr. Frosty freezing container (Thermo Scientific, Massachusetts, USA), and stored at -80°C until further analysis.

BLOOD ANALYSIS

PLASMA:

Plasma samples were analysed on an auto-analyser (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for non-esterified fatty acid (NEFA), lactate, glycerol (Randox Laboratories, County Antrim, UK) and glucose (Instrumentation Laboratory, Bedford, MA, USA) using commercially available kits. For each metabolite, 200µL of a control sample (CON) and a standard sample (CAL) were aliquoted into cuvettes and placed in the assigned tray position on the auto-analyser.

CON samples:

- *NEFA:* Human Assay Control 2 (HAC2) and HAC3
- *Lactate:* HAC2 and HAC3
- *Glycerol:* Glycerol CON
- *Glucose:* SeraChem Control Level 1 (Sera1) and Sera2

Reagents for each metabolite were made up according to the instructions supplied with the kits, and also placed in their assigned tray position. Before sample analysis, the auto-analyser was calibrated via analyses of the CON and CAL samples, and if the mean of these values was within the acceptable range (found on the instruction sheets), sample analysis could be undertaken. Prior to running the sample analysis, 200µL of each plasma sample was aliquoted into a cuvette and placed into the tray in the auto-analyser. Samples were run in duplicate, and mean values were calculated for use during data analysis.

The intra-assay (inter-assay) coefficient of variation (CV) for the above methods are as follows (based on analysis of 20 duplicate samples): NEFA, 3.34% (3.1%); lactate 2.04% (3.9%); glycerol, 2.76% (5.9%) and glucose, 1.48% (5.9%) (Robinson, 2016).

PBMC:

PBMC samples were sent away to be analysed in order to measure the NAD+ metabolome, as described by Trammell et al. (2016). However due to time constraints, the results of these analyses are not reported in this thesis.

CALCULATIONS

Total CHO and fat oxidation rates (g/min) were calculated from the rates of O_2 and CO_2 production (L/min) recorded at baseline, using the following equations proposed by Frayn (1983):

Carbohydrate oxidation (g/min) =4.55 x VCO_2 – 3.21 x VO_2

Fat oxidation (g/min) =1.67 x VO_2 – 1.67 x VCO_2

For VO_2 and VCO_2 values (L/min) obtained during SS exercise, the following equations proposed by Jeukendrup & Wallis (2005) were used, working under the assumption that protein oxidation during exercise is insignificant:

Carbohydrate oxidation (g/min) = 4.210 x VCO_2 – 2.962 x VO_2

Fat oxidation (g/min) = 1.695 x VO_2 – 1.701 x VCO_2

STATISTICAL ANALYSIS

Data analysis was performed using IBM SPSS (Version 23) software. Data are expressed as mean ± standard deviation (S.D.), and the significance level was set at $p < 0.05$. A two-way repeated-measures analysis of variance (ANOVA) was used to assess the effects of time, condition and time*condition interaction effects for all steady-state exercise data (VO_2, VCO_2, RER, fat oxidation, CHO oxidation, HR, RPM and blood variables). Time trial performance was compared using paired-sample t-tests. Ryan-Holm-Bonferroni adjustment was applied to data in which significant effects were shown.

RESULTS

WORKLOAD

The workload of 55%VO_{2max} used throughout the 60-minute steady-state exercise corresponded to an average power output of 136±11 Watts. Participants cycled at the same fixed Watts for each of their experimental trials.

OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION

During the steady-state exercise, five minute measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded at baseline, then every 15 minutes during exercise (see Table 2).

For both VO_2 and VCO_2, a main effect of time was shown ($p = .000$), yet there was no main effect of condition (NR vs. PLA) ($p = .884$ and $p = .406$, respectively) or a time*condition interaction ($p = .422$ and $p = .451$, respectively). Post-hoc analysis revealed that baseline VO_2 values (NR: 0.39±0.07, PLA: 0.40±0.04) were significantly different from all other time points during exercise (NR: 15min 1.98±0.41, 30min 2.02±0.41, 45min 2.04±0.39 and 60min 2.04±0.43, PLA: 15min 1.96±0.37, 30min 2.01±0.37, 45min 2.02±0.38 and 60min 2.06±0.38, $p < .05$). VCO_2 values at baseline (NR: 0.33±0.05, PLA: 0.34±0.03) also significantly differed from those during exercise (NR: 15min 1.79±0.33, 30min 1.79±0.33, 45min 1.80±0.32 and 60min 1.78±0.34, PLA: 15min 1.82±0.38, 30min 1.82±0.35, 45min 1.8±0.35 and 60min 1.83±0.36, $p < .05$). There were however no significant differences between exercise time points ($p > .05$).

Table 2. *Mean VO_2 and VCO_2 values for NR and placebo conditions at baseline and during steady-state exercise*

	NR		PLA	
Time	**VO_2 (L/min)**	**VCO_2 (L/min)**	**VO_2 (L/min)**	**VCO_2 (L/min)**
Baseline	0.39±0.07	0.33±0.05	0.40±0.04	0.34±0.03
15	1.98±0.41*	1.79±0.33*	1.96±0.37*	1.82±0.38*
30	2.02±0.41*	1.79±0.33*	2.01±0.37*	1.82±0.35*
45	2.04±0.39*	1.80±0.32*	2.02±0.38*	1.8±0.35*
60	2.04±0.43*	1.78±0.34*	2.06±0.38*	1.83±0.36*

Values are expressed as mean ± S.D, n=11. *$p < .001$ vs. baseline values.

RESPIRATORY EXCHANGE RATIO (RER)

A main effect of time ($p = .017$) was seen in mean RER values, indicating that RER rose significantly with time (NR: Base 0.85±0.09, 15min 0.91±0.04, 30min 0.89±0.03, 45min 0.88±0.03 and 60min 0.87±0.32, PLA: Base 0.85±0.56, 15min 0.92±0.04, 30min 0.9±0.03, 45min 0.89±0.03 and 60min 0.89±0.03, see Figure 7). At no time points during exercise did values significantly differ from baseline ($p > .05$), however a significant difference was seen between each exercise time point ($p < .05$). No interaction effect or main effect of condition was seen, however ($p = .455$ and $p = .648$, respectively), signifying that NR consumption had no effect on substrate use.

