The Physiological Effects of Nutritional Thiol Supplementation

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Abstract

Endogenous antioxidant defence systems are largely comprised of thiol antioxidants and antioxidant enzymes. Keratin protein, being high in cysteic acid, has potential to improve antioxidant status via generation of several thiol antioxidants. Common dietary antioxidants are often used by athletes aiming to reduce oxidative stress, which in turn can improve exercise performance. However, whilst studies into their action against oxidative stress are positive, the subsequent effect on exercise performance is less so. In addition to having little effect on acute performance, their non-specific ROS-targeting mechanisms may also blunt important adaptation signalling pathways. It has been proposed that improving endogenous defence systems may be of greater benefit to athletic performance, thus keratin may be an alternative to dietary antioxidant supplementation. The first study within this thesis found acute keratin supplementation to improve plasma total thiol content, while longer term supplementation increased lean body mass significantly more than casein protein. Study two suggested one week of keratin vs. taurine supplementation did not affect plasma total thiol content, while study three found that despite no significant effects on serum total thiol content, taurine improved muscle recovery following eccentric-induced muscle damage compared to a placebo. In summary, findings from this thesis suggest keratin may be a useful supplement for athletes wanting to maintain or gain lean body mass. This suggests implications for the use of keratin beyond athletes, potentially benefiting other population groups including the elderly and the sick. Results also suggest that supplementing with taurine following resistance exercise involving eccentric actions may improve recovery and subsequent performance. This research sets a platform for further investigation into the use of keratin and thiols for various areas of sporting performance.

Acknowledgements

It goes without saying that undertaking a PhD is no walk in the park. The ability to stay motivated, inspired, engaged, and driven cannot be achieved by oneself; rather, it takes a committed crew to see it through from start to finish. For that, I would like to acknowledge first and foremost my supervisory team; Professor Stephen Stannard, Dr. Matthew Barnes, and Dr. Carlene Starck. I could not have asked for a more encouraging and supportive team. Your knowledge and input over the years has not only kept me on track during this doctorate, but has challenged my thinking as a researcher. Massive thanks must also be given to my co-worker Miss Emma Crum. Without you, the research side of this doctorate would not have been possible. Also to Mr. Chris Kendrick and Ms. Felicity Jackson for their analysis of blood samples, and Ms. Anne Broomfield for running our DEXA scans. And to my participants, I am so appreciative of the effort you put in to these studies, despite it not always being easy to do so. Your willingness and enthusiasm made it so easy to work with you all – so thank you. To my family, thank you for supporting me throughout not just these past three years, but over my entire eight years of university study. The completion of this is a real testament to how you have raised me to be a determined and driven woman. Your unconditional love and encouragement to pursue my dreams has instilled a strong sense of self-belief and I can never thank you enough for that. And last but not least, to my fiancée Jordan. It is hard for me to put into words how grateful I am to have had you in my life during this time. Thank you for being an absolute rock during 2.5 years of a long-distance relationship whilst I completed this. Thank you for being my number one fan. And thank you for never letting me give up.

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List of Abbreviations

8-OHdg	8-hydroxy-deoxy-guanosine
A	
AA	Arachidonic acid
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
C	
CA	Cysteic acid
CAT	Catalase
Ca_2^+	Calcium ion
CDO	Cysteine dioxygenase
Cl^-	Chloride ion
CNS	Central nervous system
CSAD	Cysteine sulfinic acid decarboxylase
CSA	Cysteine sulfinic acid
D	
DNA	Deoxyribonucleic acid
DOMS	Delayed onset muscle soreness
E	
EIH	Exercise induced hypoxemia
EPO	Erythropoietin
ETC	Electron transport chain
F	
FADH2	Flavin adenine dinucleotide
G	
GCS	Gamma-glutamyl cysteine synthetase
GCl	Resting muscle chloride conductance
GLUT-4	Glucose transporter
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSC	γ-glutamylcysteine synthetase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H	
h	Hours
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HbO_2	Hemoglobin saturation
HIF	Hypoxia inducible factor

HOCl	Hypocholorus acid
I	
IGF-1	Insulin-like growth factor
IMP	Inosine monophosphate
IL-	Interleukin isoforms
K	
K^+	Potassium ion
L	
LBM	Lean body mass
LOX	Lipoxygenase
M	
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MPO	Myeloperoxidase
mtDNA	Mitochondrial DNA
mRNA	Messenger ribonucleic acid
N	
Na^+	Sodium ion
NAC	N-acetyl-cysteine
NAD^+	Nicotinamide adenine dinucleotide, oxidised
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NF-kβ	Nuclear factor-kappaβ
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O	
O_2	Molecular oxygen
O_2^-	Superoxide radical
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite
P	
PaO_2	Partial pressure of oxygen saturation
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PLA2	Phospholipase A2
PMRS	Plasma membrane redox system
R	
RBC	Red blood cell
RNS	Reactive nitrogen species
ROO	Peroxyl radical
ROS	Reactive oxygen species
RPM	Revolutions per minute
S	

SD	Standard deviation
SE	Standard error
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
Sub-max	Submaximal exercise

T

Tau	Taurine
TauCl	Taurine chloramine
TauT	Taurine transporter
TBARS	Thirobarbituric acid reactive substances
TNF-α	Tumour necrosis factor-alpha
tRNA	Transfer ribonucleic acid

V

VEGF	Vascular endothelial growth factor
VO_{2max}	Maximal oxygen uptake

X

XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

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Chapter 1

Introduction

The effect of oxidative stress on fatigue and subsequent performance decline in endurance athletes is well known. While previous research has investigated the use of common dietary antioxidants for athletic performance, results are controversial. High in cysteic acid, keratin protein has the potential to improve thiol antioxidant status, which may in turn benefit endurance performance. However to date, no studies have investigated these physiological measures in humans. Thus, this thesis primarily investigates keratins' potential role in endurance performance, whilst also determining the role that thiol antioxidants may have in athletes in general.

Chapter 2 extensively covers several topics relating to oxidative stress, antioxidants, and exercise performance. Specifically, the first section begins by discussing sources of exercise-induced free radical production and their acute effect on physiological processes. It also highlights their importance in long-term adaptation to exercise. The second section focuses on dietary and endogenous antioxidants and their specific exercise-related roles within the body. The final section addresses the importance of antioxidants to pro-oxidant (redox) balance and debates how common dietary antioxidants may affect this balance and what effects this can have on exercise performance and adaptation. This section also discusses the current knowledge and literature around thiol antioxidants and the roles they play in exercise performance. This leads into **Chapter 3**, where, based on information from **Chapter 2**, specific hypotheses and aims for this thesis are outlined.

The next three chapters cover one large human study, and two smaller-scale human studies. **Chapter 4** investigates whether acute and chronic intake of keratin protein affects various physiological parameters and performance in trained male cyclists compared to casein protein. **Chapter 5** then aims to assess whether supplementation with keratin protein improves plasma thiol status compared to taurine or pea protein, and how mood state is affected by taurine in healthy humans. **Chapter 6** explores how taurine may improve recovery from eccentric-exercise induced muscle damage in males.

Finally, **Chapter 7** offers a general discussion, conclusion, and limitations for **Chapters 4**, **5** & **6**. Based on findings from these studies, it also suggests potential avenues for future research.

Chapter 2

Literature Review

Publication:

McLeay, Y., Stannard, S., Houltham, S., Starck, C. (2017). Dietary thiols in exercise: oxidative stress defence, exercise performance, and adaptation. *Journal of the International Society of Sport Nutrition, 14* (12).

This literature review discusses a number of subjects relating to key thesis aims. Comprised of three sections, the first (2.1) looks into sources of exercise-induced free radical production, their acute effect on physiological processes, and their role in long-term adaptation to exercise. The second section (2.2) focuses on dietary and endogenous antioxidant and their specific exercise-related roles within the body. The third and final section (2.3) addresses the importance of redox balance and the effects of supplementing with dietary antioxidants or up-regulating endogenous defence systems on exercise performance and adaptation.

2.1 Exercise-Induced Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) is a collective term referring to a family of unstable molecules known as free radicals, and their direct products, derived from molecular oxygen (O_2). Due to one or more unpaired electrons in their outer orbital, free radicals are highly reactive and seek stabilisation by pulling electrons away from other stable molecules. In doing so, O_2 is reduced to the superoxide radical, $O_2^{\cdot-}$. Superoxide ($O_2^{\cdot-}$), is not a potent free radical as it has limited radical capacity; however due to its long half-life, it can further react with other molecules to generate secondary ROS, including the highly reactive non-radical, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$) (Fridovich, 1986). In addition, $O_2^{\cdot-}$ can react with other nitrogen-derived reactive species (RNS).

Reactive nitrogen species (RNS) refers a family of molecules derived from the free-radical nitric oxide (NO), a powerful signalling molecule and vasodilator. At rest, various isoforms of nitric oxide synthase (NOS) within skeletal muscle generate low levels of NO. During exercise however, contracting muscle upregulates endothelial NOS activity to allow for increased blood flow (Zhang et al., 2009). Additionally, immune response mechanisms to exercise-induced

tissue damage can also increase generation of NO (Schmidt, Seifert, & Böhme, 1989; Wright, Busse, & Osswald, 1989). Nitric oxide readily reacts with O_2^-, creating the powerful oxidant peroxynitrite ($ONOO^-$), which can further react with and damage cells, deoxyribonucleic acid (DNA), and proteins, compromising functionality (Ischiropoulos & Al-Mehdi, 1995; Radi, Beckman, Bush, & Freeman, 1991; Salgo, Bermudez, Squadrito, & Pryor, 1995). While RNS can contribute to oxidative stress during exercise, its contribution to this is heavily reliant on the availability of O_2^-. Thus, for the purpose of this thesis, the focus will be predominantly on ROS.

Resting muscle generates low levels of ROS which are important in respiratory signalling (Powers & Jackson, 2008), however, activity-dependent increases in ROS-generating processes result in a higher levels during exercise (Davies, Quintanilha, Brooks, & Packer, 1982; Jackson, Edwards, & Symons, 1985; McArdle, Pattwell, Vasilaki, McArdle, & Jackson, 2005). This can often lead to 'oxidative stress', a physiological state which exerts negative effects on cellular structure and function. Indeed, since ROS are crucial redox regulators, Jones (2006) has defined oxidative stress as the disruption of redox signalling and control.

2.1.1 Sources of ROS in endurance exercise

Endurance exercise primarily involves oxygen-reliant aerobic energy metabolism, placing a high demand on the cardio-respiratory system to work optimally for an extended period of time. This in turn requires increased oxygen uptake by the contracting muscle cells to regenerate adenine triphosphate (ATP), in the mitochondria. During maximal exercise, body O_2 concentration may increase 20-fold, and in skeletal muscle up to 100-fold from resting values (Powers, Talbert, & Adhihetty, 2011). It has therefore been suggested that as the duration and/or intensity of exercise increases, so does ROS production, as observed by increases in indirect indices of free radical damage such as lipid peroxidation following endurance protocols (Davies et al., 1982; Lovlin, Cottle, Pyke, Kavanagh, & Belcastro, 1987; Mastaloudis, Leonard, & Traber, 2001).

Direct measures of ROS themselves have also been seen to rise during skeletal muscle activity. Using an intracellular probe in contracting diaphragm muscle Reid et al. (1992) observed increased levels of O_2^- and H_2O_2 over time. Additionally, they observed greater fatigue in muscles not administered with antioxidant enzymes, compared to those that were. Thus, there appears to be a clear link between ROS production, consequent decreased antioxidant concentrations, and muscular fatigue.

From the early 1970's, mitochondrial respiration was considered the leading generator of exercise-induced ROS; a theory partially based on the observed linear increase of up-regulated ROS formation as oxygen concentration increased (Turrens, Freeman, Levitt, & Crapo, 1982). More recent research however (Shill et al., 2016), suggests non-mitochondrial metabolic pathways of ROS production in skeletal muscle may be greater contributors to ROS pools (see Figure 1) .

Four proposed locations of ROS generation seen during and following endurance exercise will be discussed in this section: the mitochondria, the cell membrane, the circulatory system, and skeletal muscle.

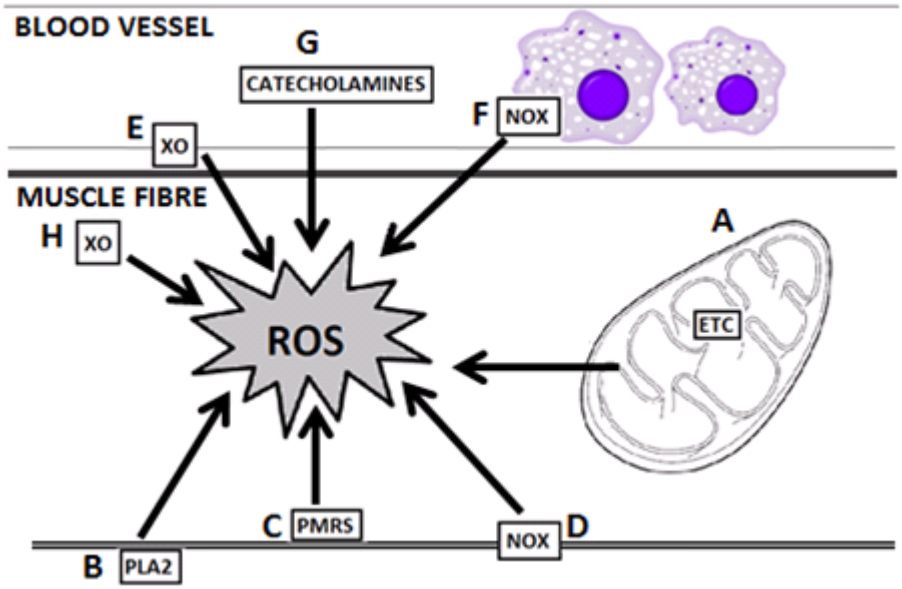

Figure 1 Sources of reactive oxygen species (ROS) generation during exercise: A) mitochondria, B) phospholipase A2 enzymes, C) the plasma membrane redox system, D) plasma membrane NADPH oxidase, E) endothelial xanthine oxidase, F) leukocyte NADPH oxidase, G) catecholamines, H) muscular xanthine oxidase. Adapted from P Steinbacher & P Eckl, 2015, *Biomolecules, 5* (2), 358.

2.1.1.1 The Mitochondria

Mitochondria are the organelles within which respiration takes place via the electron transport chain (ETC). Through a series of redox reactions whereby electrons are transferred across five "complexes", the potential energy of reduced nicotinamine adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (released primarily from the tricarboxylic acid cycle), is used to create a chemiosmotic potential across the inner mitochondrial membrane, specifically

for protons. This proton gradient is then able to rephosphorylate ADP as the proton gradient can be satisfied through the ATP-ase synthase complex.

While ATP is the crucial product of the ETC and oxygen the "terminal electron acceptor", the transfer of electrons required for the production of the chemiosmotic gradient can result in the formation of ROS. Increased rate of demand for ATP during skeletal muscle contractile activity elevates the rate of electron transfer and subsequent formation of ROS. Indeed, normoxia (normal levels of circulating oxygen) seen at rest can be dramatically altered dependent on exercise intensity, with both hyperoxic (high levels of circulating oxygen) and hypoxic (low levels of circulating oxygen) states having the ability to upregulate mitochondrial ROS production.

2.1.1.1.1 Hyperoxic ROS production

Traditionally, exercise scientists believed that the greatest source of free radical production at rest and during exercise came from mitochondrial respiration; specifically, the ETC (Figure 2.1, A). As the demand to buffer changes in ATP concentration in the myocyte intensifies during contractile activity, and further if exercise intensity and/or duration increases, oxygen uptake can dramatically increase, eliciting a hyperoxic environment. It has been suggested that the link between ROS production and the ETC is not infallible, and electrons may 'leak' predominantly from complexes I and III (St-Pierre, Buckingham, Roebuck, & Brand, 2002). A greater flux of oxygen through the ETC can lead to an increased 'leaking' of electrons, which bind to molecular oxygen to form O_2^-, a precursor of most other ROS (Turrens, 2003). These ROS then proceed to modify neighbouring cellular components, affecting cell structure and function.

Boveris and colleagues (1973; 1972) carried out some of the earliest studies observing isolated mitochondria as a source of H_2O_2. This was later confirmed to be the result of dismutation of O_2^- generated from ETC electron 'leaking' (Forman & Kennedy, 1974), with 1-5% of oxygen consumed forming O_2^- under resting conditions (Boveris & Chance, 1973; Boveris et al., 1972; Loschen, Azzi, Richter, & Flohe, 1974). However, more recent studies have concluded that at most, only around 0.15% of oxygen entering the mitochondria form O_2^- (St-Pierre et al., 2002). Furthermore, a potential decrease in ETC ROS production during exercise has been suggested based on lab observations (Boveris & Chance, 1973; Herrero & Barja, 1997) that have observed electron leakage to occur only when oxidative respiration is low and that increasing this, such as during exercise, results in a decrease in electron leak and thus lower free radical formation. If this can be supported by human studies, it may suggest other sources of ROS production to

predominate during exercise. Indeed, a recent study by Shill and colleagues (2016) suggested that exercise-induced ROS, particularly those involved with exercise-adaptation, are predominantly generated from non-mitochondrial sources.

2.1.1.1.2 Hypoxic ROS production

While hyperoxic conditions within the ETC have been shown to up-regulate ETC ROS generation, various studies have also observed a paradoxical increase in ROS formation and subsequent oxidative stress when body tissues are exposed to hypoxic conditions (Guzy et al., 2005; Turrens, 2003; Waypa & Schumacker, 2002). Hypoxia occurs when oxygen demand by the working muscles is unable to be met by oxygen transport systems, resulting in a state of low tissue oxygen. Since mitochondria rely on oxygen for energy metabolism, hypoxia has feedback effects whereby adaptive responses allow for continued mitochondrial respiration when oxygen consumption is low. Hypoxia inhibits expression of complex IV (cytochrome oxidase) of the ETC (Chandel, Budinger, Choe, & Schumacker, 1997), the terminal complex heading oxidative phosphorylation. This inhibitory effect results in increased ROS production by proximal complexes (Fukuda et al., 2007). The increase in mitochondrial ROS signal stabilization of hypoxia inducible factors (Mansfield et al., 2005; Simon, 2007). These transcription factors are principal mediators in the preservation of tissue O_2 during hypoxic conditions (Guzy et al., 2005); an important adaptive mechanism for maintaining glycolytic capacity and therefore energy production (Lum et al., 2007). Thus, cytochrome oxidase "senses" hypoxia in an attempt to adapt to lower oxygen for respiration.

2.1.1.2 Phospholipase A2 enzymes

Phospholipase A2 enzymes (PLA2) consist of 15 known isoforms that, in addition to other roles, catalyse the hydrolysis of plasma membrane phospholipid fatty acyl chains, generating fatty acids (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011). PLA2 enzymes increase during exercise in response to the muscle contraction-mediated rise in calcium (Ca^{2+}), and inhibition of these enzymes appears to impede skeletal muscle contractile properties and increase fatigue (Gong et al., 2006; Nethery et al., 2000; Nethery, Stofan, Callahan, DiMarco, & Supinski, 1999) . PLA2 indirectly produce ROS via their generation of fatty acids. Arachidonic acid (AA) is one such fatty acid that may act as a substrate for the ROS-generating enzyme system lipoxygenases (Zuo, Christofi, Wright, Bao, & Clanton, 2004). Lipoxygenases (LOX) are a family of enzymes, some of which are expressed in skeletal muscle (Oliver, Wright, Parinandi, & Clanton, 2008; Zuo et al., 2004). The breakdown of AA is catalysed by LOX, and research has suggested that PLA2's role in ROS production is in generating fatty acids for LOX metabolism (Zuo et al., 2004). The catalytic activity of LOX can generate O_2^- in resting conditions; therefore

a rise in AA during exercise may increase LOX activity, leading to up-regulated ROS production. In addition, it has been suggested that PLA2-generated AA may activate NADPH oxidase (NOX) (Mayer, Brenic, Stocker, & Glaser, 1995; Zhao, Bey, Wientjes, & Cathcart, 2002). This enzyme system is known to generate free radicals via ETC up-regulation, further contributing to ROS concentrations (see Section 2.1.1.4).

2.1.1.3 Plasma Membrane Redox System

The plasma membrane redox system (PMRS), located within the cell membrane, plays a key role in maintaining redox homeostasis in body tissues, including skeletal muscle. Using electron transfer, PMRS is able to oxidise or reduce nicotinamide adenine dinucleotide (NAD) to NAD^+ or NADH, which are utilized in further redox reactions. Activation of the PMRS appears to be contraction-induced, whereby initial muscle contraction stimulates an acute rise in NADH, activating PMRS and allowing for regeneration of NAD^+. The transfer of electrons involved in this further reduces O_2 molecules to O_2^-, which may then form secondary ROS (de Grey, 2000; Mohazzab, Kaminski, & Wolin, 1994; Morre, Lenaz, & Morre, 2000).

2.1.1.4 NADPH oxidase (NOX)

Nicotinaminde adenine dinucleotide phosphate (NADPH) is a cofactor used in many anabolic reactions. NADPH oxidases, commonly referred to as NOX, are a family of transmembrane enzyme complexes present in various isoforms, and are a major source of ROS formation during exercise. NOX use NADPH as a substrate, oxidising it to $NADP^+$ via electron transfer, subsequently forming O_2^-. Skeletal muscle NOX was the first identifiable example of a system where ROS generation was a primary function of an enzyme system, rather than a by-product (Babior, Kipnes, & Curnutte, 1973), suggesting the importance of low-level NOX generation of ROS for correct muscle function. Isoforms of NOX found within neutrophils and macrophages can also contribute to exercise-induced ROS (Peake & Suzuki, 2004).

2.1.1.4.1 Immune cells

Neutrophils make up the largest composition of leukocytes and, being the first line of defence, are essential to the innate immune response against invading pathogens (Kobayashi & DeLeo, 2009). During exercise, neutrophils are activated and released into the circulation from bone marrow in response to tissue damage; a result of muscle injury, thermal stress and re-oxygenation of hypoxic tissues (Peake & Suzuki, 2004). Several studies have observed increased engagement of neutrophil receptors, such as CD16 and CD11b, during and following acute endurance exercise (Gray, Telford, Collins, Baker, & Weidemann, 1993; Peake et al., 2004; Smith et al., 1996), as well as an increased number of circulating neutrophils (Suzuki et

al., 1996; Suzuki et al., 1999). Activation of neutrophil receptors initiates the process of respiratory burst, whereby neutrophils aim to assist the clearance of damaged tissue fragments by consuming oxygen and producing O_2^- (Baldridge & Gerard, 1932; Robinson, 2009; Segal & Abo, 1993). Generated O_2^- is then converted to H_2O_2, and further to hypochlorous acid (HOCl) which helps to destroy harmful pathogens. However, excess HOCl can further react with stable molecules including DNA and proteins, resulting in cellular damage and compromised function. It has been suggested that the ability of a neutrophil to carry out this phagocytic process may depend solely on receptor engagement, thus any attenuation of cytokine production (i.e. antioxidant administration) may hinder respiratory burst.

2.1.1.4.2 Cell Membrane

Studies within the last decade have observed that various subcellular compartments of skeletal muscle including the cell membrane and associated components (sarcolemma, transverse tubules), also express NOX enzymes (Hidalgo, Sánchez, Barrientos, & Aracena-Parks, 2006; Whitehead, Yeung, Froehner, & Allen, 2010; Xia, Webb, Gnall, Cutler, & Abramson, 2003). In addition, unlike phagocytic cells, skeletal muscle NOX are capable of utilizing either NADH or NADPH as electron donors (Rada & Leto, 2008), thus are less limited by substrate availability.

Activity of skeletal muscle NOX isoforms appear to be the result of contraction-induced protein activation (Sakellariou et al., 2012), with several in-vitro studies observing increased NOX activity and ROS following skeletal (Wang, Pan, Wang, Zucker, & Wang, 2009; Whitehead et al., 2010) and cardiac (Dudley et al., 2005) muscle stimulation .

Furthermore, there may be benefit in having NOX enzymes present in certain skeletal muscle compartments. For example, it has been shown that a low to medium level of ROS play a key role in allowing Ca^{2+} release from the sarcoplasmic reticulum, a crucial element in muscular contraction (Cherednichenko et al., 2004; Favero, Zable, & Abramson, 1995). In addition, observations using human cells suggest a strong signalling link between the mitochondria and NOX, whereby mitochondrial generated ROS further activate NOX enzyme activity, increasing ROS production (Lee, Bae, Bae, & Um, 2006). This may be a useful mechanism in activating various adaptive pathways. Thus skeletal muscle NOX enzymes appear to be more complex than was once thought.

2.1.1.5 Xanthine Oxidase

Xanthine oxidase (XO), found within skeletal muscle endothelium and the myocyte cytosol, catalyses the conversion of hypoxanthine (a product of ATP breakdown) to xanthine

(Sakellariou, Jackson, & Vasilaki, 2014) using O_2 as a cofactor. This process produces O_2^-, which can then be further oxidised to more powerful ROS.

At rest, 90% of hypoxanthine is metabolised to inosine monophosphate (IMP), with the remaining 10% being metabolised to xanthine and uric acid via XO, a process that generates ROS. Intense and/or prolonged exercise can bring about hypoxia, during which the conversion of hypoxanthine to IMP is inhibited, significantly increasing hypoxanthine levels (Saugstad, 1988). Additionally, XO requires oxygen to metabolise hypoxanthine to xanthine and then to uric acid, thus the lack of oxygen during hypoxia limits XO activity, further increasing hypoxanthine levels. Following exercise, the sudden increase in oxygen availability allows for the metabolism of high hypoxanthine levels (Vina et al., 2000), subsequently increasing ROS generation (Aitken, Buckingham, & Harkiss, 1993; Jaeschke & Mitchell, 1989; Vanden Hoek, Li, Shao, Schumacker, & Becker, 1997; Zweier, Kuppusamy, Thompson-Gorman, Klunk, & Lutty, 1994).

Exercise-induced hypoxemia (EIH), a state of low blood oxygen that can lead to hypoxia, has been observed in trained athletes (Martin et al., 1992; Powers et al., 1992; Warren, Cureton, Middendorf, Ray, & Warren, 1991); although probably only occurs briefly at maximal exercise (VO_{2max}) where the cardiac output exceeds the capacity of the pulmonary system (Sarkar, Niranjan, & Banyal, 2017). The occurrence of EIH is suggested to be a result of limited oxygen transport between blood and intracellular tissue, affecting maximal oxygen consumption by the muscle tissue (Richardson, Noyszewski, Kendrick, Leigh, & Wagner, 1995). The resulting rise in hypoxanthine and subsequent XO activity can increase ROS production and rate of fatigue.

While both animal (Gomez-Cabrera et al., 2005; VinÑa et al., 2000) and human (Gomez-Cabrera et al., 2006; Gomez-Cabrera, Pallardo, Sastre, Vina, & Garcia-del-Moral, 2003; Groussard et al., 2003) studies suggest XO to be the most important source of ROS during endurance exercise, the hypoxic model of ROS production is still controversial. Additionally, the relative contribution of XO-generated ROS to total ROS pools has yet to be determined, with studies suggesting both a minimal (Panus, Wright, Chumley, Radi, & Freeman, 1992; Poss, Hueckstead, Panus, Freeman, & Hoidal, 1996; Wadley, Nicolas, Hiam, & McConell, 2013) and potentially large contribution (Duarte, Appell, Carvalho, Bastos, & Soares, 1993; Mohanraj, Merola, Wright, & Clanton, 1998; Sahlin, Ekberg, & Cizinsky, 1991). Perhaps its relative contribution is dependent on the intensity of exercise (i.e. maximal vs. submaximal) rather than the duration. More research is needed to shed light on these unknown areas.

2.1.1.6 Catecholamines

The catecholamines adrenaline and noradrenaline are produced in situations of physical and mental stress. Under resting conditions, plasma noradrenaline is significantly higher than that of adrenaline, but during endurance exercise there is a progressive increase in both (Zouhal, Jacob, Delamarche, & Gratas-Delamarche, 2008). Acute exercise stimulates the activation of β-adrenergic receptors and the resulting increase in plasma catecholamines elevates heart rate, dilates blood vessels (Allen, Barcroft, & Edholm, 1946), up-regulates hepatic glucose release (Glabo, Holst, & Christensen, 1975) and mobilizes fatty acids (Luyckx, 1975); useful transient changes for exercise performance. At high levels however, catecholamines may contribute to ROS generation (Rodriguez-Martinez et al., 1999); although the exact process of this and its contribution to ROS pools during exercise is largely unclear (Ji, 1999). Several studies suggest catecholamines to be vulnerable to auto-oxidation under certain physiological conditions, resulting in rapid formation of O_2^- (Green, Mazur, & Shorr, 1956; Misra & Fridovich, 1972; Trautner & Bradley, 1951). It has also been proposed that catecholamines may have an effect on upregulating activity of other ROS sources (van Eeden, Granton, Hards, Moore, & Hogg, 1999).

2.1.2 Effects of ROS on exercise performance

High acute ROS concentrations can disrupt several redox-sensitive pathways important during exercise, subsequently increasing the rate of muscular fatigue and significantly impairing exercise performance (Reid, 2008). However, while negative effects on acute performance have been observed, recurring periods of oxidative stress, such as that seen with training can bring about beneficial exercise adaptations. These adaptations improve skeletal muscle's tolerance to ROS and thus fatigue.

2.1.2.1 Acute physiological effects

Whilst low levels of ROS are required for coordinated muscle contraction and release, higher levels, such as that seen with exhaustive exercise and in response to exercise-induced tissue damage, appear to acutely impact upon muscle function. ROS achieve this via interaction with various intramuscular processes as demonstrated in Figure 2. This in turn can lead to muscle fatigue, which has been defined as a decrease in the maximal force or power that an involved muscle can produce (Sogaard, Gandevia, Todd, Petersen, & Taylor, 2006).

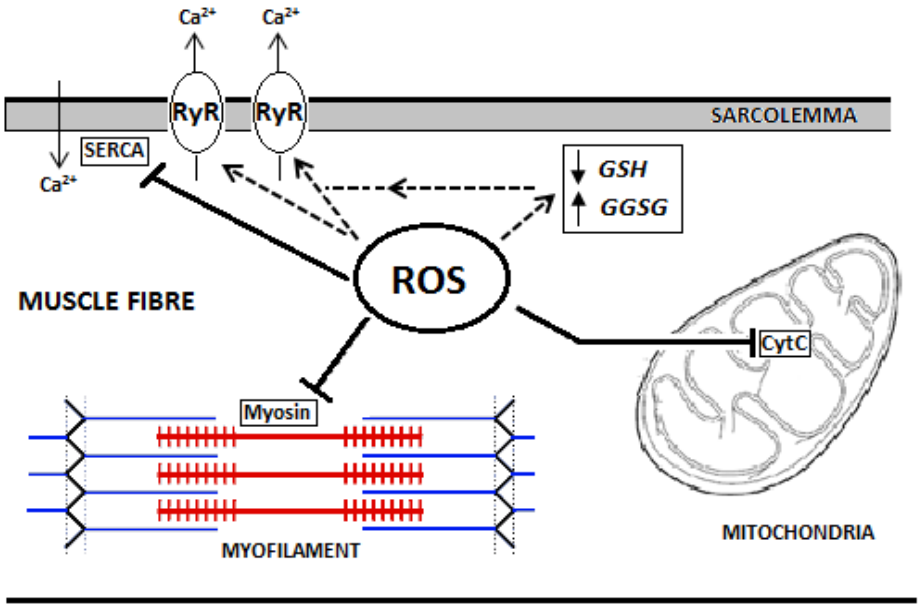

Figure 2 Acute effects of ROS on skeletal muscle processes and resulting fatigue. ROS directly blunts Cytochrome C in the mitochondria, myosin filaments, and sarcoplasmic calcium ATPase (SERCA). It stimulates ryanodine receptors (RyR) and redox imbalance via decreased GSH and increased GSSH. This further stimulates redox-sensitive RyR. Adapted from R. Nemes, 2018, *Antioxidants, 7* (85).

2.1.2.1.1 Mitochondrial complexes

Research has shown that various ROS, in addition to those produced directly by the mitochondria, can have an inhibitory effect on several ETC complexes. While the processes for this are complex (for more, see review by Murphy (2009)), the 'build-up' of unstable electrons can significantly elevate ROS production. One of ROS most notable inhibitory effects is via nitric oxide (NO), which has been shown *in vitro* to inhibit cytochrome oxidase (Cleeter, Cooper, Darley-Usmar, Moncada, & Schapira, 1994) and *in vivo* during endurance exercise (Wadley & McConell, 2007).

In addition to up-regulating ROS production, the inhibition of cytochrome oxidase essentially 'blocks' the electrochemical gradient from reaching ATP synthase, thus reducing the capacity for ATP synthesis (phosphorylation potential). Working skeletal muscles rely on the steady production and delivery of ATP to carry out the contraction-relaxation process. A reduction in phosphorylation potential can result in the transition of muscle to anaerobic energy metabolism, and a high rate of anaerobic glycolysis is associated with early onset fatigue.

2.1.2.1.2 Calcium channels

Ca^{2+} is a fundamental signalling molecule with known regulatory roles within skeletal muscle mitochondria and myofibrillar structures. For this reason, maintaining correct intracellular/extracellular Ca^{2+} levels are crucial for optimal muscle functioning. The sarcoplasmic reticulum ATP-ase (SERCA) and ryanodine receptor (RyR) are two redox-sensitive membrane-bound calcium channels involved with the transport of Ca^{2+} into and from cells respectively. High levels of ROS have been shown to disrupt redox balance by decreasing endogenous levels of reduced glutathione (GSH) and increasing oxidised glutathione (GSSG). To attain stability, GSSG and O_2^- oxidise RyR thiol groups, which enhances channel activity and resulting in a greater loss of Ca^{2+} from the muscle (Eu, Sun, Xu, Stamler, & Meissner, 2000). In a similar yet opposing way, high ROS levels inhibit SERCA channels via direct interference with its ATP binding site, resulting in decreased uptake of Ca^{2+} into the muscle (Sun, Xu, Eu, Stamler, & Meissner, 2001; Xu, Zweier, & Becker, 1997). This combined effect on calcium channels significantly reduces intracellular Ca^{2+}, negatively affecting ATP synthesis and thus skeletal muscle contraction. Early rat studies observed that disrupted Ca^{2+} channel functioning following exhaustive exercise, impaired muscle contraction, although training appeared to ameliorate this (Bonner, Leslie, Combs, & Tate, 1976). Moreover, rabbit (Favero et al., 1995) and rat (Brotto & Nosek, 1996) hindlimb muscle exposed to H_2O_2 was shown to directly stimulate Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles suggesting a link between ROS and skeletal muscle Ca^{2+} homeostasis. More recent research using biopsied human muscle tissue found a similar effect of exhaustive exercise on Ca^{2+} channels (Leppik et al., 2004).

2.1.2.1.3 Myofilament proteins

In order for myofilament proteins (actin & myosin) to carry out contraction/relaxation of skeletal muscle, cofactors Ca^{2+} and ATP must be present. Ca^{2+} works to expose myosin binding heads by binding to and changing the conformation of tropomyosin, and ATP supplies the energy required for myosin heads to then bind to actin. Whilst low levels of ROS are important for signalling muscle contraction, higher levels seen during endurance exercise can have an inhibitory effect. Several studies suggest that high concentrations of ROS may reduce myofibrillar Ca^{2+} sensitivity, limiting the ability of tropomyosin to change conformation, thus affecting contractility (Andrade, Reid, Allen, & Westerblad, 1998; Andrade, Reid, & Westerblad, 2001). In support of this, Callahan et al. (2001) showed skinned rat diaphragm muscles treated with ROS-generating endotoxins to have reduced Ca^{2+} sensitivity and muscle force-generating capacity. Certain studies have even observed changes to the contractile proteins themselves, with oxidative modifications of the myosin head seen in H_2O_2 skinned

rabbit psoas muscle, resulting in restricted activity of myosin-actin function (Prochniewicz, Spakowicz, & Thomas, 2008).

2.1.2.2 Chronic effects

While chronically high levels of ROS can exert detrimental effects on cellular structure and function, ROS species including O_2^-, H_2O_2, and the highly reactive OH^- are critical modulators of redox signalling pathways and gene expression. Chronic low to moderate increases in ROS, such as that seen with endurance training, stimulate various hormones, protein kinases, and cytokines which upregulate adaptive pathways via gene expression (see Figure 3). Physiological adaptations such as improved cellular defence mechanisms, mitochondrial biogenesis, muscle protein synthesis, angiogenesis, and enhanced fat metabolism are all pertinent for improved exercise performance.

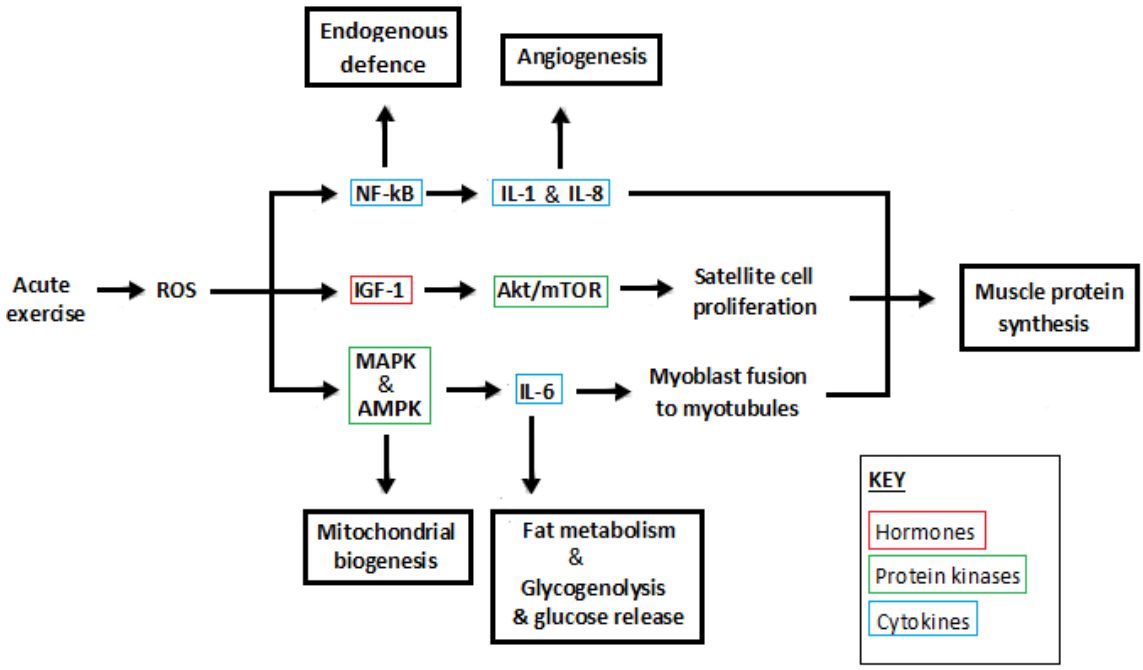

Figure 3 ROS stimulatory effects on various hormones, protein kinases and cytokines involved with physiological adaptation to improve exercise performance.

2.1.2.2.1 Up-regulation of endogenous antioxidant systems

Exercise alone appears to have antioxidant effects, upregulating endogenous antioxidant systems via redox signalling (Gomez-Cabrera, Domenech, & Viña, 2008). Various studies have observed increased activity of the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in skeletal and cardiac muscle, and the

liver following both acute exercise (Ji, 1999; McArdle, Pattwell, Vasilaki, Griffiths, & Jackson, 2001) and endurance training (Siu, Bryner, Martyn, & Alway, 2004). Acutely, activation of endogenous defence works to combat exercise-induced ROS, helping to minimise oxidative stress. Over time, adaptations to antioxidant systems allow for increased tolerance of skeletal muscle to future exercise stress, reducing susceptibility to oxidative damage, subsequently improving exercise performance (Jenkins, Friedland, & Howald, 1984; Miyazaki et al., 2001; Powers et al., 1994; Powers et al., 1993). Nuclear factor –kappa β (NF-kβ) is a key cytokine involved with endogenous antioxidant adaptation to exercise and studies have observed significantly increased levels of NF-kβ following exhaustive exercise protocols in rats (Ji, Gomez-Cabrera, Steinhafel, & Vina, 2004) and humans (Vider et al., 2001). Changes to antioxidant enzymes within muscle cells however, appear to be enzyme, muscle, and fibre type specific (Hollander et al., 1999; Leeuwenburgh, Fiebig, Chandwaney, & Ji, 1994). Furthermore, the theory of training-induced attenuation of ROS production during subsequent exercise has been disputed (Higuchi, Cartier, Chen, & Holloszy, 1985).