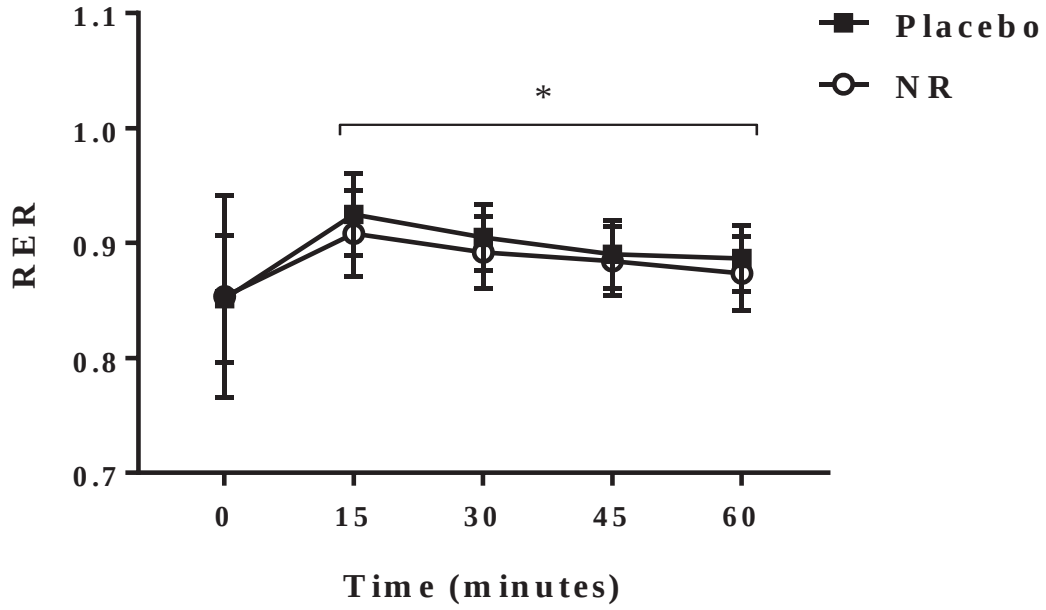


Figure 7. Mean RER values measured at baseline (0 min) and throughout the 60-minute SS exercise for NR (○) and placebo (■) conditions. *p < .05 vs. time point. Values are means ± S.D., n = 11.

FAT AND CARBOHYDRATE OXIDATION

As expected, whole-body fat oxidation increased from baseline and throughout the 60-minute steady-state exercise with a significant effect of time (p = .000, see Figure 8A), and significant differences between each time-point (NR: Base 0.10±0.06, 15min 0.31±0.19g/min, 30min 0.37±0.18 g/min, 45min 0.40±0.17 g/min and 60min 0.44±0.19 g/min, PLA: Base 0.10±0.04, 15min 0.24±0.10 g/min, 30min 0.31±0.10 g/min, 45min 0.36±0.11 g/min and 60min 0.38±0.08 g/min, p < .05). Consequently, whole-body CHO oxidation significantly decreased throughout exercise with a main effect of time seen (p = .000, see Figure 8B), however there was no significant difference shown between 45

and 60 minute time points ($p > .05$). No interaction effect ($p = .309$ and $p = .310$), nor main effect of condition ($p = .211$ and $p = .249$) was seen for either fat or CHO oxidation, respectively, indicating that NR supplementation had no significant effects on substrate utilisation in comparison to a placebo.

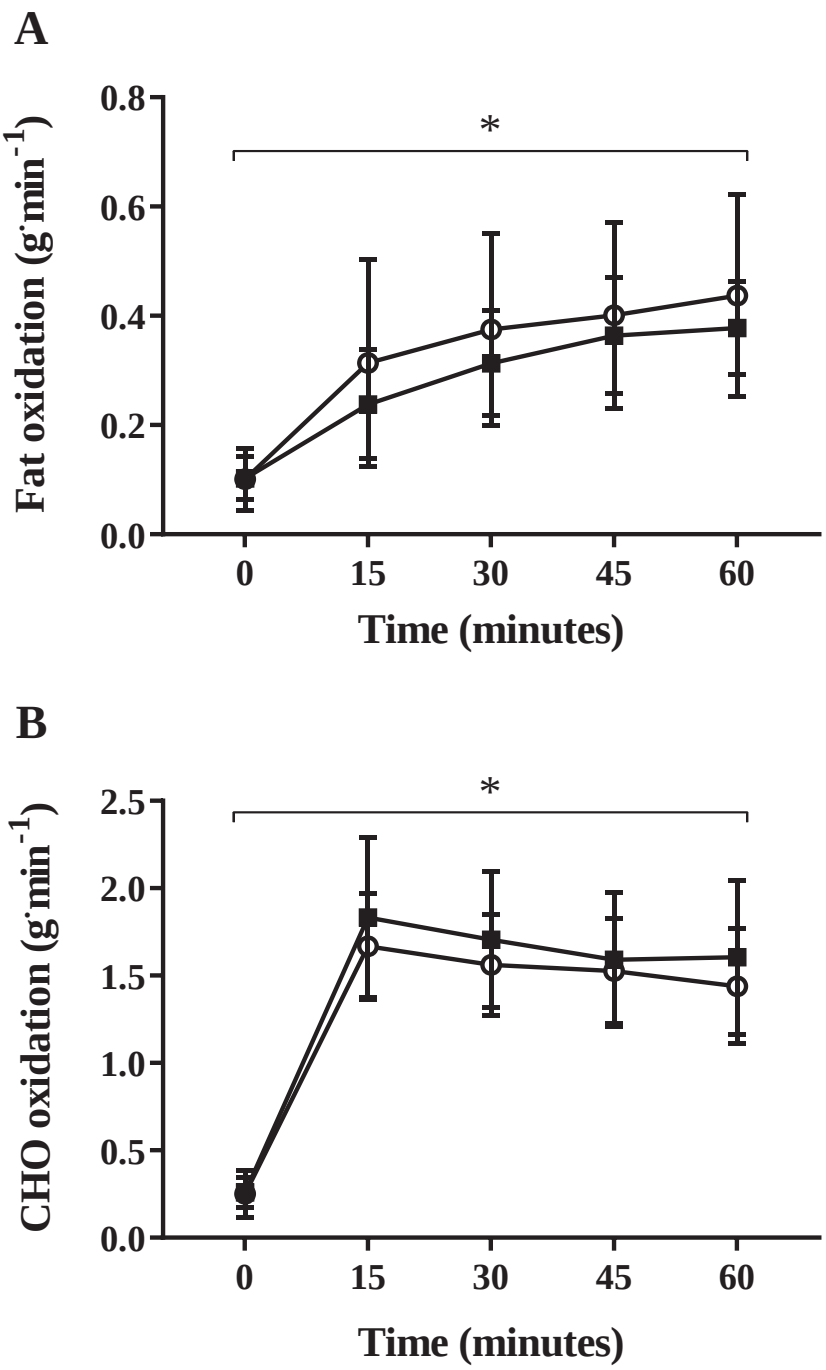


Figure 8. Fat (A) and CHO (B) oxidation rates at baseline (0 min) and throughout the 60-minute SS exercise, for NR (○) and placebo (■) conditions. *p < .05 vs time point (excluding 45 vs. 60 for CHO). Values are means ± S.D., n = 11.

The mean contribution of substrates at baseline and at each time-point during the steady-state exercise is shown in Figure 9. With NR supplementation, the overall contribution of fat to total substrate utilisation was 22.3±9.0%, and 19.6±7.3% with placebo, however this difference was not significant and therefore did not elicit a main effect of condition (p = .429) or an interaction effect (p = .647). But as expected, there was a main effect of time (p = .008), with significant differences shown between all time points (p < .05), excluding between baseline and 45 min, and baseline and 60 min.

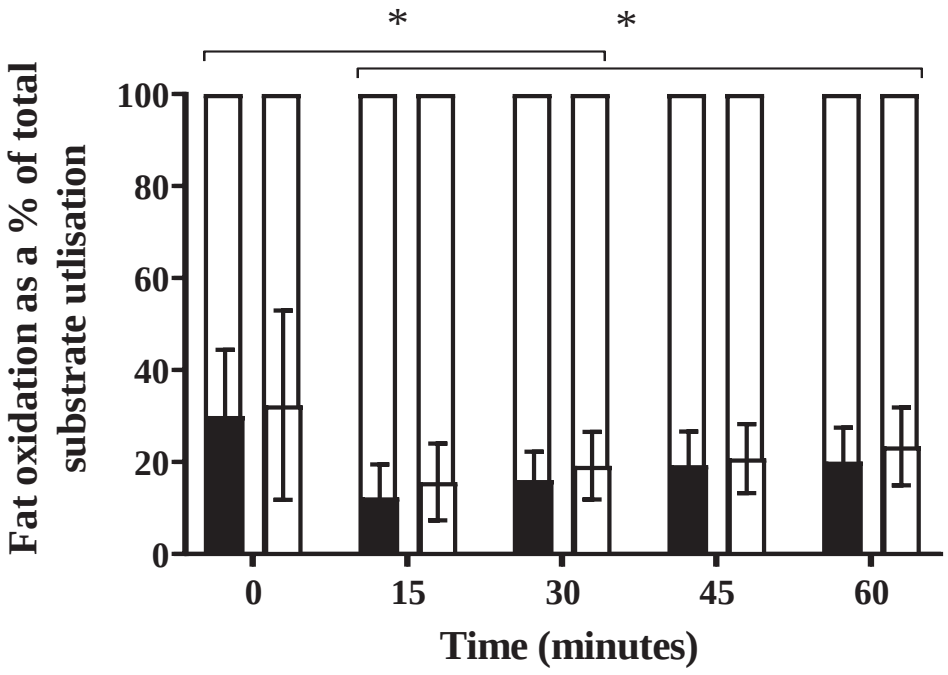


Figure 9. Mean contribution of substrate (FAT, CHO) as a percentage at baseline and throughout the 60-minute SS exercise, for NR (□) and placebo (■) conditions. *p < .05 vs time point. Values are means ± S.D., n = 11.

HEART RATE

Due to equipment malfunction, HR data is only reported for 10 out of 11 participants. A significant increase in HR occurred between baseline and all exercise time points, and between 15min and all remaining exercise time points (NR: Base 70±11 bpm, 15min 122±9 bpm, 30min 126±9 bpm, 45min 127±11 bpm and 60min 130±13 bpm, PLA: Base 68±7, 15min 123±11 bpm, 30min 126±13 bpm, 45min 128±11 bpm and 60min 131±14 bpm, $p < .05$), contributing to the main effect of time shown ($p = .000$, see Figure 10A).

The consumption of NR had no effect on HR at baseline or during exercise however, as there was no main effect of condition or an interaction effect shown ($p = .857$ and $p = .534$, respectively).

RATINGS OF PERCEIVED EXERTION

RPE values elicited a significant main effect for time ($p = .015$) in which they increased throughout exercise (Figure 10B), however no significance was shown between any specific time points during post-hoc analysis ($p > .05$). There was also no time*condition interaction effect seen ($p = .813$), nor was there a main effect of condition ($p = .267$), meaning perceived exertion was not affected by the supplementation of NR.