2.1.2.2.2 Mitochondrial biogenesis

Mitochondrial biogenesis, the process whereby cells increase their mitochondrial volume density, is mediated by exercise-induced ROS production and their activation of complex signalling pathways (Hood, 2009). Mitochondrial biogenesis underpins improvements in muscle fatigue resistance in endurance athletes, primarily through increased oxidative capacity and thus the ability to offset limited carbohydrate requirements with stored lipids. Endurance exercise-induced ROS have been shown to activate regulators involved in mitochondrial biogenesis such as mitogen-activated protein kinases (MAPK), adenine monophosphate activated protein kinase (AMPK), and several transcription factors (Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003; Mahoney, Parise, Melov, Safdar, & Tarnopolsky, 2005).

2.1.2.2.3 Muscle protein synthesis

Muscle protein synthesis refers to the anabolic process of muscle growth, or, hypertrophy, as a result of exercise-induced adaptations. The maintenance of, or gain in lean muscle mass is advantageous in endurance sport, allowing for greater muscular tension and thus power output. Several proteins, protein kinases, and cytokines are involved in signalling pathways that activate skeletal muscle protein synthesis. Insulin-like growth factor (IGF-1) is one such protein that works to increase muscle mass and prevent muscle loss (atrophy). Exercise-induced ROS have been shown to stimulate the expression of IGF-1 (Coleman et al., 1995; Devol, Rotwein, Sadow, Novakofski, & Bechtel, 1990), along with its downstream signalling pathways (Bodine et al., 2001; Ito, Ruegg, Kudo, Miyagoe-Suzuki, & Takeda, 2013; Murgia et

al., 2000; Pallafacchina, Calabria, Serrano, Kalhovde, & Schiaffino, 2002), and any disruption to this can result in decreased muscle cell size (Bohni et al., 1999; Leevers, Weinkove, MacDougall, Hafen, & Waterfield, 1996; Zhang, Stallock, Ng, Reinhard, & Neufeld, 2000). NF-kβ and MAPK are two other important regulators of the pro-inflammatory response to muscle damage (Silveira et al., 2007), and muscle protein turnover (Glass, 2005). While chronic levels of NF-kβ and MAPK are associated with muscle wasting, paradoxically, acute increases of the two play a vital role in the inflammation cascade, muscle protein turnover, cellular growth, and differentiation required for hypertrophy (Kramer & Goodyear, 2007; Roux & Blenis, 2004; Wretman et al., 2001).

ROS, specifically H_2O_2, produced during exhaustive exercise appear to activate MAPK and NF-kβ in humans (Vider et al., 2001; Williamson, Gallagher, Harber, Hollon, & Trappe, 2003) and animals (Goodyear, Chang, Sherwood, Dufresne, & Moller, 1996; Ji et al., 2004; Vina et al., 2000). In fact, the majority of studies have demonstrated exercise to enhance activity at several nodes of the NF-kβ pathway (Ho et al., 2005; Hollander et al., 2001; Ji, Gomez-Cabrera, & Vina, 2006; Ji et al., 2004).

2.1.2.2.4 Angiogenesis

Angiogenesis in response to exercise training allows for a greater volume of blood flow and thus oxygen to the working muscle, along with a greater capacity to remove muscle by-products such as lactic acid. It has been suggested that ROS function as signalling molecules within endothelial cells to induce angiogenesis via stimulation of vascular endothelial growth factor (VEGF) (Chua, Hamdy, & Chua, 1998; Ruef et al., 1997). The greatest source of these angiogenesis-stimulating ROS, in particular H_2O_2, appear to result from increased XO activity during post-exercise re-oxygenation of hypoxic tissues (Lelkes, Hahn, Sukovich, Karmiol, & Schmidt, 1998; Namiki et al., 1995; Shweiki, Itin, Soffer, & Keshet, 1992; Yasuda et al., 2000). H_2O_2 promotes endothelium-derived nitric oxide synthase (eNOS) activation, which further produces NO, a mediator of angiogenesis. In addition, exercise-induced ROS activation of NF-kβ activates other transcription factors which interact with endothelial tissue receptors to stimulate angiogenesis (Cogswell et al., 1994; Frydelund-Larsen et al., 2007; Matsusaka et al., 1993; Salven, Hattori, Heissig, & Rafii, 2002).

2.1.2.2.5 Fat metabolism

Within the body, the total amount of energy stored as fat is approximately 60-fold greater than that stored as glycogen. It is now well known that depleted skeletal muscle glycogen stores can reduce exercise performance (Bergström, Hermansen, Hultman, & Saltin, 1967) and

it appears that when glucose is readily available to muscle, there is a marked increase in time to fatigue (Coyle, Coggan, Hemmert, & Ivy, 1986; Hargreaves, Costill, Coggan, Fink, & Nishibata, 1984). Thus, the ability of muscle to utilise fat as a primary energy source during endurance exercise, and in doing so preserve glycogen, may prolong time to fatigue and therefore improve performance.

Endurance training can enhance the capacity of skeletal muscles to metabolise (oxidize) fats during submaximal exercise through ROS-mediated adaptation. This adaptation involves the upregulated gene expression of several transcription factors involved in the transport and oxidation of fatty acids across the mitochondrial membrane and into myocytes (Turcotte, Swenberger, Tucker, & Yee, 1999). Rat studies were the first to show increased capacity to oxidize fat following endurance training (Mole, Oscai, & Holloszy, 1971), while one of the first studies in biopsied human skeletal muscle found training to elicit increases in fat metabolism enzymes (Costill, Fink, Getchell, Ivy, & Witzmann, 1979). More recent studies show both high-intensity and long-duration training to significantly increase fat-oxidation transcription factors, mitochondrial oxidative enzymes, whole body and skeletal muscle fat oxidation, and reduce net glycogen use during exercise (Burgomaster, Heigenhauser, & Gibala, 2006; Burgomaster et al., 2008; Perry, Heigenhauser, Bonen, & Spriet, 2008; Talanian, Galloway, Heigenhauser, Bonen, & Spriet, 2007; Tunstall et al., 2002).

2.2 Antioxidants: Endogenous and exogenous defence

Antioxidants neutralize free radicals by accepting their highly reactive unpaired electron, becoming oxidised themselves in the process (Halliwell, 2007). Various transcription factors within the human body are activated or inhibited depending on the relative oxidant-to-antioxidant ratio, thus numerous signalling pathways are controlled by redox balance. At rest and during low-moderate exercise activity, endogenous antioxidant systems can easily maintain redox balance in healthy humans. However, during intense or long-duration exercise the capacity of these systems can be exceeded, leading to oxidative stress. As effective, endogenous defence against ROS is not infallible, we rely on dietary (exogenous) antioxidants to bridge the gap. Found naturally in food, and in higher concentrations as a supplemental form, dietary antioxidants help contribute to overall antioxidant defence, and therefore redox control.

2.2.1 Endogenous antioxidants

The endogenous defence system is comprised of antioxidant compounds and specific enzymes that catalyse their antioxidant activity. Whilst endogenous systems include several ROS-specific antioxidants such as α-lipoic acid (Packer, Witt, & Tritschler, 1995) and coenzyme Q10 (Ernster & Forsmark-Andree, 1993), the two key thiol antioxidants (GSH and taurine) are arguably the most important in maintaining redox balance. Whilst other thiol-dependent antioxidants exist, such as the family of thioredoxin proteins, due to the nature of this research, this literature review will not cover these. However, their specific roles have been reviewed in detail elsewhere (Hanschmann, Godoy, Berndt, Hudemann, & Lillig, 2013).

2.2.1.1 Thiols

Thiols play important physiological roles in processes requiring sulphur and are extremely reactive, with their –SH group being easily oxidized or reduced in the presence of a catalyst (Dhakshinamoorthy, Alvaro, & Garcia, 2010; Giles et al., 2003; Kachur, Koch, & Biaglow, 1998). Thiols can act as electron acceptors, reducing unstable free radicals by becoming oxidised, thus are potent antioxidants. Despite their high reactivity however, the antioxidant potential of thiols depend on environmental, structural, and catalytic factors (Barron, 2006; Conte & Carroll, 2013; Winterbourn & Metodiewa, 1999). Thus instead of targeting all ROS as common dietary antioxidants tend to do, thiols may instead target specific ROS. For this reason, the use of thiols may evade the 'blunting' of exercise-induced adaptation that has been observed with administration of many common dietary antioxidants such as vitamins C and E (Bjørnsen et al., 2015; Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Makanae, Kawada, Sasaki, Nakazato, & Ishii, 2013; Paulsen, Cumming, et al., 2014; Ristow et al., 2009).

Thiol redox status is tightly regulated by the body and remains relatively constant to avoid chronic oxidative stress and associated detrimental effects. Despite this, drastic oxidizing conditions, such as that resulting from intense exercise (Ji, Fu, & Mitchell, 1992), can lower thiol concentrations, allowing a pro-oxidant state to predominate.

2.2.1.1.1 Methionine

An essential amino acid, methionine must be attained from the diet. Methionine is endogenously metabolised to homocysteine and then to cysteine; the conversion of which is rate-limited by several enzymes. High plasma homocysteine concentrations have been linked to several adverse health effects including increased risk for cardiovascular damage (Refsum, Ueland, Nygård, & Vollset, 1998). However, in healthy humans, whilst high one-off intakes of methionine can increase homocysteine levels (Chambers, McGregor, Jean-Marie, Obeid, &

Kooner, 1999a) and in some cases induce endothelial dysfunction (Chambers, Obeid, & Kooner, 1999b), there appear to be no long term adverse effects. Furthermore, moderate fluctuations in dietary methionine appear to have no effect on plasma homocysteine levels (Ward et al., 2000).

2.2.1.1.2 Cysteine

Cysteine can be endogenously synthesized from methionine. As an amino acid, cysteine has important structural roles and can bind its thiol side-chains to metals such as zinc, copper and iron which are crucially important for enzymatic functions. Cysteine's thiol side-chain also allows for its incorporation into the tri-peptide thiol antioxidant GSH. Additionally, the metabolism of cysteine via the cysteine-sulphinic acid pathway can generate taurine, although being enzymatically rate-limited, this pathway is much more complicated than that of GSH (Bella, Hahn, & Stipanuk, 1999; Lee, Londono, Hirschberger, & Stipanuk, 2004). Cysteine provides both GSH and taurine with their bioactive -SH (thiol) group. Despite cysteine being important at moderate levels for cellular signalling pathways, high plasma levels have been linked to cardiovascular (El-Khairy, Ueland, Nygård, Refsum, & Vollset, 1999; Özkan, Özkan, & Şimşek, 2002) and neurological diseases (Heafield et al., 1990). Additionally, high intracellular levels can promote oxidative DNA damage via the Fenton reaction (Park & Imlay, 2003). Therefore, the homeostatic control of cysteine is crucial, and is partly achieved through the ability of the body to store cysteine as GSH.

2.2.1.2 Thiol antioxidants

GSH and taurine are arguably the most important thiol antioxidants, having numerous roles in the protection of cells from ROS attack. Both are formed endogenously from cysteine via two distinct metabolic pathways (Figure 4).

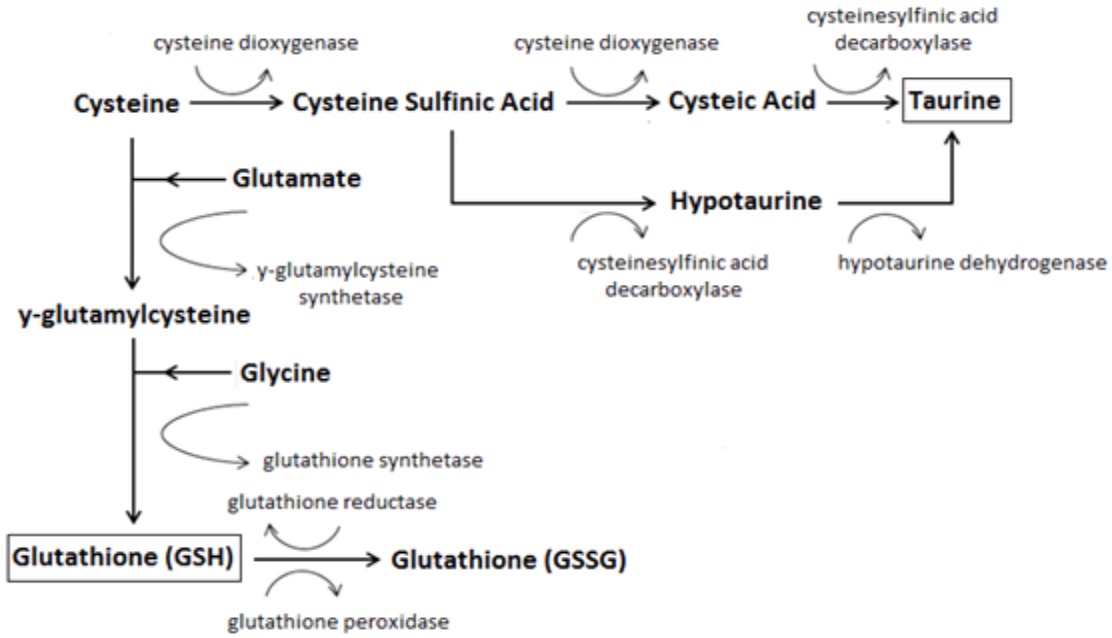

Figure 4 The metabolic pathway of glutathione and taurine synthesis. Glutathione synthesis follows the γ-glutamylcysteine pathway, while taurine synthesis follows the cysteine sulfinic acid pathway.

2.2.1.2.1 Glutathione

GSH is found abundantly throughout the human body. A tri-peptide, it is synthesized from three amino acids; glycine, L-cysteine and L-glutamate, and exists in two states; reduced (GSH) and oxidised (GSSG). Predominantly found intracellularly, the thiol-containing cysteine molecule in GSH allows it to partake in antioxidant roles, being involved in both the breakdown and removal of ROS (Hayes & McLellan, 1999). In its reduced form, GSH retains its antioxidant ability, and with GPx as a catalyst enzyme, accepts oxygen radicals to convert to its oxidised di-sulfide state, GSSG. Reduction back to GSH is mediated by the NADPH dependent glutathione reductase (GR). The cycling between these two states aids in free radical and toxic substance metabolism, with the GSH/GSSG ratio a sign of redox state and relative level of oxidative stress (Jones, 2002).

2.2.1.2.1.1 Synthesis of GSH

Approximately 90% of GSH is synthesized within the liver (Bannai & Tateishi, 1986) and exported into circulation (Kaplowitz, Aw, & Ookhtens, 1985). GSH synthesis is tightly regulated and requires several metabolic reactions (Figure 4):

1. glutamate + cysteine → γ -glutamylcysteine

   Catalyst: *γ-glutamylcysteine synthetase* (GCS)

2. γ-glutamylcysteine + glycine → GSH
   Catalyst: *GSH synthetase (GS)*

Synthesis of GSH is rate-limited in several ways. The availability of intracellular cysteine appears to significantly influence GSH synthesis (Bannai & Tateishi, 1986; Demaster, Shirota, Redfern, Goon, & Nagasawa, 1984; Tateishi, Higashi, Shinya, Naruse, & Sakamoto, 1974), thus, dietary intake of methionine, cysteine, and cystine (oxidized cysteine), their transport to and into cells, and the metabolism of methionine to cysteine all affect GSH levels (Tateishi et al., 1974; Tateishi & Sakamoto, 1983). Due to higher extracellular concentrations, cystine is preferentially transported into the cell above cysteine, although studies suggest cysteine is more *efficiently* transported into cells (Ishii, Sugita, & Bannai, 1987). Thus, cystine and cysteine uptake into cells are rate-limiting steps for GSH synthesis, with the exception of liver cells which can utilize methionine (Kaplowitz et al., 1985). The exact regulation for cystine transport is not fully understood, however some studies suggest that increased oxygen concentration and subsequent ROS formation may result in induction of cystine uptake via secondary effects (Deneke, Baxter, Phelps, & Fanburg, 1989; Miura, Ishii, Sugita, & Bannai, 1992). Depletion of intracellular GSH may also up-regulate cystine transport into the cell and thus GSH synthesis as a protective mechanism, with increased electrophilic activity showing enhanced cystine transport in human fibroblasts (Bannai, 1984).

2.2.1.2.1.2 Distribution and regulation of GSH

Most cellular GSH (85-90%) resides within the cytosol, with the remaining 10-15% being found in various organelles including the mitochondria and peroxisomes (Lu, 2009). While GSH concentrations in different cell types can vary up to 10-fold, the concentration within any given cell type tends to remain fairly consistent in order to maintain redox balance. Regulation of GSH is primarily based on availability of cysteine, and activity of catalyst enzymes GCS and GS. The enzymatic activity of GCS is a key determinant of GSH synthesis, generally dictating GSH levels and biosynthetic capacity. Oxidative stress has been shown to regulate GCS activity via both transcriptional regulation of the two GSC subunits, and post-translational modification of either one, or both subunits (Krzywanski et al., 2004; Yang, Magilnick, Ou, & Lu, 2005). The resulting increase in GCS activity up-regulates GSH synthesis. GCS activity is also dependent on the availability of cysteine, which in turn is reliant on methionine, homocysteine, and several metabolic co-factors, within the liver (Ubbink, Vermaak, van der Merwe, & Becker, 1993). Several studies have shown depleted hepatic GSH concentrations when methionine metabolism is impaired (Lee, Sadda, et al., 2004; Vendemiale et al., 1989). GCS also appears to

be feedback inhibited by GSH, whereby GSH competes with glutamate at the active site of the GCS-C subunit (Misra & Griffith, 1998; Yang et al., 2007). Thus, high concentrations of GSH inhibit the activity of GCS and synthesis of GSH, whilst low levels allow for increased synthesis rates in the presence of glutamate. The regulation of GSH/GSSG ratio is dependent on catalyst enzymes GPx, and GR, which oxidise GSH to GSSG and reduce back to GSH, respectively (Meister, 1995).

2.2.1.2.1.3 Roles

The GSH/GSSG redox system play roles in various general processes including immune function (Dröge & Breitkreutz, 2000), hormonal regulation (Howie, Walker, Åkesson, Arthur, & Beckett, 1995), and apoptosis (Circu & Yee Aw, 2008), however, those important for endurance exercise include cysteine storage, skeletal muscle glucose uptake, gene expression, and notably, antioxidant activity (Figure 5).

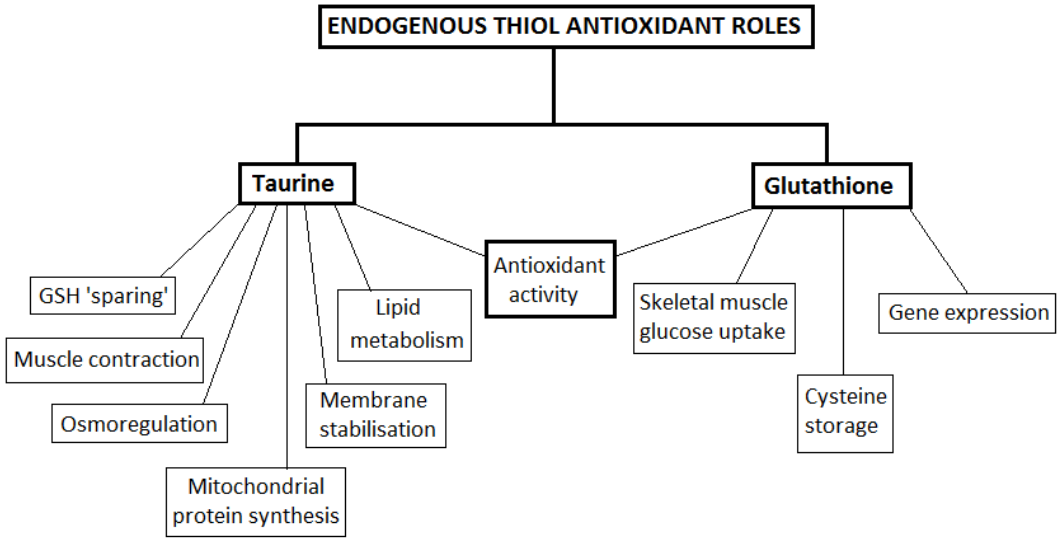

Figure 5 Roles of the endogenous antioxidants taurine and glutathione. Whilst having specific actions, both have powerful antioxidant activity that act to reduce ROS and their potential damaging effects.