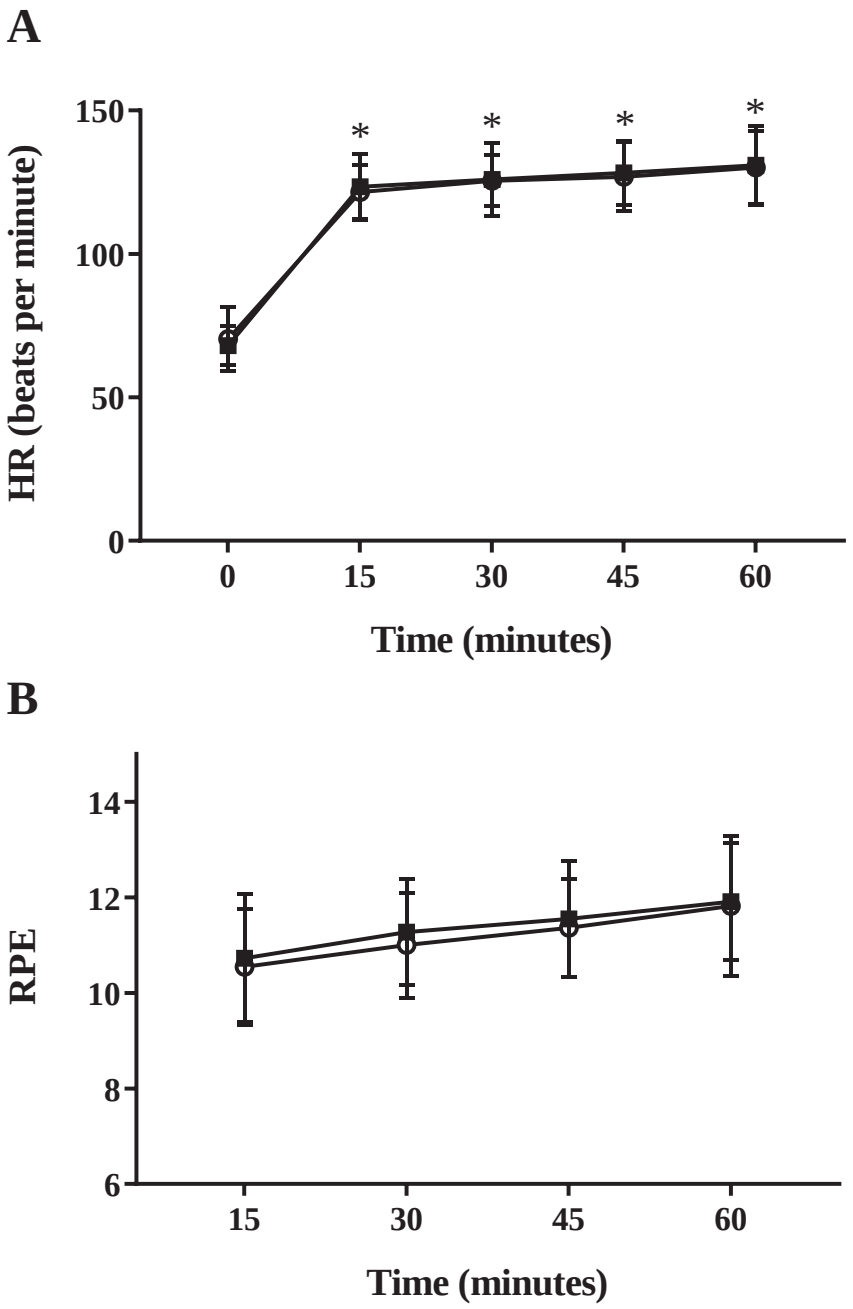


Figure 10. Mean HR values (A) measured at baseline (0 min) and throughout the 60-minute SS exercise (n=10) and mean RPE values (B) throughout exercise (n=11), for NR (○) and placebo (■) conditions. *$p < .05$ vs baseline and 15 min time points. Values are means ± S.D.

TIME TRIAL PERFORMANCE

T-test analysis indicated that there was no significant differences in TT performances between the participants' first and second experimental trials (p = .85). This confirms that the two familiarisation TTs undertaken by all participants prior to these trials were sufficient at eliminating a learning effect.

One participants' TT data was excluded from the results, due to equipment malfunction. The average time to completion of a 5km TT following the consumption of placebo and NR were 823.4±250.0 and 819.5±239.8, respectively (Figure 11). Hence, the supplementation of NR had no effect on performance as no statistical difference was seen between these results (p = .72).

The large variability between participants is clear when studying the individual data points also presented in Figure 11, although individuals did show no significant differences in time to completion between trials.

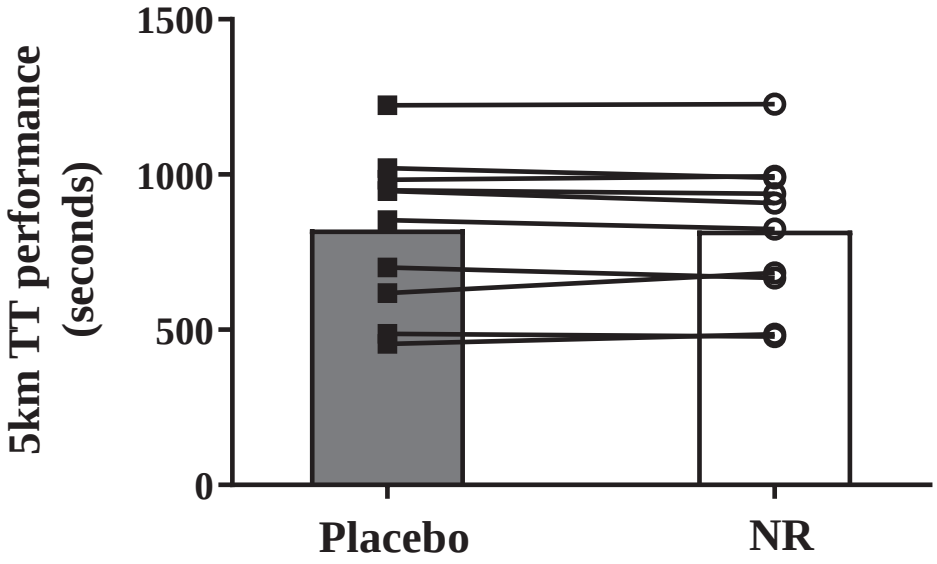


Figure 11. Mean 5km TT performance times for NR and placebo conditions, with individual participant data also displayed. Values are means ± S.D., n = 10.

PLASMA METABOLITES

Figure 12 shows the effects of both time and condition (NR or placebo) on plasma NEFAs, glycerol, glucose and lactate. Due to some unattainable samples from one participant, all blood data from this participant was not included in analyses.

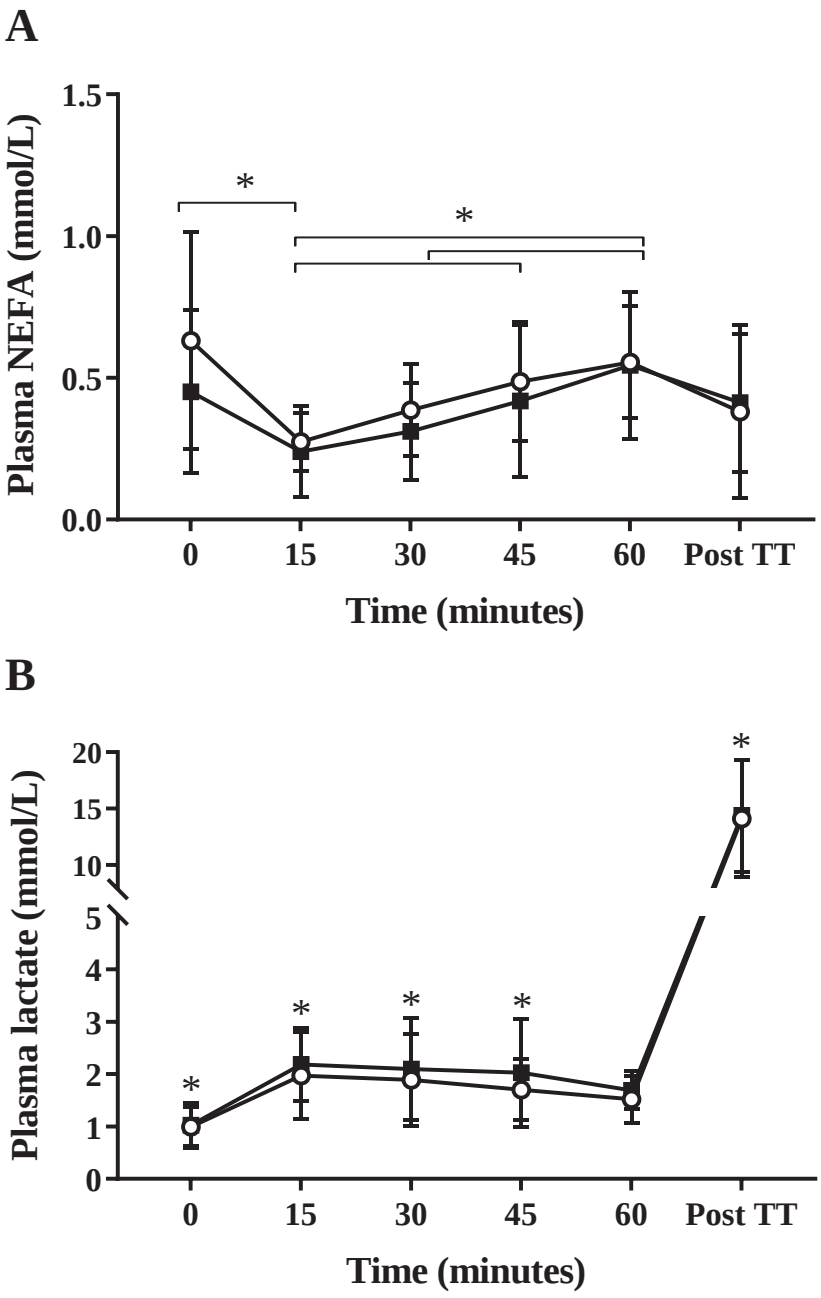
Plasma NEFA, lactate, glycerol and glucose concentrations were all unaffected by NR supplementation, as there was no significant main effect of condition (p = .435, p = .521, p = .162 and p = .811, respectively) or a time*condition interaction (p = .237, p = .838, p = .620 and p = .756, respectively) shown for any metabolite.

However, there was a main effect of time shown for plasma NEFA, lactate and glycerol (p = .002, p = .000 and p = .000, respectively). Average NEFA concentration decreased

from baseline with the onset of steady-state exercise with a significant difference seen between baseline and 15 min ($p < .05$), then increased with time during exercise, with significant differences shown between 15 and 45, 15 and 60, and 30 and 60 min time points ($p < .05$). Immediately following the TT, NEFA concentrations tended to decrease again, although this was not significant (Figure 12A, $p > .05$).

Plasma lactate concentration was significantly different between baseline and all remaining time points ($p < .05$), but did not differ throughout the 60-minute steady-state exercise ($p > .05$). However, following the high-intensity 5km TT, lactate concentration significantly increased (Figure 12B), with differences shown between post-TT and all other time points, ($p < .05$). These differences together contributed to a main effect of time ($p = .000$).

There was an increase in plasma glycerol concentration with time during steady-state exercise, and this continued to rise post-TT. A main effect of time was seen ($p = .000$), with significant differences shown between all time points excluding between baseline and 15 min (Figure 12C, $p < .05$). Finally, plasma glucose concentrations did not differ with time or supplement, with no main effect of time ($p = .068$) nor condition ($p = .811$) shown despite there being significant differences shown between baseline and both the 30 and 45 min time points (Figure 12D, $p < .05$).


A
Plasma NEFA (mmol/L)
1.5
1.0
0.5
0.0
*
*
0
15
30
45
60
Post TT
Time (minutes)
B
Plasma lactate (mmol/L)
20
15
10
5
4
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2
1
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Time (minutes)