2.2.1.2.1.3.1 Cysteine source

One of the major functions of GSH is to act as a cysteine 'reservoir'. Cysteine is extremely unstable outside the cell, and rapidly auto-oxidises to cystine. As it does so, electrons are released which proceed to form ROS (Meister, 1988). 'Storing' cysteine in the form of GSH reduces the rate of ROS production, while still providing a source of cysteine when needed. The process of GSH synthesis and breakdown is known as the gamma γ-glutamyl cycle. As GSH

is broken down, it releases cysteine, while in reverse, the majority of imported cysteine is incorporated into GSH, although some is degraded to taurine (Lu, 1999). Therefore the γ-glutamyl cycle allows GSH to serve as a continual source of cysteine (Orlowski & Meister, 1970).

2.2.1.2.1.3.2 Skeletal muscle glucose uptake

During endurance exercise, glucose availability within skeletal muscle is particularly important, and the ingestion and consequent uptake of glucose into these cells via a combination of insulin action (Klip & Paquet, 1990) and skeletal muscle contraction (Hayashi, Hirshman, Kurth, Winder, & Goodyear, 1998) have been shown to prolong time to fatigue (Coggan & Coyle, 1987; Coyle et al., 1986; Wright, Sherman, & Dernbach, 1991). The action of insulin on skeletal muscle glucose uptake appears to be partly controlled by hepatic GSH levels. Guarino and colleagues (2003) found that hepatic insulin-sensitizing substance (HISS), a hormone responsible for insulin sensitivity, was decreased when hepatic GSH was depleted, thereby decreasing sensitivity of skeletal muscle to insulin and reducing glucose uptake into cells. On the other hand, excess GSH can also interfere with insulin sensitivity, with McClung *et al.* (2004) finding an overexpression of GPx, the enzyme required for GSH oxidation, to induce insulin resistance in mice, thereby also decreasing glucose uptake. This reiterates the importance of GSH redox balance within the body.

2.2.1.2.1.3.3 Gene expression

GSH plays a key role in regulating transcription factors involved in gene expression. Transcription factors interact with the promoter region on a wide variety of genes involved in oxidative stress and cellular response mechanisms (Lavrovsky, Schwartzman, Levere, Kappas, & Abraham, 1994; Zhou, Johnson, & Rando, 2001). Acute exercise has been shown to up-regulate genes involved in several adaptive processes including energy metabolism (Safdar et al., 2011), muscle protein synthesis (Coleman et al., 1995), and angiogenesis (Frydelund-Larsen et al., 2007) via ROS modulated up-regulation of certain transcription factors (see Figure 3). While small amounts of ROS are required for gene expression and subsequent skeletal muscle function and adaptation, higher levels such as that seen in oxidative stress can result in altered gene expression. This in turn can affect exercise processes, potentially affecting acute performance. GSH/GSSG homeostasis is therefore crucially important in gene expression (Arrigo, 1999).

2.2.1.2.1.3.4 Antioxidant activity

The ability of GSH to act as an antioxidant stems from its thiol-containing cysteine moiety. GSH is involved in both the first and second line of ROS defence, and requires GPx enzymes to catalyse the breakdown of H_2O_2 via reduction of GSH to GSSG. The most extensively studied GPx (GPx1) is selenium dependent (Rotruck et al., 1973) and is found in the kidneys and mitochondria (Esworthy, Ho, & Chu, 1997). Four other GSH peroxidases (GPx2-GPx5) have also been discovered, with evidence of antioxidant properties *in vivo* (Arai et al., 1999). Detoxified metabolites resulting from GPx defence, are eliminated from the cell via a glutathione S-conjugate transporter (Hayes & McLellan, 1999). Thus GSH is central to detoxification of ROS; although without an enzyme system to catalyse these detoxification reactions, along with the ability to catalyse the reduction of GSSG, it could not function as an intracellular antioxidant.

2.2.1.2.1.3.5 Regeneration of other antioxidants

GSH appears to indirectly regenerate vitamin E back to its antioxidant state via restoration of vitamin C (Chan, 1993; Vatassery, Smith, & Quach, 1989). GSH deficiency in new-born rats, produced by administration of a GSH enzyme inhibitor, has been shown to decrease vitamin C levels within the kidney, liver, brain, and lung (Martensson, Meister, & Mrtensson, 1991), while GSH administration has been shown to increase both vitamins C and E (Chan, Tran, Raynor, Ganz, & Chow, 1991; Niki, Tsuchiya, Tanimura, & Kamiya, 1982). Interestingly, studies show vitamin C deficiency to significantly lower blood GSH levels (Henning, Zhang, McKee, Swendseid, & Jacob, 1991) while supplementation appears to regenerate GSH (Jain et al., 1992; Johnston, Meyer, & Srilakshmi, 1993). Similarly, vitamin E deficiency appears to significantly decrease blood GPx activity and GSH levels (Chow, 1976) while supplementation appears to enhance GSH levels (Costagliola et al., 1986; Jain, McVie, & Smith, 2000). Thus it seems both endogenous and exogenous antioxidants work together to maintain redox balance.

2.2.1.2.2 Taurine

In addition to GSH, cysteine can be metabolised to the intracellular sulfonic acid, taurine, via the cysteine-sulfinic acid pathway. Taurine, or 2-aminoethanesulfonic acid, is found abundantly throughout the human body. Lacking a carboxyl group, it is unable to be incorporated into proteins thus it is theoretically a non-amino acid (Gilbert & Martin, 2010), although it is usually referred to as one. As a result of taurine's non protein-bound state, it remains free in the plasma and intracellular tissue of mammals (Brosnan & Brosnan, 2006). Excitable tissues that produce higher levels of ROS appear to have increased taurine levels, as well as tissues where other potentially toxic substances such as bile acids and xenobiotics are

found (Wright, Tallan, & Lin, 1986). High levels are also found in white blood cells and platelets (Franconi, Di Leo, Bennardini, & Ghirlanda, 2004), thus taurine's most notable function is as an antioxidant.

2.2.1.2.2.1 Synthesis

Synthesis of taurine from cysteine requires three main metabolic reactions:

1. cysteine → cysteine sulfinic acid →cysteic acid
   Catalyst: *cysteine dioxygenase (CDO)*

2. cysteine sulfinic acid →hypotaurine
   cysteic acid →taurine
   Catalyst: *cysteinesulfinic acid decarboxylase (CSAD)*

3. hypotaurine →taurine
   Catalyst: *hypotaurine dehydrogenase (HDH)*

Synthesis occurs primarily in the liver, with hepatocytes having the greatest capacity for taurine production due to the highest level of CDO-mRNA (Bella, Hirschberger, Kwon, & Stipanuk, 2002; Tsuboyama et al., 1996). Liver CDO responds rapidly to increased dietary sulphur amino acid intake (Bella et al., 1999; Kwon & Stipanuk, 2001). While taurine is the most abundant amino acid in cardiac and skeletal muscle, taurine biosynthesis within these tissues is very limited (Ito, Kimura, Uozumi, Takai, Muraoka, Matsuda, Ueki, Yoshiyama, Ikawa, Okabe, et al., 2008; Warskulat et al., 2004), indicating a very strong taurine transporter uptake system. Uptake is dependent therefore on adequate plasma levels which are regulated by the liver. Once synthesised, taurine is actively transported in the circulation by platelets (Ahtee, Boullin, & Paasonen, 1974), and is taken up into cells by the transmembrane taurine transport protein, TauT; a sodium- and chloride dependent active transporter that is specific for the β-amino acids taurine, hypotaurine and β-alanine.

Taurine synthesis is rate limited by three main factors. The availability of the taurine precursors methionine and cysteine are key determinants of endogenous taurine, thus adequate dietary intake is crucial. Rats have demonstrated increased liver, kidney, and urine taurine after administration of methionine (Lombardini & Medina, 1978; Stipanuk, Londono, Lee, Hu, & Yu, 2002), while cysteine administration appears to increase heart, muscle, liver, brain, and spleen taurine levels (Awapara & Wingo, 1953; Dawson Jr, Liu, Eppler, & Patterson,

1999; Stipanuk et al., 2002). Cysteine-sulfinic acid pathway enzyme activity is also crucial to taurine synthesis. Increased CDO activity and taurine levels have been observed in humans and monkeys when given a diet high in cysteine or methionine (Bagley & Stipanuk, 1995; Helms, Storm, Christensen, Hak, & Chesney, 1999). The availability of co-factors vitamins B6 (Shin & Linkswiler, 1974) and B12 (Roman-Garcia et al., 2014), and folate (Fenech, 2001; Takeyama, Hatch, & Buchanan, 1961), can also significantly affect taurine synthesis. Factors such as age (Sturman & Gaull, 1975), gender (De la Rosa & Stipanuk, 1985) , and disease states (Di Leo et al., 2002) can also affect synthesis of taurine to a lesser extent.

2.2.1.2.2.2 Distribution and regulation

The largest mass of taurine (70%) is found in skeletal muscle (Hayes & Sturman, 1981; Huxtable, 1992) and is also highly concentrated in cardiac muscle (Jacobsen & Smith, 1968). In humans, skeletal muscle taurine appears to be more abundant in type-1 (oxidative) than in type-2 (non-oxidative) muscle fibres (Airaksinen et al., 1990). In fact, within the vastus lateralis muscle, taurine concentration appears to be four times higher in type-1 than type-2 fibres (Harris, Dunnett, & Greenhaff, 1998). Endurance exercise relies predominantly on type-1 fibres, therefore maintaining muscle taurine levels may allow for greater performance.

Skeletal muscle taurine appears to be tightly regulated by TauT (Galloway, Talanian, Shoveller, Heigenhauser, & Spriet, 2008; Tappaz, 2004) which are primarily located within the kidneys; the organs responsibly for whole body taurine regulation (Han, Budreau, & Chesney, 2000). However, TauT are also found in the cell membranes of various organs, allowing for uptake and use of taurine in cells, and are highly expressed in skeletal muscle cells (Ramamoorthy et al., 1994). Studies using TauT-knockout mice support TauT as the primary transport of taurine into cells, with significantly decreased cardiac, hepatic and skeletal muscle taurine concentrations along with substantial detrimental effects on the retina, heart, kidney, brain and reproductive development and health in those with disrupted gene coding for TauT (Heller-Stilb et al., 2002; Ito, Kimura, Uozumi, Takai, Muraoka, Matsuda, Ueki, Yoshiyama, Ikawa, Okabe, et al., 2008; Warskulat et al., 2006; Warskulat et al., 2004).

The kidneys respond to circulating taurine levels by up- or down-regulating TauT activity, which in turn determines whether taurine is reabsorbed or not (Tappaz, 2004). Increased dietary taurine up-regulates taurine flux through the liver and circulation, thereby lowering TauT activity and kidney reabsorption. Conversely, low dietary taurine down-regulates flux through the liver, resulting in lower circulating levels and therefore increased TauT activity and kidney reabsorption. Thus TauT activity and subsequent cell uptake is very reliant on

circulating substrate and optimal liver and kidney function. When taurine levels are in excess, and kidney reabsorption is low, there is an increase in urinary taurine excretion (Thompson & Vivian, 1977). Similarly, low taurine levels resulting in increased renal reabsorption is often measured through decreased urinary taurine excretion (Chesney, Gusowski, & Dabbagh, 1985; Han, Patters, Jones, Zelikovic, & Chesney, 2006). This conservative mechanism allows for tissue taurine concentrations to stay relatively constant in the majority of species (Chesney et al., 1985).

Taurine regulation can be both directly and indirectly affected by external osmolarity. Hyperosmolarity up-regulates the promoter region of TauT-mRNA (Ito et al., 2004), inducing TauT activity and allowing uptake of taurine into cells to assist in cell volume regulation (Guizouarn, Motais, Garcia-Romeu, & Borgese, 2000). Additionally, CDO and CSAD genes appear to be sensitive to hyperosmolarity, although less so than that of the TauT gene (Beetsch & Olson, 1998; Bitoun, Levillain, & Tappaz, 2001).

2.2.1.2.2.3 Roles

Taurine is critical right from conception (Casslen, 1987; Holmes, Goodman, Shihabi, & Jarow, 1992) through to aging (Militante & Lombardini, 2004; Schaffer, Azuma, & Mozaffari, 2009). Normal plasma taurine levels in healthy humans sit around 44 mumol/L, whilst whole blood levels sit within the range of 164-318 mumol/L(Trautwein & Hayes, 1990)) With its most notable role being antioxidant activity and contribution to tissue antioxidant status (Aruoma, Halliwell, Hoey, & Butler, 1988; Gürer, Özgünes, Saygin, & Ercal, 2001; Oudit et al., 2004; Vohra & Hui, 2001; Wright et al., 1986), taurine also appears to be involved in mitochondrial protein synthesis (Suzuki, Suzuki, Wada, Saigo, & Watanabe, 2002), electrical stabilization (Pierno, De Luca, Camerino, Huxtable, & Camerino, 1998), calcium homeostasis (Schaffer, Jong, Ramila, & Azuma, 2010), osmoregulation (Guizouarn et al., 2000), and lipid metabolism (Hardison, 1978; Rabin, Nicolosi, & Hayes, 1976; Vessey, 1978) (see Figure 5).

2.2.1.2.2.3.1 Antioxidant activity

Taurine appears to act more as an indirect antioxidant, with limited or no ability to directly scavenge and react with common ROS (Aruoma et al., 1988). Instead, taurine may up-regulate endogenous antioxidant activity via increased antioxidant enzymes such as GPx (Nandhini, Balakrishnan, & Anuradha, 2002; Vohra & Hui, 2001) and SOD (Nandhini et al., 2002). In addition, taurine may indirectly increase endogenous GSH levels. Endurance exercise can significantly increase circulating lipid peroxidation products, which are often associated with a decline in GSH (Noeman, Hamooda, & Baalash, 2011; Trevisan et al., 2001). However, taurine

supplementation has been shown to reduce lipid peroxidation and maintain GSH levels, suggesting its action against oxidative stress (Anand, Rajakumar, Jeraud, Felix, & Balasubramanian, 2011; Oudit et al., 2004; Zhang, Izumi, et al., 2004). Since GSH is an antioxidant that decreases with oxidative stress, it seems plausible that taurine's potent antioxidant activity may improve endogenous antioxidant defence, thereby mitigating the consumption of GSH. Furthermore, taurine's metabolic precursors, cysteine sulphinic acid (CSA) and hypotaurine, also show apparent indirect antioxidant roles, with *in vitro* scavenging of HOCl and the OH^- (Aruoma et al., 1988), along with inhibition of DNA oxidation (Messina & Dawson Jr, 2002).

Taurine may also inhibit the formation of free radicals (Schaffer et al., 2009). During endurance exercise, there is an increase in neutrophil activity, thus, an increase in HOCl formation (see Section 2.1.1.4.1). The amino group of taurine is a direct scavenger of HOCl (Schaffer et al., 2009), and in the presence of myeloperoxidase (MPO), reacts with the acid to produce a less toxic oxidant known as taurine chloramine (TauCl) (Schuller-Levis & Park, 2003). Since neutrophils contain high levels of taurine, the formation of TauCl can continue as long as there is sufficient turnover of taurine. TauCl not only plays a role in antioxidant systems by reducing HOCl levels, but also inhibits production of O_2^- and pro-inflammatory mediators in neutrophils (Schuller-Levis & Park, 2004) and macrophages (Marcinkiewicz, Grabowska, Bereta, & Stelmaszynska, 1995). Park *et al.* (2003) observed further anti-inflammatory properties of TauCl, with its inhibition of inflammatory markers nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α). While also important at low levels for cell signalling, these inflammatory markers can interact with other free radicals to form secondary ROS, and act as messengers in ROS-generating pathways respectively, thus contributing to oxidative stress.

2.2.1.2.2.3.2 Mitochondrial Protein Synthesis

Mitochondrial proteins, such as those required by the ETC, are produced primarily within the cell nucleus and are then transported to the mitochondria via protein translocases. It has been suggested that regulation of ETC ROS formation can be affected by mitochondrial protein synthesis (Schaffer et al., 2009). Taurine can conjugate with uridine, to form 5-taurinomethyluridine in the wobble position of mitochondrial tRNA, which then further modulates mitochondrial protein synthesis (Suzuki et al., 2002). With mitochondrial proteins being functional subunits of respiratory complexes, reduced expression of 5-taurinomethyl uridine, and thus certain mitochondrial proteins, can decrease the activity of ETC subunits. Consequently, rather than being used by the ETC, a flux of electrons may divert to other acceptors such as oxygen, forming ROS. Indeed, studies have observed a probable link

between 5-taurineomethyluridine deficiency and mitochondrial diseases such as MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke like episodes) (Kirino, Goto, Campos, Arenas, & Suzuki, 2005; Scheper, van der Knaap, & Proud, 2007; Yasukawa, Suzuki, Ishii, et al., 2000; Yasukawa, Suzuki, Ueda, Ohta, & Watanabe, 2000).

2.2.1.2.2.3.3 Muscle function

Taurine is crucially involved in muscle function via its roles in membrane stabilization and calcium homeostasis. Taurine can directly or indirectly (through protein kinase C (PKC)-mediation) modulate ClC-1 chloride channel and other ion channels (K^+) that control muscle excitability (Conte-Camerino et al., 1987). In order for an action potential to be initiated, and subsequent muscle contraction to occur, the resting muscle chloride conductance (gCl) must exceed the resting potassium conductance. ClC-1 is the major chloride (Cl^-) channel in the sarcolemma of mammalian skeletal muscle that is responsible for restoration of gCl and muscle membrane repolarization following contraction. If there is a reduction in gCl due to altered ClC-1 activity, skeletal muscle function is affected. In aged rats, there is an observed decrease of taurine in fast-twitch muscle along with a reduction in gCl (Pierno et al., 1998). However, taurine supplementation appears to increase muscle taurine levels and improve muscle function.

Taurine also modulates intracellular calcium homeostasis, important for controlling muscle fibre contractility. Skeletal muscle SR Ca^{2+} ATPase is the transport protein responsible for the re-uptake of Ca^{2+} into the SR post-contraction. ATPase appears to be inhibited by oxidative stress (Park, Kanekal, & Kehrer, 1991), with one study suggesting a direct attack of OH^- on its ATP binding site of ATPase (Xu et al., 1997) . If ATPase is unable to function, Ca^{2+} cannot exit the cell, thus compromising muscle cell contractibility. Taurine appears to modulate Ca^{2+} levels (Sawamura, Sada, Azuma, Kishimoto, & Sperelakis, 1990) in a biphasic pattern in which it seems to exert multiple effects on Ca^{2+} transport, via both cellular uptake and loss (Schaffer, Ballard, & Azuma, 1994). Taurine applied directly to the SR of isolated rat skeletal muscle has been shown to slow the rate of loss of Ca^{2+} transport through increased ATPase activity (Huxtable & Bressler, 1973) while in the rat retina (Militante & Lombardini, 1998), and hippocampal neurons (Zhao, Huang, & Cheng, 1999), taurine supplementation increases Ca^{2+} cell uptake via membrane Ca^{2+} channels; although there appears to be uptake differences between acute and chronic supplementation (Bkaily et al., 1997). There may even be a strong link between taurine's role in membrane stabilization and Ca^{2+} homeostasis, with results from later studies suggesting taurine's effect on membrane stabilization, and thus the initiation of an action potential, may partly regulate these cellular changes in Ca^{2+} (De Luca, Pierno, &

Camerino, 1996; Pierno et al., 1998). In support of this, several studies have shown taurine-increased membrane polarisation (Conte-Camerino et al., 1987; Gruener & Bryant, 1975) and intracellular membrane stability (Huxtable & Bressler, 1973). Taurine also appears to have an effect on increasing the sensitivity of contractile filaments to Ca^{2+} in rodent muscle fibres (Bakker & Berg, 2002; Hamilton, Berg, Easton, & Bakker, 2006).