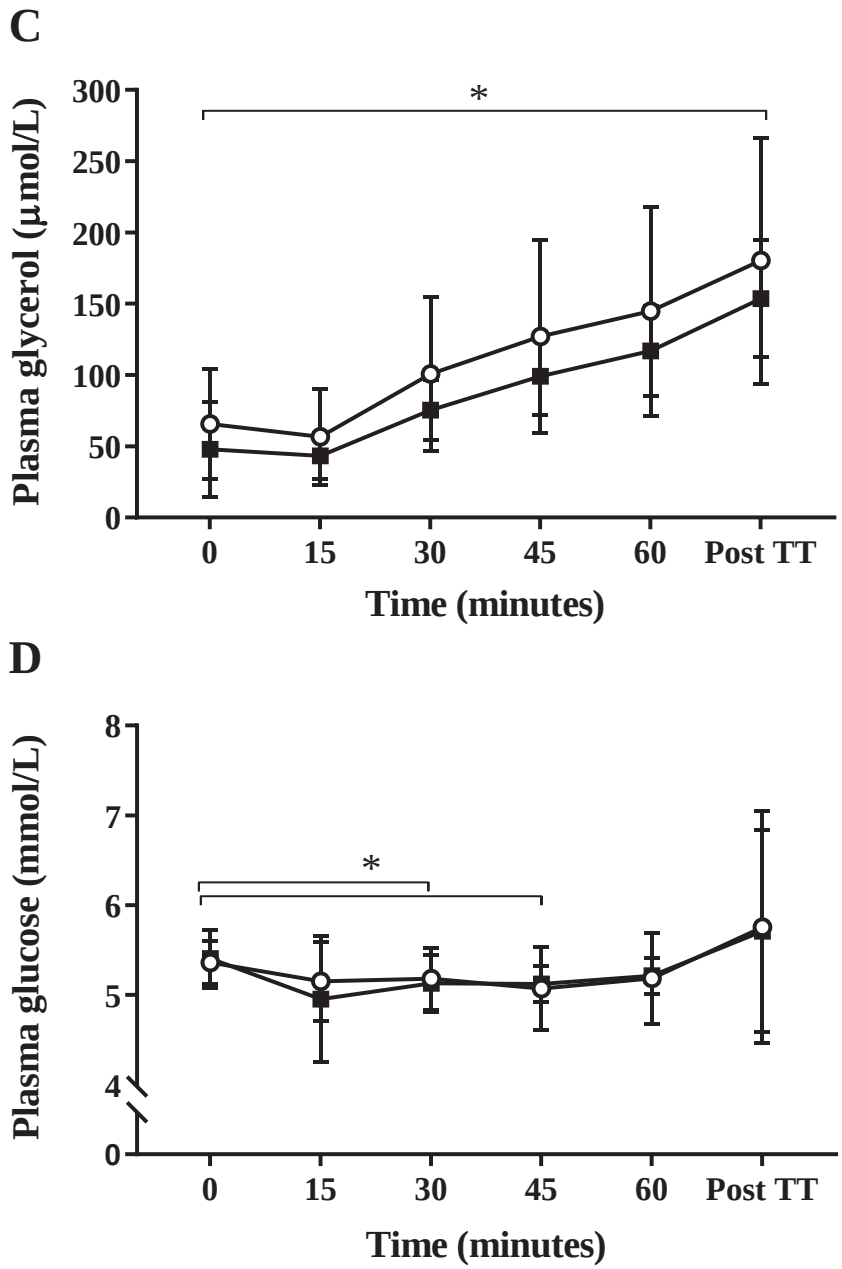

Figure 12. Mean plasma concentrations of NEFA (A), lactate (B) glycerol (C) and glucose (D) at baseline (0 min), throughout the 60-minute SS exercise and immediately post-TT, for NR (○) and placebo (■) conditions. *$p < .05$ vs time points as described in text. Values are means ± S.D.. n = 10.

DISCUSSION

The present study is the first to investigate the effects of Nicotinamide Riboside supplementation on humans during exercise, with a specific focus on substrate metabolism and performance. This study expands on the limited research surrounding NR consumption in humans, as the effects of this substance during exercise have only been examined in rodents thus far (Canto et al., 2012; Kourtzidis et al., 2016). The primary aims of this study were to investigate whether the consumption of a single dose of NR alters substrate utilisation during moderate-intensity exercise, and performance during a high-intensity 5km TT in recreationally-active males, in comparison to a placebo.

It was shown that the consumption of NR had no effect in the present study, with no significant main effects of condition or time*condition interactions shown for any outcome measure. Although main effects of time were reported for almost all measures, NR in no way enhanced these effects, opposing the hypotheses. The intensity and duration of the steady-state exercise were sufficient to alter substrate utilisation, with a significant increase in fat oxidation and parallel decrease in CHO oxidation seen throughout exercise (see Figure 8). Both blood analysis and RER results support these findings, with plasma NEFA and plasma glycerol concentrations significantly increasing (see Figure 12), and RER values significantly decreasing with exercise (see Figure 7), indicating that the use of fats as an energy source was enhanced with time. Steady-state exercise also gave rise to significant increases in both HR and RPE, however there were no differences seen between the NR and placebo conditions for any

of these measures (see Figure 10). No significance was shown between the two conditions when comparing TT performance either, hence confirming that NR consumption does not improve performance in a 5km TT following a bout of steady-state exercise (see Figure 11).

As the first study of its kind, and one of very few trials to investigate NR in human participants, the hypotheses proposed in the present study are based on limited evidence. Hence, the results of this study should not be seen in a negative light, as they in fact provide a wider insight into the effects of NR in humans - specifically in exercise - and also offer a foundation for future research to build upon. Modifications to the design of this study are necessary to test the full potential of NR with exercise, whether that be altering the exercise intensity, duration or mode, changing the dosing strategy or combining supplementation with long-term exercise training.

During prolonged steady-state cycling, a gradual depletion of muscle glycogen usage and a shift towards fat oxidation occurs (Watt et al., 2002). Hence, reducing the intensity but increasing the duration of exercise would likely promote fat oxidation more so than during the 60-minute bout in the present study.

It is possible that in order to see an effect of NR supplementation, intra-muscular NAD+ needs to be altered. The dosing strategy used in the present study is known to influence the NAD+ profile in the blood (Trammell et al., 2016), however no data exists as to how this relates to skeletal muscle thus far. It is possible that longer term NR supplementation would be required to alter intra-muscular NAD+ levels.

Finally, the combination of exercise training and long-term NR supplementation has the potential to further enhance the physiological adaptations seen from training alone, due to the positive effect both components can have on the activity of sirtuins.

SUBSTRATE UTILISATION

It was hypothesised that fat oxidation may increase more so during steady-state exercise in the NR condition than with a placebo, due to the downstream effects of enhanced deacetylation of SIRT3, in response to heightened NAD+ levels (Hirschey et al., 2010). However, as no significant differences were seen between conditions, it appears this was not the case. As the first human study to investigate the effects of NR on substrate metabolism, this hypothesis was based on the evidence that NR has the ability to boost SIRT3 activity in mice (Canto et al., 2012), which upregulates various metabolic enzymes, subsequently increasing fat oxidation. In this study by Canto et al. (2012) however, NR was administered long-term, in contrast to the single dose consumed in the present study.