2.2.1.2.2.3.4 Osmoregulation

Osmoregulation is important for ion transport, cell volume regulation, and maintaining cell structure and function. The most well documented effect of osmotic imbalances on exercise performance is seen with exercise-induced changes in plasma volume. Endurance exercise can significantly decrease plasma volume through hypo-hydration (Costill & Fink, 1974; Sawka, Young, Francesconi, Muza, & Pandolf, 1985), a state achieved through sweat loss and increased respiration, causing cell shrinkage and intracellular dehydration (Reinehr & Häussinger, 2006). Short term increases in plasma osmotic pressure (hyperosmolarity) are important for extracellular signalling and the regulation of target genes such as those involved in amino acid transport (Alfieri et al., 2001), and in fact may be necessary for training adaptations (Convertino, Brock, Keil, Bernauer, & Greenleaf, 1980). However, prolonged hyperosmolarity can have significant impact on exercise performance via decreased cardiac output and subsequent reduction of blood flow to the working muscles, resulting in reduced energy production (González-Alonso, Calbet, & Nielsen, 1998). In addition, a decline in blood flow to the skin has been observed (Gonzalez-Alonso, Mora-Rodriguez, Below, & Coyle, 1985; Nadel, Fortney, & Wenger, 1980; Sawka et al., 1985), reducing heat dissipation thus increasing core body temperature further reducing exercise performance through 'central fatigue' (Montain & Coyle, 1992; Tucker, Rauch, Harley, & Noakes, 2004).

Cell hyperosmolarity up-regulates the promoter region of TauT mRNA (Ito et al., 2004), inducing TauT activity in the kidneys (Bitoun et al., 2001; Uchida, Nakanishi, Kwon, Preston, & Handler, 1991), brain (Bitoun & Tappaz, 2000), and liver (Warskulat, Wettstein, & Haussinger, 1997). The subsequent uptake of taurine into the cell regulates cell volume (Guizouarn et al., 2000), protecting against hyperosmotic stress and related tissue damage. During endurance exercise, increased plasma osmolarity has been observed along with increased plasma taurine levels, a possible result of taurine efflux from muscle cells (Matsuzaki et al., 2002). Because this increase in taurine does not appear to correlate to the changes in plasma osmolarity, it has been suggested that increased plasma taurine during exercise is related to osmotic regulation (Cuisinier et al., 2002). In addition, the decline in muscle taurine does not appear to correlate to this increased plasma taurine (Matsuzaki et al., 2002). It may therefore be assumed that

while some muscle taurine is released into the plasma to combat osmotic stress, the majority of muscle taurine is used to combat exercise-induced cellular stress. Thus, low taurine levels within the body may result in the inability of the cell to regulate hyperosmotic stress within the plasma and tissues, potentially affecting exercise performance.

2.2.1.2.2.3.5 Lipid metabolism

One of the earliest researched uses of taurine was its role in bile salt formation. Along with glycine, taurine is used by the liver to produce bile salts, a crucial component in lipid metabolism (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Taurine conjugates with cholic acid, a bile acid formed from cholesterol, to form TauCl. Endurance exercise places a large reliance on fat as a fuel source in order to preserve muscle glycogen stores; thus the ability of the body to extract fat from food and store it in cells for later use begins with optimal absorption of lipids from the intestine into the blood stream, a process that is dependent on the formation of TauCl, and therefore, taurine availability. While research in this area is limited, low birth-weight infants supplemented with taurine have shown increased intestinal fat absorption (Verner, Craig, & McGuire, 2007; Wasserhess, Becker, & Staab, 1993), while supplementation in overweight adults have shown improved serum lipid profiles (Zhang, Bi, et al., 2004). Moreover, increased faecal fat excretion (steatorrhoea), a sign of incomplete fat absorption, has been observed with a low taurine to glycine conjugate ratio; an issue that appears to be rectified with taurine supplementation (Darling, Lepage, Leroy, Masson, & Roy, 1985; Smith et al., 1991; Thompson, 1988). Thus it appears that low body taurine concentrations may affect fat uptake into cells and the subsequent utilization for energy production.

The importance of using fat as a source of fuel during endurance exercise has been well documented over the years. While muscle glycogen is used during the initial stages of exercise, there is a limited amount stored within muscle, and as exercise duration and/or intensity increases, there is a greater reliance on fat oxidation by the mitochondria to provide ongoing energy to the working muscles. Endurance training appears to increase total fat oxidation during prolonged exercise (Horowitz, Leone, Feng, Kelly, & Klein, 2000; Martin et al., 1993; Romijn, Klein, Coyle, Sidossis, & Wolfe, 1993), while decreasing plasma glucose oxidation (Coggan, Kohrt, Spina, Bier, & Holloszy, 1990). In addition, research suggests that endurance athletes following a high fat diet may have enhanced resistance to fatigue (Lambert, Speechly, Dennis, & Noakes, 1994), a result of greater fat oxidation sparing muscle glycogen (Miller, Bryce, & Conlee, 1984). Thus, taurine's ability to enhance fat uptake from the intestine may reduce fatigue by providing substrate for cellular fat oxidation. In support of this, taurine

intake prior to endurance exercise in humans has been shown to significantly increase total fat oxidation (Rutherford, Spriet, & Stellingwerff, 2010), while increasing time to fatigue in rats (Yatabe, Miyakawa, Miyazaki, Matsuzaki, & Ochiai, 2003).

Additionally, fat-soluble vitamins are dependent on lipids for transport across intestinal epithelial cells, thus if TauCl is low, the resulting decrease in lipid absorption limits vitamin uptake. Vitamins A, D, and E all play various antioxidant roles within the body, thus have the ability to lower oxidative stress.

2.2.2 Exogenous antioxidants

Due to our endogenous antioxidant systems not being completely efficient, and a large amount of environmental, physical, and dietary stressors, exogenous (dietary) antioxidants are required to assist in combatting excess ROS production. The most well researched dietary antioxidants in exercise studies are vitamins C and E.

2.2.2.1 Vitamin C

A reducing agent, vitamin C is a powerful antioxidant, and can neutralize free radicals by donating electrons. Being essential, vitamin C must be obtained through dietary means, and whilst predominantly found in fruits in vegetables, it can be also obtained through supplementary means.

Regulation of vitamin C within plasma is tightly controlled up to a steady-state concentration of approximately 80μM which Levine et al. (2001) found corresponded to a daily intake of ≥200-400mg. The kidneys regulate vitamin C reabsorption in the proximal tubule via the vitamin C transporter SVCT1 (Savini, Rossi, Pierro, Avigliano, & Catani, 2008). As transport reabsorption approaches maximal velocity, excess vitamin C is excreted within urine. Vitamin C cannot be stored within cells, thus daily intake is crucial to maintain adequate plasma levels.

2.2.2.1.1 Roles

Whilst vitamin C plays a key role in various physiological systems within the human body, it is also crucial for a number of exercise-related processes. In addition to potent antioxidant activity, vitamin C is necessary for iron absorption, carnitine synthesis, catecholamine production and collagen synthesis.

2.2.2.1.1.1 Antioxidant activity

Vitamin C's most notable role is its powerful antioxidant activity. While it has long been known that vitamin C can neutralize harmful free radicals such as O_2^-, OH^- and H_2O_2, it can also

neutralize reactive non-radicals such as HOCl, nitrosating compounds, and transition metal-mediated reactions involving copper and iron (Padayatty et al., 2003). Vitamin C is also crucial in restoring oxidized vitamin E (α-tocopheroxyl), a secondary radical, back to its reduced active form (Bruno et al., 2006). Vitamin C is therefore vital for overall redox balance.

2.2.2.1.1.2 Iron absorption

Vitamin C increases iron absorption within the small intestine by keeping it in a reduced form (Hallberg, Brune, & Rossander, 1988). Iron, as part of the oxygen-transport protein haemoglobin in red blood cells, is crucial for the adequate transport of oxygen in circulation. With oxygen playing a critical role in energy production, optimal absorption of iron in endurance athletes may prevent fatigue-related symptoms. Several studies have shown supplemental vitamin C to increase iron absorption (Davidsson, Walczyk, Morris, & Hurrell, 1998; Hunt, Mullen, Lykken, Gallagher, & Nielsen, 1990), particularly in those consuming plant-based diets (Mao & Yao, 1992; Sharma & Mathur, 1995) compared with meat eaters (Cook, Watson, Simpson, Lipschitz, & Skikne, 1984). Endurance athletes are known to have higher bodily iron loses through sweating (DeRuisseau, Cheuvront, Haymes, & Sharp, 2002), gastrointestinal bleeding (Fisher, McMahon Jr, Ryan, Larson, & Brand, 1986), and hematuria (Mclnnis, Newhouse, von Duvillard, & Thayer, 1998), thus vitamin C is vital to ensure dietary iron can be optimally absorbed and utilized.

2.2.2.1.1.3 Carnitine synthesis

Carnitine, an amino acid derivative produced from the breakdown of lysine and methionine, is involved in lipid metabolism. Specifically, carnitine works by transporting long-chain acyl groups from fatty acids into the mitochondria, where they are used for energy production (Bremer, 1990). Several catalyst enzymes involved in carnitine synthesis require vitamin C as a co-factor, and studies using vitamin C deficient guinea pigs have observed reduced enzyme activity and hepatic carnitine concentrations (Nelson, Pruitt, Henderson, Jenness, & Henderson, 1981; Thoma & Henderson, 1984). It has been argued that unlike guinea pigs, humans have tight regulation of both vitamin C and carnitine, and to reduce enzyme activity to a level that would restrict the normal rate of carnitine synthesis would require severe restriction of vitamin C (Rebouche, 1991). However, a study by Johnston and colleagues (2006) observed reduced fat oxidation during submaximal exercise in young adults with a marginal vitamin C status compared to those with adequate levels, suggesting that even low vitamin C levels may affect carnitine activity.

2.2.2.1.1.4 Catecholamine production

Norepinephrine, or noradrenaline, is a sympathetic nervous system catecholamine synthesized within the adrenal medulla of the brain from the amino acid tyrosine. Plasma norepinephrine has been shown to increase vastly during exercise, playing important roles in the increase of blood pressure and heart rate (Calbet, 2003), redistribution of blood flow to working skeletal muscle (Kenney & Ho, 1995), and triggering the release of glucose into plasma (Sigal, Fisher, Halter, Vranic, & Marliss, 1996). The metabolism of tyrosine to dopamine (La Du & Zannoni, 1961), and dopamine to norepinephrine (May, Qu, Nazarewicz, & Dikalov, 2013), is vitamin C dependent, and several animal studies have observed significantly reduced brain norepinephrine concentration when vitamin C is deficient (Deana, Bharaj, Verjee, & Galzigna, 1974; Hoehn & Kanfer, 1980). Due to the highly invasive procedures to determine brain dopamine levels, such an effect of vitamin C deficiency in humans is unknown. However, human studies have observed increased dopaminergic-related behaviours with high vitamin C supplementation, such as increased libido and reduced anxiety (Brody, 2002; Dolske, Spollen, McKay, Lancashire, & Tolbert, 1993), and inhibition of norepinephrine uptake appears to decrease exercise performance (Roelands et al., 2008).

2.2.2.1.1.5 Collagen production

Collagen is the main structural component of connective tissue, forming bone, tendons, cartilage, ligaments, and skin. It also constitutes about 1-2% of muscle tissue, serving as a major component of the endomysium. Collagen is synthesized from the hydroxylation of proline and lysine, and requires vitamin C as a cofactor. Endurance exercise can increase collagen turnover, with mechanical and ROS-related damage causing a breakdown of connective tissue; however, studies show significant increases in collagen synthesis rates within skeletal muscle, tendons, and ligaments that peak around 72 h post strenuous exercise (Langberg, Skovgaard, Asp, & Kjaer, 2000; Miller et al., 2005). This up-regulation of collagen synthesis is crucial for recovery and adaptation, reiterating the importance of adequate vitamin C intake in athletes.

2.2.2.2 Vitamin E

Vitamin E, a fat-soluble vitamin, includes eight compounds known as tocopherols and tocotrienols. Of these compounds, alpha-tocopherol (α-tocopherol) has the highest biological activity and is the most comprehensively studied. It plays important antioxidant roles within cellular membranes, and is the major lipid-soluble antioxidant in plasma (Ingold et al., 1987; Machlin & Bendich, 1987). Foods containing the highest concentrations of vitamin E include nuts, seeds, and oils, and while vitamin E supplements do not appear to have significant side

effects at high doses, there is little evidence that supplementation has any benefit in healthy individuals (Bendich & Machlin, 1988).

2.2.2.2.1 Roles

Vitamin E plays many roles within the human body, being involved in processes such as reproduction, neuromuscular health, and vascular protection. It also has exercise-specific roles as an antioxidant and being involved in cell signalling.

2.2.2.2.2.1 Antioxidant activity

Vitamin E's arguably most important role is its powerful antioxidant action. As a free radical scavenger, vitamin E readily reacts with lipid-membrane damaging peroxyl radicals (ROO), breaking the chain of lipid peroxidation. In fact, various *in vitro* studies have shown α-tocopherol to have close to optimal properties for trapping ROO (Burton, Hughes, & Ingold, 1983; Burton & Ingold, 1981; Burton, Le Page, Gabe, & Ingold, 1980). It has been suggested that vitamin E is incorporated into membranes, stabilizing and directly protecting them from oxidative damage (Atkinson, Epand, & Epand, 2008). Interestingly, a study carried out by Howard *et al.* (2011) found that muscle cells exposed to oxidants were unable to carry out membrane repair, however, when administered with vitamin E, membrane repair was enhanced. Additionally, vitamin E reacts directly with O_2^-, preventing the formation of other damaging ROS. Research strongly suggests that supplementation with vitamin E may prevent exercise-induced increases in markers of lipid (Satoshi, Kiyoji, Hiroyo, & Fumio, 1989), protein (Reznick, Witt, Matsumoto, & Packer, 1992) and DNA (Hartmann, Nieβ, Grünert-Fuchs, Poch, & Speit, 1995) oxidation within plasma. Interestingly, while vitamin E appears to be useful in reducing such markers during exercise, in individuals not under oxidative stress, vitamin E supplementation shows no effect on markers of oxidative damage (Meagher, Barry, Lawson, Rokach, & FitzGerald, 2001), suggestive of an efficient endogenous antioxidant system defence.

2.2.2.2.2.2 Cellular signalling

Vitamin E, specifically α-tocopherol, plays an important role in cellular signalling. PKC is a signalling molecule involved in many organ system pathways. Within blood vessels, α-tocopherol can have an inhibitory effect on PKC, which, via a complex inhibitory pathway, can impair the production of vascular O_2^- and NO (Azzi, Ricciarelli, & Zingg, 2002). This in turn can reduce circulating ROS and resulting tissue damage.

2.3 Redox balance and antioxidants: To supplement or to not?

Reduction-oxidation, or redox, refers to the balance of antioxidants to pro-oxidants (free radicals), and is vitally important to almost every physiological process within the human body. Redox balance controls the activation and inhibition of various signalling molecules that work to maintain optimal functioning of body systems. Endurance exercise is well known to shift redox balance toward a more oxidized state, whereby free radicals and their resulting ROS begin to dominate. Early studies looking at exercise-induced ROS alluded to their potential effect on muscular fatigue, reducing the ability to maintain performance over time. This formed the idea that antioxidant supplementation may delay fatigue via reduction of ROS, thus enhancing performance. However, whilst studies (Hartmann et al., 1995; Rokitzki, Logemann, Huber, Keck, & Keul, 1994; Rokitzki, Logemann, Sagredos, et al., 1994; Sumida, Doi, Sakurai, Yoshioka, & Okamura, 1997) clearly show the ROS-reducing effects of antioxidant supplementation, the effects of such on exercise performance are less clear. Furthermore, there appears to be significant differences in the effect of short-term vs. long term antioxidant supplementation on performance and training.

2.3.1 Exercise effects on redox balance

Being a physiological stressor, exercise can significantly alter redox balance, the extent of which is dependent on exercise duration, type, and intensity. The majority of research has assessed redox changes by comparing blood concentrations of various dietary and endogenously produced antioxidants and antioxidant enzymes, prior to and following exercise. Other studies have looked at muscle tissue concentrations of certain antioxidants and/or excretion of metabolites within urine, faeces, and sweat to determine changes in tissue redox status and total antioxidant loss, respectively.

2.3.1.1 Dietary antioxidants

2.3.1.1.1 Vitamin C

Vitamin C turnover in athletes is high due to the physiological stress of exercise. Although most athletes display plasma vitamin C levels within the normal range (Duthie, Robertson, Maughan, & Morrice, 1990; Gleeson, Robertson, & Maughan, 1987; Weight et al., 1988), several studies show exercise to reduce concentrations over time in certain athletic populations; notably in highly trained athletes under heavy and frequent training loads (Namyslowski, 1956; Namyslowski & Desperak-Secomska, 1955; Schröder, Navarro, Tramullas, Mora, & Galiano, 2000). A number of studies have observed an initial rise in plasma vitamin C

levels during and/or immediately following exercise, often followed by a significant decline for up to 48 h (Camus et al., 1994; Gleeson et al., 1987; Nieman et al., 2002; Quindry, Stone, King, & Broeder, 2003; Thompson, Williams, McGregor, et al., 2001). This initial response may be due to ROS stimulated up-regulation of adrenocorticotrophic hormone and subsequent release of vitamin C from the adrenal glands (Cinar, Mogulkoc, Baltaci, & Polat, 2008; Wilson et al., 2013) whilst the subsequent reduction for up to 48 h post-exercise may indicate the level of oxidative stress reached for a given workload. Indeed, Umegaki and colleagues (2000) observed a negative correlation between post-exercise rises in plasma vitamin C and adrenal levels, while Camus *et al.* (1994) noted decreased plasma vitamin C levels correlated to increased malondialdehyde (MDA) concentrations, suggesting reduced antioxidant capacity. In contrast, several studies have observed increased plasma vitamin C concentrations following intense endurance exercise, although its argued this may be due to a decreased plasma volume (Duthie et al., 1990; Quindry et al., 2003).

2.3.1.1.2 Vitamin E

Although few studies have assessed the vitamin E status of athletes (Cohen, Potosnak, Frank, & Baker, 1985; Guilland et al., 1989; Weight et al., 1988), dietary intake of vitamin E in athletes is considered to be more than sufficient (Clarkson, 1991). In fact, there is little evidence that acute exercise or training significantly alters tissue or serum levels of vitamin E in humans. While several earlier studies in men (Camus et al., 1990; Pincemail et al., 1988) observed significantly increased plasma α-tocopherol levels during intense cycle ergometer exercise, this was suggested to be a result of mobilization of adipose tissue stores and plasma volume changes were not accounted for. In contrast, a study that did adjust for plasma volume changes found exhaustive cycling to significantly reduce serum vitamin E in both supplemented and non-supplemented smokers (SuÈrmen-GuÈr, OÈztuÈrk, GuÈr, Pündük, & Tuncel, 1999). Whilst human studies appear to focus on plasma changes in vitamin E, Bowles *et al.* (1991) found a single bout of submaximal treadmill running in rats to significantly deplete skeletal muscle vitamin E, suggestive of its antioxidant role within muscle tissue.

2.3.1.2 Endogenous thiol antioxidants

2.3.1.2.1 Glutathione

Endogenous changes in the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) are commonly used to determine redox status. GSH is oxidised to GSSG as it neutralizes ROS, thus GSSG efflux from cells into the plasma is considered indicative of oxidative stress. Research suggests short durations of maximal exercise have no measurable effect on blood

GSH, while endurance exercise does (Gohil, Ciguie, Stanley, Brooks, & Packer, 1988; Revan et al., 2010). In addition, type I oxidative muscle fibres, which are primarily used during endurance exercise, appear to contain significantly higher levels of GSH compared to type II fibres (Ji et al., 1992). Endurance exercise can shift the GSH/GSSG ratio toward the oxidised state where GSH concentrations are lower and GSSG higher. In humans, a number of studies have reported significant rises in GSSG concentrations in whole blood (Gohil et al., 1988; Laaksonen et al., 1999; Medved, Brown, et al., 2004a; Tessier, Margaritis, Richard, Moynot, & Marconnet, 1995) and serum (Sastre et al., 1992) following endurance exercise while other studies have observed declines in liver (Gohil et al., 1988; Leeuwenburgh & Ji, 1995; Lew, Pyke, & Quintanilha, 1985; Sen, Marin, Kretzschmar, & Hänninen, 1992) and plasma (Ji, Katz, Fu, Griffiths, & Spencer, 1993; Lew et al., 1985) GSH. This suggests the 'dumping' of GSH from the liver into the plasma in an attempt meet the increased ROS generation of working muscles, whilst also attempting to maintain plasma GSH/GSSG balance, thus redox control.