Based on the results of Trammell et al. (2016), it can be assumed that NAD+ levels in the blood of participants in the present study were enhanced following the consumption of NR. Yet based on the lack of significant differences between conditions, it appears that this heightened NAD+ level in the blood is not great enough to have an effect on substrate utilisation. It is possible that an increased NAD+ level in tissue, such as muscle or liver, is required for NR to alter substrate use. Chronic NR supplementation in rodents has been seen to increase NAD+ levels in both liver and muscle tissue (Canto

et al. 2012), suggesting that a longer supplementation period may be necessary to induce any significant changes to health and/or performance.

Despite the significant increase in fat oxidation throughout steady-state exercise in the present study, and consequent decrease in CHO oxidation, it is likely that the duration and/or intensity of this exercise did not lead to maximal rates of fat oxidation. The intensity of 55%VO_{2max} used in the present study, based on previous Fat_{max} studies (Achten, Gleeson & Jeukendrup, 2002a; Venables, Achten & Jeukendrup, 2005; Randell et al., 2017; Fletcher et al., 2017) should be sufficient for promoting near maximal fat oxidation. Hence, it seems that the duration of steady-state exercise undertaken was not optimal. In a study by Watt et al. (2002), substrate utilisation was monitored throughout a four hour cycle at 55%VO_{2max} in seven endurance-trained men. It was shown that fat oxidation rates surpassed those of CHO only after participants had been exercising for over 120 minutes, and by the end of the four hour cycle, the rate of fat oxidation was markedly higher than that of CHO. These results were supported by the considerable changes seen in substrate use throughout exercise - both plasma FFA and plasma glycerol use increased progressively as time went on, whereas muscle glycogen decreased substantially. Hence, it would appear that the 60-minute exercise bout undertaken in the present study was not a powerful enough stimulus to promote fat oxidation to the desired level. It is possible that if fat oxidation had been enhanced more so - by increasing the duration of the steady-state exercise - a difference between the NR and placebo conditions may have been shown.

The utilisation of substrates is controlled by numerous hormonal, metabolic and neural mechanisms (Esséen, 1977). Whilst it cannot be ruled out entirely, it appears that NR

does not have the capacity to alter hormonal activity, unlike other ‘fat burning’ supplements, such as caffeine and green tea. Although results regarding an alteration in substrate utilisation in humans are ambiguous, there is evidence that caffeine works to enhance sympathetic nervous system activity, heightening adrenaline levels which can hence increase lipolytic rate and FA availability (Jeukendrup & Randell, 2011). Green tea works in a similar manner, due to its high capacity of catechin polyphenols (Jeukendrup & Randell, 2011). These catechins directly inhibit the enzyme catechol-O-methyltransferase, which works to lower noradrenaline levels. This hence has the potential to increase FA oxidation, by acutely enhancing the stimulation of the sympathetic nervous system (Jeukendrup & Randell, 2011). The way in which these substances work appears to differ greatly from the way in which NR does, as a change in NAD+ levels does not directly alter catecholamine secretion. It seems that the speculated molecular changes alone brought about by acute NR supplementation, if any, were not potent enough to alter substrate utilisation in the present study.

TIME TRIAL PERFORMANCE

The supplementation of NR did not affect 5km TT performance following a 60-minute bout of moderate-intensity steady-state exercise, with mean performance times of 819.5±239.8 seconds in the NR condition, and 823.4±250.0 seconds with the placebo. The hypothesis that TT performance would be improved following NR supplementation was based on the premise that fat oxidation would be increased during the 60-minute steady-state exercise, sparing muscle glycogen - the dominant fuel source at higher intensity exercise (Van Loon et al., 2001) - and hence allowing participants to exert a

higher power output for longer during the TT. However, fat oxidation was not enhanced by NR consumption during the steady-state exercise, supporting the non-significant difference between TT performances. Based on the results of the aforementioned study by Watt et al. (2002), it appears that the duration of the steady-state cycling in the present study was not long enough to promote maximal rates of fat oxidation, allowing for the attenuation of muscle glycogen use in order for it to later be spared in the TT. In fact, it seems the steady-state exercise would have needed to double in duration for this mechanism to work, with Watt et al. (2002) reporting a decline in CHO oxidation 120 minutes into exercise, corresponding with the decrease in muscle glycogen use also seen.

Although sparse, previous studies also investigating the effects of NR supplementation on exercise performance in rodents have produced contrasting results (Kourtzidis et al., 2016; Canto et al., 2012). Following a three week NR supplementation period, rats tended to perform worse in an incremental swimming test compared to a control group (Kourtzidis et al., 2016). Conversely, in high-fat diet fed mice, endurance performance in a treadmill exercise test was significantly improved after long-term NR supplementation (Canto et al., 2012). An alternative approach to increasing the NAD+ pool was employed by Frederick et al. (2014), in which the enzyme NAMPT was overexpressed in mice, who were then assessed via exercise tolerance, voluntary running and grip strength tests. Despite increasing the NAD+ content of skeletal muscle by 50%, no alterations in oxygen consumption, running capacity, voluntary running activity nor strength were observed in mice with NAMPT overexpression. Hence, the improved performance seen in Canto et al. (2012) is unlikely to be due to purely muscle-specific increases in NAD+.

The discrepancy in the results of the above studies could partly be due to methodological differences, such as type of exercise, the animal model used, or the way in which the supplement was administered. However, as Kourtzidis et al. (2016) also discuss, there appears to be a possible explanation for a decreased exercise performance following NR consumption, in the form of early Na studies (Murray et al., 1995; Pernow & Saltin, 1971). NR and Na are similar in that both are NAD+ precursors, and hence work to enhance NAD+ levels. There is evidence however that Na blunts the exercise-induced rise in plasma FFAs, therefore limiting fat oxidation throughout exercise and accelerating the onset of fatigue (Murray et al., 1995; Pernow & Saltin, 1971). The effects of Na consumption during a steady-state bout of cycling at 68%VO_{2peak} followed by a 3.5 mile performance test were investigated by Murray et al. (1995). It was reported that the increase in FFAs during the pre-load exercise was blunted, with participants eliciting a higher RER post Na ingestion. Despite a lack of significance between the water placebo trial and the water+Na trial, the authors propose that Na administration may have actually been detrimental to performance, supporting the results of Kourtzidis et al. (2016). In the present study however, plasma NEFA levels were comparable between groups, suggesting the blunting of plasma FFAs did not occur following acute NR consumption.