A number of rodent studies have shown reduced skeletal muscle GSH/GSSG ratio post endurance exercise, suggesting the inability of GSH to keep up with antioxidant demand (Duarte, Carvalho, Bastos, Soared, & Appell, 1994; Sen, Atalay, & Hanninen, 1994; Sen et al., 1992). Others however, show increased GSH and GSSG, suggesting uptake by the muscle to combat ROS (Ji & Fu, 1992; Ji et al., 1992). Still others have found no effect of endurance exercise on blood GSH or GSSG (Camus et al., 1994; Marin, Hänninen, Müller, & Klinger, 1990). It is apparent that effects on muscle GSH status and relative enzymes are directly related to exercise intensity and duration (Ji et al., 1992; Powers et al., 1994), which may explain the large variance in GSH/GSSG responses to exercise. Furthermore there appears to be a training adaptation on skeletal muscle GSH levels. Endurance trained animals show greater GSH and GPx activity within specific muscle tissue and fibre types (Leeuwenburgh et al., 1997; Salminen & Vihko, 1983; Sen et al., 1992). In humans, endurance training has been shown to increase GPx activity and hepatic GSH levels, whilst also improving maximal oxygen uptake (VO_{2max}) (Sen, 1999; Tessier et al., 1995). This suggests an adaptive effect on endogenous GSH activity, which increases tolerance to exercise-induced ROS, allowing for improved VO_{2max}.

2.3.1.2.2 Taurine

Studies into skeletal muscle and plasma taurine changes during and following exercise are limited. Further, results are varied and appear to be determined by a number of study design factors. In rats, increased skeletal muscle taurine content has been observed following eccentric-exercise (Dawson, Biasetti, Messina, & Dominy, 2002; Goodman et al., 2009) while treadmill running to exhaustion has resulted in decreased muscle taurine, particularly in fast

twitch fibres (Dawson et al., 2002; Matsuzaki et al., 2002; Yatabe et al., 2003). In recreationally active men, two hours of submaximal cycling had no effect on muscle taurine (Galloway et al., 2008), with the same result being observed in untrained men following a similar protocol (MacLean, Spriet, Hultman, & Graham, 1991). However, endurance-trained men appear to have higher resting and post-exercise muscle taurine content than untrained subjects, suggesting an exercise-induced up-regulation of taurine stores (Graham, Turcotte, Kiens, & Richter, 1995).

In trained athletes, increased plasma taurine has been observed following endurance exercise (Cuisinier, Ward, Francaux, Sturbois, & de Witte, 2001; Decombaz, Reinhardt, Anantharaman, von Glutz, & Poortmans, 1979). Ward *et al.* (1999) were the first to show that such an increase correlated with exercise intensity and not duration, with greater intensity giving rise to higher plasma taurine. Various studies have attempted to explain the mechanism behind the loss of skeletal muscle taurine and the increase in plasma taurine resulting from exercise. Some suggest it is to counteract osmolarity changes (Cuisinier et al., 2002) and/or neutralize circulating exercise-induced ROS (Ørtenblad, Young, Oksbjerg, Nielsen, & Lambert, 2003).

2.3.2 Effects of antioxidant supplementation on exercise-induced oxidative stress

Increases in ROS generation seen during exercise are thought to work as endogenous mediators of acute muscle fatigue; a hypothesis based on numerous studies that have found antioxidant supplementation attenuates fatigue (McKenna et al., 2006; Medved, Brown, et al., 2004a; Miyazaki et al., 2004; Reid, Stokić, Koch, Khawli, & Leis, 1994; Richardson & Allen, 1983), improves exercise performance (Aguiló et al., 2007; Freedson & Kohl, 1991; MacRae & Mefferd, 2006), and reduces muscle recovery time (Freedson & Kohl, 1991; Reid et al., 1994); although some studies fail to support this (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Nielsen et al., 1999; Patil, Chaudhuri, & Dhanakshirur, 2009; Sharman, Down, & Sen, 1971). It is now clear however, that whilst ROS may negatively affect acute performance, they also play crucial roles in redox signalling pathways. Changes to the levels and expression of exercise-induced ROS, as a result of dietary antioxidant supplementation, can impact significantly on cellular redox potential and subsequent homeostasis, which in turn can disrupt important physiological processes. However, it appears that despite their importance at low-moderate levels for exercise-related processes, high levels of ROS can give rise to oxidative stress, subsequently damaging cells, DNA, and proteins (Sun et al., 2001). Thus, dietary antioxidants and endogenous antioxidant-forming thiol donors are often used in the attempt to balance redox status during exercise and prevent ROS-induced damage.

2.3.2.1 Dietary antioxidants

Several studies have reported an attenuation of circulating markers of lipid peroxidation, DNA and protein oxidation, and muscle damage following acute exercise with administration of dietary antioxidants. Satoshi *et al.* (1989) found that supplementing men for 4 weeks with 300mg.day^{-1} vitamin E reduced MDA levels following exhaustive exercise, while Cannon *et al.* (1990) showed seven weeks of vitamin E supplementation to accelerate the return of creatine kinase (muscle damage marker, CK) levels to baseline following 45 minutes of downhill running. Rokitzki and colleagues (1994) observed lower CK levels in runners following a marathon after 4 weeks of vitamin E+C supplementation, while Hartman *et al.* (1995) found vitamin E to prevent DNA damage following a single bout of exhaustive exercise. Other studies have failed to observe such protective effects with dietary antioxidants (Francis & Hoobler, 1986; Helgheim, Hetland, Nilsson, Ingjer, & StrØmme, 1979). In fact, a number of studies have noted increased markers of oxidative stress in athletes following antioxidant supplementation (Bailey, Williams, Betts, Thompson, & Hurst, 2011; Knez, Jenkins, & Coombes, 2007; Nieman et al., 2004), supporting the notion of their potential pro-oxidant action (Rietjens et al., 2002).

2.3.2.2 Endogenous antioxidants

2.3.2.2.1 Taurine

Due to its powerful antioxidant abilities, a number of studies have used taurine supplementation to investigate any effect on oxidative stress. However, the majority of research appears to focus on diseased populations due to high resting oxidative states. In iron overloaded mice, intraperitoneal injection with taurine for 13 weeks has been shown to increase myocardial taurine levels by 45% and reduce plasma MDA levels (Oudit et al., 2004). Balkan *et al.* (2002) found taurine to decrease diet-induced rises in plasma MDA in rabbits fed a high-cholesterol diet, while diabetic rats consuming taurine in drinking water have significantly decreased lipid peroxidation within the retina compared to controls (Di Leo et al., 2002). These results indicate the potential for taurine's use in diseased human population groups including diabetics and those with heart/vascular disease. Indeed, taurine has been found to reduce serum low density lipoprotein (LDL) cholesterol (Mizushima, Nara, Sawamura, & Yamori, 1996) and improve cardiac function (Azuma, Sawamura, & Awata, 1992) in those with coronary heart disease. Further, it appears to reduce platelet aggregation in diabetics (Franconi et al., 1995)

Taurine may also protect endurance athletes against exercise-induced oxidative stress, although research into this area is scarce. Zhang *et al.* (2004) found seven days of oral taurine

supplementation to increase plasma taurine and reduce serum levels of the lipid peroxidation product thiobarbituric acid reactive substances (TBARS) in sedentary males following exhaustive exercise. A later study (Silva et al., 2011) reported two weeks of taurine supplementation to reduce plasma levels of CK and O_2^- following 90-minutes downhill running. Furthermore, there was an increase in plasma total thiol content, suggesting a 'sparing' effect of other thiols. Indeed, several studies have observed a potential sparing effect of GSH by taurine both with (Zhang, Izumi, et al., 2004) and without exercise (Anand et al., 2011; Oudit et al., 2004).

2.3.2.2.2 Glutathione/NAC

Studies looking into the direct effect of oral administration of GSH on GSH status, and impact on oxidative stress during exercise are scarce, as GSH is primarily degraded to its constituent amino acids glycine, cysteine and glutamate before it reaches the cell (Hinchman & Ballatori, 1994). Rodent studies (Aw, Wierzbicka, & Jones, 1991; Hagen, Wierzbicka, Sillau, Bowman, & Jones, 1990), have reported increased plasma GSH following oral GSH supplementation, which appeared to be the result of intact GSH uptake rather than from GSH metabolism and re-synthesis. In healthy humans, four weeks of taurine supplementation at $1g.day^{-1}$ had no effect on plasma GSH levels or oxidative stress markers compared to controls (Allen & Bradley, 2011). However, the same dose for 3-6 months (Richie Jr et al., 2015) significantly increased plasma levels (31% above pre-values), whilst halving this dose ($500mg.day^{-1}$) over the same time period had no significant effect compared to placebo. This suggests effect is both dose and time-dependent. At this point however, there appears to be no research looking at the effects of GSH supplementation on exercising humans. Furthermore, it cannot be ruled out that the increase in plasma GSH is a result of the metabolism and re-synthesis of GSH, rather than direct absorption from the gastrointestinal tract. In this instance, exogenous GSH precursors may be a more bioavailable option for increasing GSH status, via endogenous GSH synthesis.

N-acetyl-cysteine (NAC) is a supplemental GSH precursor. NAC reduces cystine to yield two cysteine molecules, providing substrate for GSH synthesis. Various studies have observed NAC-induced GSH increases in the plasma (Bridgeman, Marsden, MacNee, Flenley, & Ryle, 1991), liver and brain (Aydin, Ozaras, Uzun, Belce, & Uslu, 2002), and skeletal muscle (Medved, Brown, et al., 2004a), particularly when levels were originally depleted. A study by Matuszczak *et al.* (2005) showed NAC infusion prior to fatiguing handgrip exercise in healthy humans resulted in a lower rate of GSH oxidation, suggesting the attenuation of oxidative stress. Supporting this, Medved *et al.* (2004) found that NAC infusion before and during cycling to exhaustion reduced GSH levels, while total GSH (GSH + GSSG) remained steady.

2.3.3 Effects of antioxidant supplementation on exercise performance

2.3.3.1 Dietary antioxidants

2.3.3.1.1 Vitamins C and E

Despite research showing acute antioxidant supplementation reduces ROS or oxidative damage markers, this does not appear to correlate with improved (Gomez-Cabrera, Domenech, & Viña, 2008; Powers, DeRuisseau, Quindry, & Hamilton, 2007; Ristow et al., 2009). One of the only studies to use a one-off antioxidant dose prior to exercise found that a single intramuscular injection of 300mg.kg^{-1} vitamin E significantly increased swim time to fatigue in mice (Novelli, Bracciotti, & Falsini, 1990). The majority of literature however investigates longer periods of antioxidant supplementation, and even then most fail to observe any exercise performance benefit (Akova et al., 2001; Avery et al., 2003; Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Telford, Catchpole, Deakin, Hahn, & Plank, 1992). Richardson and Allen (1983) were one of the very limited number of studies to observe positive effects of antioxidant supplementation on exercise performance. They found that administration of 30mg vitamin C orally for 30 days in rats increased muscle endurance when induced by electrical stimulation, compared to non-supplemented counterparts. A later study by Devi *et al.* (2003a) found 8 weeks of daily vitamin E supplementation in rats significantly increased swim endurance capacity, but did not improve swim velocity compared to their non-supplemented counterparts.

Human studies on the other hand have only shown beneficial performance effects of antioxidant supplementation at altitude (see Table 1). Kobayashi (1974) found that daily supplementation with 400mg vitamin E for six weeks improved submaximal cycling performance in men exercising at an altitude of 5000 feet. Furthermore, those supplemented showed lower oxygen debt and blood lactate, suggestive of a protective antioxidant effect on cellular membranes and red blood cells. In support of this, Simon-Schnass and Pabst (1987) observed lipid peroxidation to be lower and endurance performance higher in mountain climbers supplemented with vitamin E compared to those who were not. Altitude exposure increases lipid peroxidation, which is further exacerbated by exercise (Askew, 2002), thus it isn't surprising that results seen with vitamin E supplementation at altitude do not appear to occur in non-altitude environments (Akova et al., 2001; Bryer & Goldfarb, 2006; Lawrence, Bower, Riehl, & Smith, 1975; Paulsen, Cumming, et al., 2014; Sharman et al., 1971; Shephard, Campbell, Pimm, Stuart, & Wright, 1974; Thompson, Williams, Kingsley, et al., 2001; Yfanti et al., 2010).

The majority of positive benefits resulting from antioxidant administration are in fact seen through enhanced antioxidant status and decreased oxidative stress, and not in exercise performance (see Section 2.3.2.1). However, depleted antioxidant concentrations have been shown to affect optimal performance (Lukaski, 2004) and it has been suggested that studies observing positive performance responses with antioxidant supplementation are a result of low initial antioxidant levels (Buzina et al., 1982; Sharman et al., 1971; Takanami, Iwane, Kawai, & Shimomitsu, 2000). Vitamin E deficiency in rats has been shown to impair muscular endurance and alter contractile properties (Coombes et al., 2002), while those deficient in vitamin C have shown decreased VO_{2max} (Buzina et al., 1982). Indeed, in humans it appears that vitamin supplementation increases endurance capacity in those who have inadequate levels, yet once saturation is achieved there is no further improvement (Takanami et al., 2000). Certainly in rats, vitamin E deficiency has been shown to induce muscular fatigue during exercise (Coombes et al., 2002), whilst supplementation above 'normal' levels appears to have no significant effect on performance (Sharman et al., 1971).

Dietary antioxidants therefore, may only be beneficial in maintaining adequate antioxidant levels. It is also becoming clear that, whilst contributing to acute fatigue, ROS are crucial modulators of exercise processes. For example exercise-induced ROS help to activate GLUT-4 channels thus improving glucose uptake into cells (Sandstrom et al., 2006; Toyoda et al., 2004), and any disruption to this may affect energy production. Similarly, Ca^{2+} channels are also dependent on certain levels of ROS for activation (Sun et al., 2001), and any interference with this may result in reduced muscle contraction. Indeed, a depressive effect on force production in unfatigued rat skeletal has been observed following a diet high in vitamin E (Coombes et al., 2001). Therefore, supplementing with vitamin E in those who already have adequate intake as determined by plasma levels, may detrimentally affect exercise performance. This suggests the need to closely evaluate athlete vitamin E status prior to administering supplements.

Table 1 Effect of dietary antioxidants vitamins C and E on exercise performance. ↑ denotes performance improvement versus control. Majority show little to no effect of antioxidant supplementation on exercise performance.

Reference	Treatment	Subjects; design	Performance protocol	Performance outcome
Richardson & Allen, 1983	30mg.day-1 vitamin C for 30 days	30 male rats; controlled	Strength and time to fatigue during electrical stimulation	↑19% time to fatigue, p < 0.05, no change in strength
Kobayashi, 1974	400mg.day-1 vitamin E for 6 weeks	12 trained males; controlled	Submaximal cycling performance at 5000 feet	↑14% aerobic capacity (p value not reported)
Simon-Schnass and Pabst, 1988	400mg day-1 vitamin E for 4 weeks at altitude	12 highly trained climbers; controlled trial	Anaerobic threshold power during cycle incremental test at altitude	↑18% p <0.01
Novelli et al., 1990	300mg.kg-1 vitamin E via intramuscular injection	92 male mice; controlled trial	Swim time to fatigue	↑139% p <0.0001
Yfanti et al., 2010	500mg.day-1 vitamin C + 270mg day-1 vitamin E for 16 weeks	21 moderately trained endurance males; controlled trial	Maximal power output and VO2max during incremental cycle test	No sig. difference between groups
Akova et al., 2001	300mg.day-1 vitamin E for 42 days	18 healthy sedentary women; controlled trial	VO2max and maximal total work of knee extensors	No sig. difference between groups
Shephard et al., 1974	800mg.day-1 vitamin E for 85 days	14 trained male swimmers; controlled trial	Maximal oxygen uptake and grip strength	No sig. difference between groups
Thompson et al., 2001	400mg.day-1 vitamin C for 12 days	16 healthy male students; controlled trial	Muscle function over 3-days following 90min shuttle running	No sig. difference between groups
Bryer & Goldfarb, 2006	3g.day-1 vitamin C for 2 weeks	18 untrained males; controlled trial	Muscle function over 4-days post 70 eccentric elbow flexor contractions	No sig. difference between groups
Sharman et al., 1971	400mg day-1 vitamin E for 6 weeks	26 schoolboy swimmers; controlled trial	Time to run 1 mile, 400m swim, and motor fitness	No sig. difference between groups
Lawrence et al., 1975	600mg day-1 vitamin E for 6 months	48 competitive swimmers; controlled trial	Time to swim repetitions, 100 yards x 10	No sig. difference between groups
Paulsen et al., 2014	1000mg day-1 vitamin C + 235mg day-1 vitamin E for 11 weeks	50 healthy men and women; controlled trial	VO2max and 20m shuttle sprint time following endurance training	No sig. difference between groups

2.3.3.2 Thiol donors

While many studies suggest that antioxidant vitamins C and E do not positively affect exercise performance (Avery et al., 2003; Bryer & Goldfarb, 2006; Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Oostenbrug et al., 1997), thiol donors appear to delay fatigue under certain conditions (Diaz, Brownstein, & Clanton, 1994; Kelly, Wicker, Barstow, & Harms, 2009; Lands, Grey, & Smountas, 1999; Medved, Brown, et al., 2004b; Reid et al., 1994; Shindoh, DiMarco, Thomas, Manubay, & Supinski, 1990). The various physiological processes that thiols are involved in may be the reason behind such performance benefits.

Whilst dietary antioxidants such as vitamin C and E are generalized, non-target scavengers of all ROS, endogenous regulation of redox systems allows for discrete, controlled and compartmentalized production of specific ROS (Jones & Young-Mi, 2010). The use of thiol antioxidants may therefore permit ROS-signalled adaptations to occur, despite increased antioxidant activity potential, promoting performance improvement.

2.3.3.2.1 Taurine supplementation

Depletion of tissue taurine appears to evoke a number of effects on exercise parameters. In their earlier study, Takashi *et al.* (2008) found that TauT knockout mice not only exhibited cardiomyocyte atrophy, mitochondrial and myofibril damage and cardiac dysfunction, but also skeletal muscle structural defects and reduced exercise endurance capacity. Taurine depletion in skeletal muscle has been shown to alter electrical and contractile properties in aging rodents (Brooks & Faulkner, 1988; Dawson Jr et al., 1999; Thompson & Brown, 1999) and humans (Doherty & Brown, 1997; Vandervoort & Mccomas, 1986). It appears that supplementing with taurine may not only increase taurine status, but may reverse the observed negative effects on muscle function (Pierno et al., 1998). Thus the inability of cells to take up taurine, leading to a taurine-depleted state could have significant impacts on physiological processes and exercise parameters, which may in turn negatively affect exercise performance.

Previous studies have shown endurance-trained individuals to have higher taurine muscle content compared to their untrained counterparts (Blomstrand & Saltin, 1999; Graham et al., 1995), and others have observed negative impacts on exercise performance when taurine status is not optimal (Ito, Yoshikawa, Schaffer, & Azuma, 2014; Warskulat et al., 2004). This apparent role of taurine in human exercise performance has prompted several studies to investigate this area. However, while a number of these studies have looked at the effects of taurine-containing drinks on exercise performance (Baum & Weiss, 2001; Forbes, Candow,

Little, Magnus, & Chilibeck, 2007; Geiss, Jester, Falke, Hamm, & Waag, 1994), research into acute supplementation of isolated taurine in humans is still extremely scarce. In middle-distance male runners, 1 gram (g) of taurine ingested two hours prior to a running time-trial, was shown to significantly increase performance compared to placebo (Balshaw, Bampouras, Barry, & Sparks, 2013), while in endurance trained cyclists, 1.66g of taurine one hour prior to a cycling time trial did not improve performance compared to controls (Rutherford et al., 2010). Several other studies have shown longer-term dosage of taurine to have beneficial performance effects (da Silva et al., 2013; Dawson et al., 2002; Imagawa et al., 2009; Zhang, Izumi, et al., 2004) .

2.3.3.2.2 Glutathione/NAC supplementation

As mentioned previously, oral supplementation of GSH does not appear to be the best way to enhance GSH status, due to its degradation in the gut mucosa (Lash, Hagen, & Jones, 1986; Mårtensson, Jain, & Meister, 1990). Any potential performance effects are therefore more likely to be seen with GSH precursor supplements such as NAC.