IMPLICATIONS

Based upon the results of the majority of previous NR research, this substance appears to work optimally when supplemented long term, and in populations with a deficiency in NAD+ prior to consumption. The results of the present study support this, as no

significant differences were seen in any measure between NR and placebo conditions following consumption of a single dose of NR in young, healthy participants. In rodent studies, the chronic administration of NR in clinical populations has proven extremely successful, with significant improvements seen in various diseased models, including rodents with Alzheimer's (Gong et al., 2013), mitochondrial myopathy (Khan et al., 2014) and muscular dystrophy (Ryu et al., 2016). Many of the diseases targeted by NR research are age-related, and ageing is associated with an accumulation of DNA damage (Brace et al., 2016). Hence, NAD+ levels are decreased due to heightened PARP activity (Malanga & Althaus, 2005), possibly providing NR with capacity to build upon these levels, unlike in healthy participants such as those used in the present study. This expanding body of research provides an exciting insight into the potential uses of NR in clinical human populations, and ageing in general.

FUTURE DIRECTION

As the first study of its kind, there is huge scope to expand on this in order to investigate fully the potential benefits of NR supplementation in humans. The effects of a single dose of NR were explored in the present study finding no significant results, and hence it would be logical for a longer supplementation period to be implemented in future research. 1,000mg NR was administered daily for seven consecutive days in the first human study of NR (n=1) (Trammell et al., 2016). Although PBMC NAD+ concentration peaked 7.7 hours post-ingestion of the first dose, subsequent measurements – one prior to the second dose and one 24 hours following the seventh and final dose – show that NAD+ concentration was on the rise between these two

points. The same pattern was shown with Nam and NAAD, and also with MeNam, Me4PY and Me2PY, indicating enhanced NAD+-consuming activity. It is plausible that a supplementation period in excess of seven days may help to continue this rise in NAD+ metabolome concentrations. A chronic and sustained increase in NAD+ levels in the blood prior to a single bout of exercise should, in theory, give rise to more pronounced differences in substrate utilisation in comparison to the single dose administered in the present study. Building upon the n=1 study by Trammell et al. (2016) by recruiting a larger group of participants and administering them with NR for an extended period of time (e.g. four to six weeks) would allow for the establishment of the dose:response relationship of NR consumption. Determining the effects of long-term NR supplementation on the NAD+ metabolome would be extremely beneficial for the future of NR research.

Supported by research from Watt et al. (2002), a steady-state exercise bout of between 90-120 minutes would likely promote higher levels of fat oxidation and decreased levels of CHO oxidation, compared to those reported after 60 minutes of exercise in the present study. Future research should investigate the effects of NR on substrate metabolism during longer bouts of exercise, as it may be possible that with higher rates of fat oxidation, a difference between the placebo and NR condition would be seen. With the protocol used in the present study, whether or not any differences in performance were shown between conditions in the 5km TT was largely dependent on whether a main effect of condition was shown for substrate utilisation during the steady-state exercise. With an alternative protocol in which the duration of steady-state exercise is increased, the possibility of muscle glycogen sparing occurring with NR

supplementation may be enhanced. Hence, the likelihood of NR positively affecting TT performance would also be heightened.

The future of NR research should also include the combination of long-term supplementation and exercise training. Alone, repeated bouts of exercise are capable of inducing a variety of physiological adaptations that together work to improve both performance and health (Jones & Carter, 2000). One of these adaptations is the increased utilisation of fat as a fuel source during both rest and exercise (Jeukendrup & Aldred, 2004), partly due to the enhanced activity of various TFs, co-activators and enzymes - including sirtuins. As SIRT1 and SIRT3 are NAD+-dependent, long-term NR supplementation should further increase deacetylation of these energy sensors in various tissues, as seen in mice in the study by Canto et al. (2012). Hence, the synergistic effects of supplementation and training together have the potential to further enhance the adaptations – including increased fat oxidation - seen from exercise training alone.

In the present study, diet one day prior to the first experimental trial was recorded, then replicated the day prior to the second trial. However, when investigating a supplement which is found naturally in foods, the habitual diet of its consumers is likely to have an impact on the proficiency of the supplements' effects. Hence, the recording of habitual diet and a longer period of dietary control prior to experimental trials, where meals were provided by the experimenters, would have enhanced reliability. However, this was outside the scope of this study. In future NR research, it would be advantageous to monitor and control dairy intake especially, as NR is found naturally in cow's milk (Bieganowski & Brenner, 2004). The completion of a two week food diary prior to the onset of a study would allow for the exclusion of participants who consume above-

average levels of dairy products, as these individuals may have naturally higher levels of NR present in their blood and/or tissues.

CONCLUSION

To conclude, the present data suggests that a single 1,000mg dose of NR has no effect on substrate utilisation in humans, during a 60-minute bout of steady-state cycling, or on 5km TT performance. Despite the significant increase in fat oxidation and subsequent decrease in CHO oxidation seen throughout exercise, no significant differences were shown between conditions, with both RER and blood plasma measures supporting this. It is probable that a single dose of NR is insufficient in heightening NAD+ levels in tissues such as skeletal muscle and liver, and hence is unable to enhance any mechanisms in which NAD+ plays a role – such as increasing the deacetylation of sirtuins. Additionally, unlike other 'fat burning' substances that appear to act via enhancing the activity of the sympathetic nervous system (Jeukendrup & Randell, 2011), NR does not appear to have the capacity to alter hormonal activity. Alterations to the present study design such as a longer-term supplementation period, an increased duration of steady-state exercise or the incorporation of exercise training into the protocol would be beneficial to further test the capacity of NR in humans during exercise, and as a possible ergogenic aid. Furthermore, any insight into the way in which NR works in humans is advantageous - with evidence demonstrating its success in rodents with diabetes, Alzheimer's, mitochondrial myopathy and more, it seems NR has huge potential for use within therapeutic and clinical settings.

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