Various studies have observed that NAC can upregulate GSH production (Aydin et al., 2002; Bridgeman et al., 1991; Medved, Brown, et al., 2004a), which may reduce various indices of muscular fatigue, improving exercise performance. One way in which this can occur is via the regulation of skeletal muscle sodium/potassium (Na^+/K^+) pump activity. Elevated ROS during exercise have been shown to increase plasma K^+ levels, disrupting Na^+/K^+ ratio's and therefore pump activity (McKenna et al., 2006). This in turn can significantly affect muscle contraction, increasing the rate of muscular fatigue. A study by McKenna *et al.* (2006) found that in addition to an attenuated rise in plasma K^+, NAC administration prior to submaximal exercise delayed time to fatigue compared to placebo. It was suggested that this delay in fatigue may have resulted from continued plasma K^+ regulation achieved through antioxidant action against ROS. Similar effects of NAC on plasma K^+ regulation have been seen in untrained men during prolonged cycling (Medved, Brown, Bjorksten, & McKenna, 2004).

A number of studies have observed acute supplementation of NAC to have beneficial effects on exercise performance. In healthy subjects, pre-treatment of intravenous NAC at 150mg.kg^{-1} improved time to fatigue during involuntary contractions of the leg (Reid et al., 1994), with a similar effect seen in rats exposed to increasing inspiratory load (Supinski, Stofan, Ciufo, & DiMarco, 1997). Matuszczak *et al.* (2005) showed that an infusion of NAC at 150mg.kg^{-1} prior to fatiguing handgrip exercise in healthy, active participants increased repetitive handgrip performance by 32% compared to a saline infusion. Additionally, NAC reduced the rate of GSH

oxidation, suggesting attenuation of oxidative stress. Supporting this, Medved *et al.* (2004) found NAC infusion before and during a fatiguing cycling protocol to increase time to exhaustion by 26% compared to placebo. Furthermore, total GSH (GSH + GSSG) was maintained, supporting previous study observations (Medved et al., 2003). The use of oral NAC supplementation has also been investigated in humans. In healthy men, Kelly *et al.* (2009) found 1800mg of oral NAC 45 minutes prior to exhaustive exercise significantly reduced respiratory muscle fatigue compared to placebo, while Corn *et al.* (2011) found 70mg.kg^{-1} of oral NAC one hour prior to a fatiguing cycling protocol significantly increased time to fatigue by 20% compared to placebo. In contrast, Trewin *et al.* (2013) found an oral dose of 100mg.kg^{-1} 30 minutes prior to a 10km cycle time trial in elite male cyclists elicited a 4.9% decrease in mean power output compared to placebo. In general, results from these studies suggest that a dose of NAC prior to exercise may have a positive effect on performance, however, various factors including training status, dose amount and exercise protocol appear to dictate the extent of this effect.

Table 2 Effect of thiol donors (NAC, GSH, TAU) on exercise performance. ↑ denotes performance improvement versus control; ↓ denotes performance impairment versus control. Majority of studies suggest performance benefits with thiol supplementation.

Reference	Treatment	Subjects; design	Performance protocol	Performance outcome
Rutherford et al, 2010	1.66g Taurine 1h before trial	11 endurance-trained male cyclists; crossover	Time to complete time trial	No difference in performance compared to placebo
Balshaw et al, 2013	1g Taurine 2h before trial	8 middle distance male runners; crossover	Maximal exercise performance during a simulated 3km treadmill time-trial (TT)	↑1.7% TT , p = 0.013
Matuszczak et al. 2005	150mg.kg-1 (IV) NAC before trial	18 untrained mixed gender; controlled trial	Number of repetitive handgrip exercises until fatigue	↑32% number repetitions, p < 0.01
Medved et al. 2004	Prior to: 125mg.kg-1h-1 for 15min; during: 25mg.kg-1h-1 (IV) NAC	7 trained males; crossover	45min submax cycle followed by time to fatigue at 92% VO2max	↑26% time to fatigue, p < 0.05
Reid et al, 1994	150mg.kg-1 (IV) NAC before trial	10 untrained males; crossover	Force output during 30min repetitive tetanic stimulations	15% less decline in force output , p < 0.01
Corn et al, 2011	70mg.kg-1 (oral) NAC 1h before trial	27 healthy males; crossover	Power during cycle time to fatigue	↑20% time to fatigue, p = 0.03
Trewin et al, 2013	100mg.kg-1 (oral) NAC 30min before trial	9 elite trained male cyclists; crossover	Mean power during 10km cycle time trial following a preload (10min)	↓4.9% in mean power output, p > 0.05
Kelly et al, 2009	1800mg (oral) NAC 45min before trial	8 active healthy males; crossover	Respiratory muscle fatigue following 6x 5min submaximal cycling	14% less respiratory muscle fatigue, p < 0.05

2.3.4 Effects of antioxidant supplementation on exercise adaptation

2.3.4.1 Dietary antioxidants

While antioxidants are commonly used by athletes aiming to prolong the onset of fatigue bought about by exercise-induced ROS, there is increasing evidence that long-term supplementation with dietary antioxidants such as vitamins C and E may hamper useful training adaptations, by reducing important ROS-activated transcription factors (Gomez-Cabrera et al., 2005; Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Schreck, Meier, Mannel, Droge, & Baeuerle, 1992; Schreck, Rieber, & Baeuerle, 1991). During exercise, these activated transcription factors and their downstream signalling pathways, play a role in the modulation of exercise adaptations. Additionally, several studies have even suggested long-term antioxidant supplementation contributes to exercise-induced oxidative stress via pro-oxidant effects (Rietjens et al., 2002; Yfanti et al., 2010; Yfanti et al., 2012).

2.3.3.1.1 Mitochondrial biogenesis

Several rodent studies suggest antioxidant administration may hamper exercise-induced increases in mitochondria by reducing crucial ROS-regulators involved in the biogenesis signalling pathway (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Strobel et al., 2011). Supplementation with vitamin C during long-term endurance training has been shown to suppress rises in mitochondrial biogenesis markers and prevent gains in endurance capacity compared to controls (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008). A similar effect has been seen with vitamin E supplementation (Strobel et al., 2011). Other studies however, have found supplementation to have no effect on exercise-induced increases in mitochondrial biogenesis markers (Higashida, Kim, Higuchi, Holloszy, & Han, 2011; Wadley & McConell, 2010).

Human research in this area is limited, however, several studies have found long-term supplementation with vitamin C and E to reduce training-induced increases in VO_{2max} compared to controls, suggesting blunted adaptation (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008). Other studies however, have found no effect on VO_{2max}, despite attenuation of mitochondrial enzyme up-regulation (Paulsen, Cumming, et al., 2014). The wide variation in results could be due to differences in study design, particularly study length, dose amount, and the specific exercise protocol used.

2.3.4.1.2 Muscle protein synthesis

Eccentric based endurance sports (i.e. running), and those of long-duration/high intensity can result in muscle tissue damage and high muscle protein turnover. ROS can be both the cause

and effect of this, and while very high ROS levels can further damage muscle tissue, moderate levels play crucial signalling roles in muscle protein synthesis pathways that repair, replace, and further strengthen damaged tissue. ROS-activated transcription factors involved in muscle protein synthesis such as MAPK and NF-kβ are activated during and following eccentric based exercise (Irrcher et al., 2003; Ji et al., 2004; Vider et al., 2001), and several studies have found antioxidant supplementation to blunt activation of these, adversely affecting strength gains (hypertrophy) (Makanae et al., 2013; Paulsen, Hamarsland, et al., 2014). Again, this appears to be dependent on several factors, such as age, length of training, and antioxidant dose. Further, NO producing NOS inhibition has been shown to significantly attenuate hypertrophy in rodent muscle (Ito et al., 2013), supporting a more direct role of ROS; in this case of NO, in adaptive pathways.

2.3.4.1.3 Endogenous antioxidant systems

Endurance training appears to reduce oxidative stress during subsequent training sessions at the same intensity, via ROS-induced up-regulation of endogenous antioxidant systems (Miyazaki et al., 2001). A number of rodent studies show vitamins C/E supplementation to attenuate exercise-induced up-regulation of antioxidant enzymes including GPx and SOD, thereby reducing cell tolerance to subsequent exercise stress (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Meier, Renga, Hoppeler, & Baum, 2013; Ristow et al., 2009; Strobel et al., 2011). Other studies suggest vitamin C/E supplementation has no effect on increases in SOD protein expression with short-term (3-days) training (Higashida et al., 2011), but increases in endogenous antioxidant enzyme activity over longer periods of training do appear to be affected (Chang, Huang, Tseng, Hsuuw, & Tso, 2007). Still, others show vitamin C/E supplementation to improve endogenous defences as well as endurance exercise capacity (Devi et al., 2003a; Devi, Prathima, & Subramanyam, 2003b).

In contrast to animals, few human studies have investigated the effect of vitamin C/E supplementation on endogenous antioxidant systems. One study reported vitamin C/E to attenuate increases in skeletal muscle SOD, and GPx mRNA levels following four weeks of endurance training in both trained and untrained men (Ristow et al., 2009), while another found no effect on such parameters following 12 weeks of training (Yfanti et al., 2010). Yet another reported no effect of either vitamin C/E or exercise training on SOD or GPx expression (Cumming et al., 2014). It has been suggested that in some cases, the negative effect of antioxidants on acute exercise response and long-term adaptation, may be overcome by training (Merry & Ristow, 2016). This may explain the wide variability in study outcomes which may be dependent on various protocol factors.

2.3.4.1.4 Angiogenesis

Several studies have shown antioxidant supplementation to have a protective effect on vascular cells following endurance exercise (Balestrieri et al., 2008; Fiorito et al., 2008). Men supplemented with mixed antioxidants one week prior to exhaustive exercise appeared to show improved exercise-induced adaptive mRNA angiogenic responses (Hellsten et al., 2007). However, human studies are limited, and *in vitro* research has found antioxidants to inhibit angiogenic responses in endothelial cells (Mousa & Mousa, 2005; Polytarchou & Papadimitriou, 2004; Tang & Meydani, 2001). One suggested mechanism behind this, is the down-regulation of eNOS expression and activity (Polytarchou & Papadimitriou, 2004, 2005). eNOS synthesizes nitric oxide; an important mediator of exercise-induced angiogenesis (Murohara et al., 1998).

2.3.3.1.5 Fat metabolism

The ability of skeletal muscle to efficiently utilize fat as a primary energy source can have significant performance benefits relating to prolonged time to fatigue (Coyle et al., 1986; Lambert, Hawley, Goedecke, Noakes, & Dennis, 1997). Endurance training has been shown to enhance the capacity of skeletal muscles to metabolise fats during submaximal exercise (Martin et al., 1993). This ROS-mediated adaptation involves upregulated gene expression of several transcription factors involved in the transport and oxidation of fatty acids across the mitochondrial membrane and into myocytes (Mole et al., 1971; Turcotte et al., 1999). Enhanced fat oxidation allows the body to better deliver and utilize fatty acids. Several human studies have observed an increase in fat oxidation transcription factors following endurance training (Burgomaster et al., 2008; Tunstall et al., 2002) and increases in levels of muscular enzymes involved in fat metabolism (Costill et al., 1979). The effect of antioxidant supplementation on fat metabolism in humans is limited, however Roberts *et al.* (2011) found vitamin C supplementation did not affect fat oxidation or exercise performance in young active males. In contrast, supplementation with high-dose vitamin C mixed with co-enzyme Q10 and NAC during training in mice significantly reduced expression of the lipid metabolism transcription factor, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), compared with controls (Meier et al., 2013). Similar effects of antioxidant supplementation on PGC-1α have also been observed in other human studies (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Ristow et al., 2009).

Table 3 Effect of vitamin C and E supplementation on adaptations and exercise performance. ↓denotes performance impairments versus control; ↑ denotes performance improvement versus control.

Reference	Treatment	Subjects; design	Performance protocol	Performance outcome
Gomez-Cabrera et al, 2008	500mg.kg.day-1 vitamin C for 6 weeks	36 adult male rats; controlled	Running endurance after 6 weeks of training	↓39% running endurance gains, p = 0.014, ↓skeletal muscle Mn-SOD, GPx,PGC-1, cytochrome C
Gomez-Cabrera et al, 2008	1000mg.day-1 vitamin C for 8 weeks	14 healthy sedentary men; controlled	VO2max after 8 weeks of training	↓12% gains in VO2max, non significant (p-value not given)
Paulsen et al, 2014	1000mg.day-1 vitamin C + 235mg.day-1 vitmain E for 11 weeks	54 young men and women; controlled	11-week endurance training	↓72% markers mitochondrial biogenesis, p <0.03. No effect on VO2
Strobel et al, 2011	1000IU.kg diet.day-1 vitamin E + 1.6g.kg diet.day-1 α-lipoic acid for 14 weeks	48 male rats ; controlled	14-week treadmill training	↓ SOD p = 0.08 and citrate synthase, Cyt C, COXIV p < 0.05
Meier et al, 2013	140mg/L vitamin C + 12mg/L coenzyme Q10 and 1% NAC in water for 4 weeks	32 male rats; controlled	4-week treadmill training	↓27% up-regulation of SOD-1 mRNA, and SREBF-1c p < 0.01, no effect on peak power
Paulsen et al, 2014	1000mg.day-1 vitamin C + 235mg.day-1 vitmain E for 10 weeks	32 recreationally strength trained men and women; controlled	10-week strength training	↓9.5% bicep curl 1RM gain p = 0.04, ↓ MAPK p = 0.01 no difference in muscle mass gains
Makanae et al, 2013	500mg.kg.day-1 vitamin c for 2 weeks	24 male rats	Mechanical overload on skeletal muscle hypertrophy	↓11% muscle hypertrophy, p < 0.01
Ristow et al, 2009	1000mg.day-1 vitamin C + 400IU.day-1 vitamin E for 4 weeks	19 untrained and 20 pretrained healthy young men	4-week training program	↓antioxidant enzymes p < 0.05
Yfanti et al, 2010	500mg.day-1 vitamin C + 400IU.day-1 vitamin E for 4 months	21 young healthy active men; controlled	12 weeks cycling training	No effect on antioxidant enzymes
Roberts et al, 2011	1g.day-1 vitamin C for 4 weeks	18 young active males; controlled	4-week high-intensity interval running training	No effect on VO2max and fat oxidation
Higashida et al, 2011	750mg.kg.day-1 + 150mg.kg.day-1 vitamin C + E for 8 weeks	Male rats (n =unknown); controlled	Swimming training during last 3-weeks of supplementation	No significant effect on SOD and GLUT-4 increases p > 0.05
Wadley & McConell, 2010	500mg.kg.day-1 vitamin C for 1 week	24 male rats; controlled	60-minute treadmill running	No significant effect on mitochondrial biogenesis markers p > 0.05
Devi et al, 2003	45mg.kg.day-1 vitamin E for 8 weeks	12 male rats, controlled	Endurance swimming training	↑26% swimming endurance, greater SOD activity p >0.05

2.3.4.2 Thiol donors

Whilst the generalized, non-target ROS scavengers vitamins C and E may have detrimental effects on signalling pathways involved in exercise adaptions, these effects cannot be extrapolated to non-generalised antioxidants such as thiols. Antioxidants are not biochemically and functionally homogenous, and the types of ROS vitamins E and C interact with, and the way in which they do so *in vivo*, are not the same as endogenous antioxidant enzymes (Murphy et al., 2011). Indeed, in contrast to universal ROS-scavenging antioxidants, endogenous regulation of thiols allows for discrete, localized control of specific ROS (Powers & Jackson, 2008); a balance that may be disrupted by over-supplementation with exogenous generalized antioxidants. Whist some studies using thiol donors show the blunting of exercise-induced rises in several signalling molecules, unlike common dietary antioxidants, this does not appear to affect long-term improvement in exercise performance.

2.3.4.2.1 Taurine

Determining the ideal dose of taurine that may positively benefit exercise performance in humans is difficult, with many factors influencing its digestion, absorption, and utilization (Hayes & Sturman, 1981). In rats however, Matsuzaki *et al.* (2004) found that the optimal amount of oral taurine to maintain tissue taurine concentration and increase exercise performance during a two-week transient exercise protocol was between 100 and 500mg.kg.day^{-1}, supporting earlier findings by Yatabe and colleagues (2003). Extrapolated to humans, at an average weight of 60kg, this would amount to 6-30g of taurine per day. However to date, no human studies have supplemented above 10g.day^{-1} (Azuma et al., 1982; Beyranvand et al., 2011; Brons, Spohr, Storgaard, Dyerberg, & Vaag, 2004; Durelli, Mutani, & Fassio, 1983); thus an upper limit for safe and effective supplementation has yet to be determined.

While there appears to be no current literature investigating the effect of taurine on exercise adaptations, several rodent studies have investigated longer-term taurine supplementation on exercise performance. In rats, two weeks of taurine supplementation prior to exhaustive treadmill running not only attenuated loss of muscle taurine, but significantly increased time to fatigue during exercise (Yatabe et al., 2003); in some cases by up to 50% (Miyazaki et al., 2004). Dawson *et al.* (2002) found one month of taurine supplementation in rats to significantly reduce markers of oxidative stress and increase running performance, while Imagawa *et al.* (2009) observed two weeks of administered taurine in mice increased running distance to exhaustion by 51%.

Humans studies are very limited, however, sedentary males had reduced serum TBAR's, increased plasma taurine, and a significant increase in VO_{2max}, exercise time to exhaustion, and maximal workload following seven days of taurine supplementation at 6g.day^{-1} (Zhang, Izumi, et al., 2004). Supplementation two weeks prior to eccentric-exercise in men was shown to reduce post-exercise serum TBARS and increase strength compared to placebo (da Silva et al., 2013). Furthermore, supplementation did not appear to affect exercise-induced increases in antioxidant enzymes or pro-inflammatory markers, suggesting no blunting effect on adaptive pathways.

Table 4 Effect of thiol donors on adaptations and exercise performance. ↓denotes performance impairment versus control; ↑ denotes performance improvement versus control

Reference	Treatment	Subjects; design	Performance protocol	Performance outcome
Yatabe et al, 2003	5% taurine soultion in water for 2 weeks	40 male rats; controlled	Treadmill running to exhaustion	↑34% time to fatigue, p <0.05
Miyazaki et al, 2004	0.2%, 1%, or 5% taurine solution in water for 2 weeks	34 male rats; controlled	Treadmill running to exhaustion	↑50% time to fatigue with 5% taurine, p <0.05
Dawson et al, 2002	3% taurine solution in water for 4 weeks	40 male rats; controlled	Treadmill running performance	↑ running performance, p < 0.05
Imagawa et al, 2009	15mg.day-1 taurine (via feeding tube) for 2 weeks	48 male mice; controlled	Running distance to exhaustion	↑51% running distance, p < 0.01
Zhang et al, 2004	6g/day Taurine for 7 day	11 young sedentary males; crossover	VO2max and cycling time to exhaustion	↑VO2max and exercise time to exhaustion, p<0.05
Leelarungrayub et al, 2011	2x 600mg/day NAC for 7 days (oral)	29 sedentary men; controlled	VO2max and fatigue index	↑7% VO2max, p < 0.01
Slattery et al, 2014	1200mg.day-1 NAC (oral) for 9 days	8 trained male triathletes; crossover	Repeated cycle sprint performance	↑performance, p < 0.001, ↓IL-6, ↑NF-kβ p = 0.002,
Michailidis et al, 2013	20mg.day-1 NAC (oral) immediately after, and for 8 days following exercise	10 recreationally trained males; crossover	300 eccentric contractions of the quadriceps muscle group.	↓force output at 8 days p = 0.012, ↓IL-6, IL-8, MAPK, NF-kβ & Akt phosphorylation p < 0.05
Petersen et al, 2012	125mg.kg-1.hour for 15min, 25mg.kg-1.hour constant (IV) for 20min + 45min NAC	8 endurance trained men; crossover	45min cycling, then to fatigue	↓ exercise-induced rises in MnSOD p < 0.01, ↓JNK & NF-kB phosphorylation p < 0.05

2.3.4.2.2 GSH/NAC

As previously mentioned, exercise studies using supplemental GSH are not common due to degradation into its constituent amino acids during digestion (Meister, 1991), and the use of NAC is more commonly researched. However, as with taurine, few studies have looked at long-term NAC intake on exercise adaptations in humans. In trained men, Petersen *et al.* (2012) found an infusion of NAC prior to and during an acute bout of cycling to significantly reduce expression of several transcription factors involved in adaptive pathways, including MnSOD and NF-kβ. In contrast, 9 days of oral NAC in triathletes actually increased post-exercise NF-kβ activity, whilst also improving sprint-cycle performance; however, there was an observed blunting of plasma IL-6 (Slattery, Dascombe, Wallace, Bentley, & Coutts, 2014). Similarly, Leelarungrayub *et al.* (2011) found seven days of NAC to significantly improve VO_{2max} and ratings of fatigue during a graded cycle test in sedentary men, compared to controls. However, a study looking at recovery from eccentric exercise-induced muscle damage found NAC supplementation immediately following exercise, and for a further eight days, significantly attenuated NF-kβ phosphorylation, blunted the increase in several adaptive pathway signalling molecules, and increased time to recovery compared to placebo (Michailidis et al., 2013). This suggests a potential difference between endurance and resistance responses to NAC supplementation, although it is important to note, timing of dose (i.e. prior to vs. following exercise) differed between the studies.

In general, whilst taurine supplementation seems to have little to no effect on adaptations and performance long term, several studies suggest NAC may do so (see Table 4). This sets a platform for other exogenous thiol donors to be developed and investigated with the aim to upregulate endogenous defence without affecting long-term performance.

2.4 Keratin: a novel dietary thiol donor

Keratins are animal proteins naturally high in the sulphur amino acid cysteine. The thiol groups of cysteine produce strong covalent disulphide bonds, forming highly stable, insoluble structures. This highly stable structure of keratin allows for its function as a structural protein, forming hair, nails, hooves, horns, feathers, wool, and skin. The greater the level of sulphur within keratin, the harder (and stronger) it is, while softer keratins such as those found in skin, have less sulphur and are therefore weaker. In addition to its structural role, keratin may have the potential to increase GSH concentrations within the body through the provision of cysteine. Furthermore, evidence exists to suggest that oxidation of cysteine within keratin and

the resulting formation of cysteic acid may metabolise to the all-important antioxidant, taurine.

2.4.1 Keratin as a Food Source

In its natural form, keratin is comprised of many covalently-linked cysteine molecules. Due to being a thiol, cysteine plays several important roles within the body. While for the most part it can be endogenously generated from its pre-cursor thiol amino acid methionine, dietary sources of cysteine are necessary to maintain adequate body concentrations. For years, highly keratinous chicken feathers have been used as feed for poultry and livestock with the assumption that cysteine, along with keratins nutritionally sound amino acid profile, would be beneficial. However, whilst being a great way to dispose of feather waste, the digestibility of natural feather keratin is low (El Boushy, Van der Poel, & Walraven, 1990), and therefore has not added great nutritional value to animal diets. In the last decade, various processing methods have been used to increase digestibility of feather keratin, opening the door for the use of keratin as a food source in humans (Bertsch & Coello, 2005; Riffel, Lucas, Heeb, & Brandelli, 2003; Suntornsuk & Suntornsuk, 2003). Many human supplements marketed today aim to increase cysteine status within the body; however the use of dietary keratin to do so has not been addressed due to the body's inability to digest its previously insoluble cysteine bonds. Furthermore, the addition of cysteine or its precursors to food sources in the aim to increase endogenous cysteine has proved relatively pointless, with methionine being unpalatable to humans, cysteine being toxic if routinely used, and cystine, the product of cysteine oxidation, being fairly insoluble (Baker, 2006). Thus, while in theory keratin supplementation suggests potential benefits, studies in this field have been limited.

2.4.2 Keratin supplementation: Safety and benefits

Despite its naturally low digestibility, the recent discovery of keratin hydrolysis appears to yield valuable peptides and amino acids. Furthermore, these products have a level of digestibility that is comparative to other dietary proteins (Kelly & Marsh, 2010). Cysteic acid, the metabolic product of oxidised cysteine, is also produced via hydrolysis of keratin, and is able to be digested, metabolised, and utilised by the body. This method has thus paved the way for keratin as a potentially beneficial food source. Cysteic acids' likely role in up-regulating generation of GSH and taurine may help reduce circulating free radicals within the body.

In the first of its kind, a study using rats found that supplementation with a keratin/casein mix for four weeks resulted in higher liver taurine concentrations compared with casein-only, or pea protein (Wolber, McGrath, Jackson, Wylie, & Broomfeild, 2016). Furthermore, GSH levels were maintained in all but the pea protein group, suggesting a possible cysteine sparing effect

of keratin for conversion to GSH. Following on from this, the first study to assess palatability and digestibility of a the same keratin protein within humans found no adverse gastrointestinal effects during a two-week supplementation period, of up to 40 $g.day^{-1}$, supporting the safe use of dietary keratin in humans (Houltham, Stark, & Stannard, 2014). With GSH being a powerful antioxidant, additional research into hydrolysed keratin and its effects on humans is required to assess bioavailability and bioactivity of cysteic acid and its direct metabolites such as taurine. Moreover, further research may suggest any beneficial outcomes of keratin to increase these endogenous antioxidants on various population groups that exhibit high levels of oxidative stress, such as endurance athletes. With oxidative stress contributing to exercise fatigue via various physiological mechanisms, keratin's potential to lower oxidative stress may delay fatigue, benefiting performance. Furthermore, due to its metabolism into endogenous thiol antioxidants, compared to commonly consumed dietary antioxidant supplements such as vitamins C and E, keratin may reduce oxidative stress and improve acute performance without blunting important exercise adaptations.

Chapter 3

Hypotheses and Aims

3.1 Hypotheses

Information gathered during the literature review provides a platform for the investigation of a thiol-containing food, keratin, on exercise performance. This thesis presents one large- scale study, and two related side studies that use keratin, or its metabolic antioxidant products, to explore various hypotheses.

Specific hypotheses:

1) Acute (1 day) keratin supplementation at 0.8 gram per kilogram of body weight ($g^{-1}.kg^{-1}$ BW.day^{-1}) will increase endogenous thiol concentrations compared to casein protein (**Chapter 4**)

2) Chronic (4 weeks) keratin supplementation at 0.8 $g^{-1}.kg^{-1}$BW.day^{-1} will improve endogenous antioxidant systems by elevating thiol levels compared to casein protein (**Chapter 4)**

3) Chronic (4 weeks) keratin supplementation at 0.8 $g^{-1}.kg^{-1}$BW.day^{-1} will increase exercise performance as determined by VO_{2max} compared to casein protein (**Chapter 4**).

4) Chronic (4 weeks) keratin supplementation at 0.8 $g^{-1}.kg^{-1}$BW.day^{-1} will prevent the decline in circulating thiols during a sub-maximal exercise test compared to casein protein (**Chapter 4**)

5) Chronic (4 weeks) keratin supplementation at 0.8 $g^{-1}.kg^{-1}$BW.day^{-1} will increase lean body mass compared to casein protein (**Chapter 4**).

6) Keratin supplementation at 0.8 $g^{-1}.kg^{-1}BW.day^{-1}$ for one week will show similar increases in plasma total thiol content compared to an equivalent dose of taurine (**Chapter 5**)

7) Taurine supplementation and keratin supplementation will increase plasma total thiol content compared with pea protein (**Chapter 5**)

8) Taurine supplementation at 0.1 $g^{-1}.kg^{-1}BW.day^{-1}$ taken for three days post eccentric-induced muscle damage will increase serum thiol concentrations and speed up time to recovery as determined by exercise performance measures (**Chapter 6)**

3.2 Aims

The general aim of this thesis is to investigate the effect of keratin protein on physiological parameters that may contribute to enhanced exercise performance. In order to explore the previously stated hypotheses, the following aims were determined:

1) Determine whether a one-off intake of keratin increases endogenous thiol concentrations as observed by increased plasma total thiol content (**Chapter 4**)

2) Explore whether chronic keratin supplementation has any effect on improving endogenous antioxidant defence as determined by plasma total thiol content (**Chapter 4**)

3) Determine whether chronic keratin supplementation prevents, to any extent, oxidative stress during sub-maximal exercise at 65% of maximal oxygen uptake (VO_{2max}) as indicated by total thiol content (**Chapter 4**)

4) Identify whether chronic keratin supplementation increases exercise performance as shown by a change in VO_{2max} (**Chapter 4**)

5) Determine whether chronic keratin supplementation increases lean body mass as measured by Dual-energy X-ray absorptiometry (DEXA) (**Chapter 4**)

6) Investigate whether one week of keratin supplementation increases total thiol content compared to taurine and pea-protein (**Chapter 5**)

7) Determine whether taurine supplementation following eccentric-exercise induced muscle damage has an effect on total thiol content and muscular force recovery (**Chapter 6**)

Chapter 4

Chronic keratin supplementation does not improve plasma total thiol content or exercise performance, but increases lean body mass in endurance-trained men

4.1 Abstract

Endurance exercise is known to up-regulate reactive oxygen species production (ROS) which can elicit a state of oxidative stress and subsequently increase the rate of fatigue. Hydrolysed keratin protein is high in cysteic acid, whose main metabolic product, taurine, may improve overall thiol antioxidant status, reducing oxidative stress and subsequently improve exercise performance. Rats supplemented with a feather-based keratin protein for four weeks showed a significant increase in liver taurine and maintenance of GSH levels compared to their casein or pea protein fed counterparts. Further, lean body mass (LBM) significantly improved. Two week supplementation of this same keratin in humans found no adverse effects at intakes of up to 40 grams per day ($g.day^{-1}$). Since athletic performance predominantly depends on optimal lean muscle mass and the rate of muscular fatigue, this study aimed to investigate the effects of an acute and 4-week keratin (KER) vs. casein (CAS) supplementation (treatment) on plasma total thiol content (TTC), LBM and exercise performance of male cyclists. Fifteen trained male cyclists completed a blinded, randomized, cross-over trial; KER vs. CAS supplementation. Two 4-week interventions were separated by a 2-month washout period. Nutritional intake was replicated across both trials, and training sessions were monitored. Plasma TTC, LBM, and performance measures (maximal oxygen uptake (VO_{2max}) & maximal power output (MPO)) were taken before and after the acute and chronic phase. Protein was given daily at 0.8g per kg of body weight in the form of bars and powder. TTC responded differently between treatments with acute supplementation; KER showed improved TTC, whilst CAS showed decreased TTC (p = .028). Chronic supplementation of either treatment however had no effect on TTC (p = .478), nor were there differences between treatments (p = .368). Mean MPO increased from pre- to post- in both trials (p = .012), with KER showing a greater increase (p = .027). Supplementation with either treatment had no effect on VO_{2max} (p >.05). LBM significantly improved with KER compared to CAS (p = .033). These results suggest keratin supplementation improves LBM in trained cyclists but does not significantly improve TTC or exercise performance.

4.2 Introduction

It is well known that during endurance exercise there is significant increase in skeletal muscle oxygen consumption which is associated with a rapid rise in free radical generation (Gibala, MacLean, Graham, & Saltin, 1998). This constant production of free radicals requires the buffering capacity of an endogenous defence system, and a multitude of physiological mechanisms have evolved to detect and respond to elevated oxidant production. The action of these systems determines the overall endogenous antioxidant capacity, and if this capacity is exceeded, oxidative stress ensues, potentially eliciting damage to cell membranes, functional proteins, and DNA (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003; Mateos, Lecumberri, Ramos, Goya, & Bravo, 2005; Tsai et al., 2001). In turn, such damage can disrupt a number of cellular processes, impairing physical performance.

Paradoxically, the redox activities of ROS play critical roles in cell signalling and exercise adaptation, a phenomenon summarized by the concept of hormesis, where low levels of stress promote adaptation to, and thus protection from, subsequent stress (Mattson, 2008). Various exercise studies have observed improved endogenous defence in rodents following training (Criswell et al., 1993; Higuchi et al., 1985; Hollander et al., 2001; Powers et al., 1994), along with reduced ROS production at the same absolute exercise workload during subsequent training (Leeuwenburgh et al., 1994; Miyazaki et al., 2001). However, at very high concentrations, ROS can move beyond being advantageous, and have detrimental effects on performance. Thus balance between pro-oxidants and antioxidants is crucial.

Antioxidants work as potent free-radical quenchers within body tissues, and numerous studies have observed their ability to reduce oxidative stress and subsequent cellular damage (Cannon et al., 1990; Hartmann et al., 1995; Rokitzki, Logemann, Sagredos, et al., 1994; Sumida et al., 1997). As a result, the consumption of non-specific free radical targeting dietary antioxidants such as vitamins C and E are commonplace amongst many athletes involved in endurance sports as they attempt to reduce oxidant load to optimise performance and enhance recovery. In times of heavy training, when endogenous antioxidant capacity cannot meet the increasingly high ROS generation, dietary antioxidants may be of benefit. However, the exact 'balance' of antioxidant supplementation to ROS levels that may enhance performance without blunting adaptive pathways is currently unknown. Additionally, while in theory acute antioxidant supplementation may enhance exercise in humans (Romano-Ely, Todd, Saunders, & Laurent, 2006), the majority of studies suggest no performance benefit (Akova et al., 2001; Bryer & Goldfarb, 2006; Paulsen, Cumming, et al., 2014; Sharman et al., 1971; Shephard et al.,

1974; Thompson, Williams, McGregor, et al., 2001; Yfanti et al., 2010). Furthermore, as the role of ROS in cellular signalling and exercise adaptation becomes more apparent, there is concern that chronic antioxidant supplementation may hamper performance progression by interfering with the essential endogenous antioxidant response to ROS (Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; Ristow et al., 2009). Instead, improving endogenous antioxidant defence systems by way of dietary thiol donors may reduce free-radical load in a more specific manner, reducing the risk of hampering performance gains.

Several thiol donors including GSH, cysteine, and taurine have generated interest for the improvement of exercise recovery and performance, with their ability to boost endogenous antioxidant defence in a way that generalized antioxidants cannot. However, the use of these substances is limited by poor supplement bioavailability and several side effects (Go & Jones, 2011; Zhang, Forman, & Choi, 2005). Keratin is a structural protein found in various tissues such as hair, nails, hooves, feathers, and skin. Comprised of strong cysteine di-sulphide bonds, keratin is a potent thiol donor, however due to these bonds it has previously been insoluble and thus non-digestible within the human body. The recent development of keratin hydrolysis oxidizes these cysteine bonds, yielding cysteic acid; an intermediate metabolic product in the taurine-generation pathway. A recent study in rats (Wolber et al., 2016) observed increased liver taurine following four weeks of keratin supplementation, derived from poultry feathers, along with the maintenance of GSH levels. A later study tested the same keratin protein in humans over two weeks using a ramped dose protocol. Starting at ten grams of keratin per day (10 $g.day^{-1}$) for three days, the supplementation period finished with an intake of 40 $g.day^{-1}$ for five days; a level at which there were still no adverse physiological effects reported (Houltham et al., 2014). These two studies may have implications for the use of keratin-based thiol supplementation in endurance athletes; however, research into this area is scarce.

Additionally, as well as having positive effects on liver thiol status, Wolber *et al.* (2016) found keratin to significantly improve LBM. Athletic performance is partly dependent on attainment and maintenance of optimal LBM. Generally speaking, greater lean mass allows for greater power output and slower fatigue rates at the same absolute workload. In endurance sports, high lean to fat mass ratio is desired, thus keratin may be an alternative to other protein supplements. However, studies thus far have only investigated keratin's effect on LBM in the absence of exercise training.

Our study therefore aimed to determine the effect of keratin on plasma total thiol content (TTC) after an acute (one off) and chronic (four week) intake compared to casein. We were

also interested in whether chronic keratin intake would improve LBM and performance as measured by VO_{2max} and MPO, and whether it would maintain TTC following sub-maximal cycling.

4.3 Methods

4.3.1 Subjects

Fifteen trained male cyclists aged 18-50 inclusive (mean age 34.8 ± 12.2 years, weight 82.8 ± 14.9 kg, height 179 ± 7 cm, mean initial VO_{2max} 62 $ml.kg^{-1}.min^{-1}$) were recruited via word-of-mouth, poster advertising, and social media. Each individual was screened using a Health Screening Questionnaire designed to exclude those whom were at risk physically, culturally, or religiously in following the study protocol. Additionally, participants were included in the study if they had an initial VO_{2max} test above 45 millilitres of oxygen uptake per kilogram of bodyweight per minute ($ml.kg-^{1}.min^{-1}$). Once deemed suitable, participants were asked to sign consent and confidentiality forms.

Approval for this study was granted by the Massey University Human Ethics Committee, Southern A Application (approval number 14/79) (see Appendix A)

4.3.2 Pre-testing procedures

Three weeks preceding the first trial, participants were asked to refrain from taking any form of supplement, with a full list being included in the information sheet. Any prescribed supplements and/or medication were discussed with the researchers to deem whether they would have any potential effect on study measures. Two weeks prior to the first trial, participants were asked to record their training activity using 'Training Peaks'® (Training Peaks, Boulder, CO), and one week prior, were asked to record their dietary intake using 'MyFitnessPal' (UnderArmor, Baltimore, MD). This was done to monitor diet and exercise over the study period and allow participants to replicate their food intake and exercise during their second trial.

4.3.3 Protocol overview

The study was designed as a two-armed, randomized, blinded crossover protocol. Each trial involved an acute supplementation phase of 24 hours, followed by a chronic phase of four weeks, with a two-month washout period between each trial (Figure 6). This washout period was chosen to ensure any exercise-induced adaptations returned to participant 'baseline'

levels prior to the next trial (Maldonado-Martin, Camara, James, Fernandez-Lopez, & Artetxe-Gezuraga, 2017; Mujuka & Padilla, 2000) Initial performance measures were carried out prior to the acute phase. Participants underwent a fasted VO_{2max} test during the morning, and the following morning completed a two-hour sub-maximal cycle ride at 65% of calculated VO_{2max}. Fasted venous blood samples were taken prior to and following the submaximal tests to determine plasma TTC. Three days later, participants began their acute supplementation phase, where they attended the lab in the morning, fasted, for venous blood samples. They were then given either a keratin-based protein supplement or a 'control' gold-standard casein supplement; both in the form of two bars and powder at 0.8 grams of protein per kilogram of bodyweight per day (0.8 g.protein.kg^{-1}BW.day^{-1}). Participants were instructed to consume the supplement in equal amounts in the morning and evening. Participants returned to the lab the following morning for a fasted venous blood sample. One week later, they began their chronic phase. This involved attending the lab in the morning for a fasted DEXA scan and venous blood sample. Participants were given a week's worth of supplements, the same as what they were on during their acute phase, protein content at 0.8 g.protein.kg^{-1}BW.day^{-1} and were advised to split it into two intakes over each day. They were required to attend the lab twice a week for set training sessions on the Lode cycle ergometer (Excalibar Sport, Lode, Netherlands) or wind-trainer. At the end of the chronic phase, participants underwent another DEXA scan, VO_{2max} test and submaximal test at 65% VO_{2max}. They also consented to having their venous blood sample taken. After a two-month washout period, participants repeated the trial again. Those that had taken casein during the first trial were given keratin, whilst those who had taken keratin during the first trial were given casein.

4.3.4 Treatment

During the acute phase, participants were given a one-off amount of either a keratin-based protein, KER (Kerapro; Keraplast Research Ltd, Christchurch NZ; patent application number US 13/381,766), derived from poultry feathers, or a "gold standard" casein protein (CAS) (sodium caseinate 180, Morrinsville, NZ) at 0.8 g.protein.kg^{-1} BW.day^{-1}. This equated to approximately a third to one half of their recommended daily protein intake (Tarnopolsky, 2004), the rest being met by normal dietary intake. KER or CAS were given as two protein bars (set protein amounts; 21g per casein bar, 22g per keratin bar), with the remaining supplemented protein (to meet 0.8 g.protein.kg^{-1} BW.day^{-}1) given in powder form. During the chronic phase, participants were given the same protein as their acute phase, also at 0.8 g.protein.kg^{-1} BW.day^{-1}. They were allowed one day off from supplementation each week, on a day that suited them best. Protein content of the two supplements varied (casein = 92%, keratin =

87%), thus calculations were carried out to ensure the same amount of protein was being consumed in both trials, dependent on body weight (see Appendix F).

4.3.5 Exercise performance

4.3.5.1 VO2 max

Maximal oxygen uptake was determined using a VO_{2max} on a Lode cycle ergometer. Participants completed a sub-maximal phase consisting of four seven-minute stages of increasing resistance followed by a ramp protocol. At the end of each sub-maximal stage, heart rate (HR) and rate of perceived exertion (RPE) (Borg, 1998) were recorded and expired gas was collected and analysed (AEI Technologies Inc., Pittsburgh, USA) using the Douglas bag technique, described elsewhere (Douglas, 1911). At the completion of the fourth stage, participants began a ramp protocol, starting at a resistance of 100 watts (W) and increasing by 25W/min until cadence dropped suddenly, indicating voluntary fatigue and the attainment of VO_{2max}. Gas collection was carried out toward the end of the test until VO_{2max} was attained. Maximal power output (MPO) at attainment of VO_{2max} was also recorded.

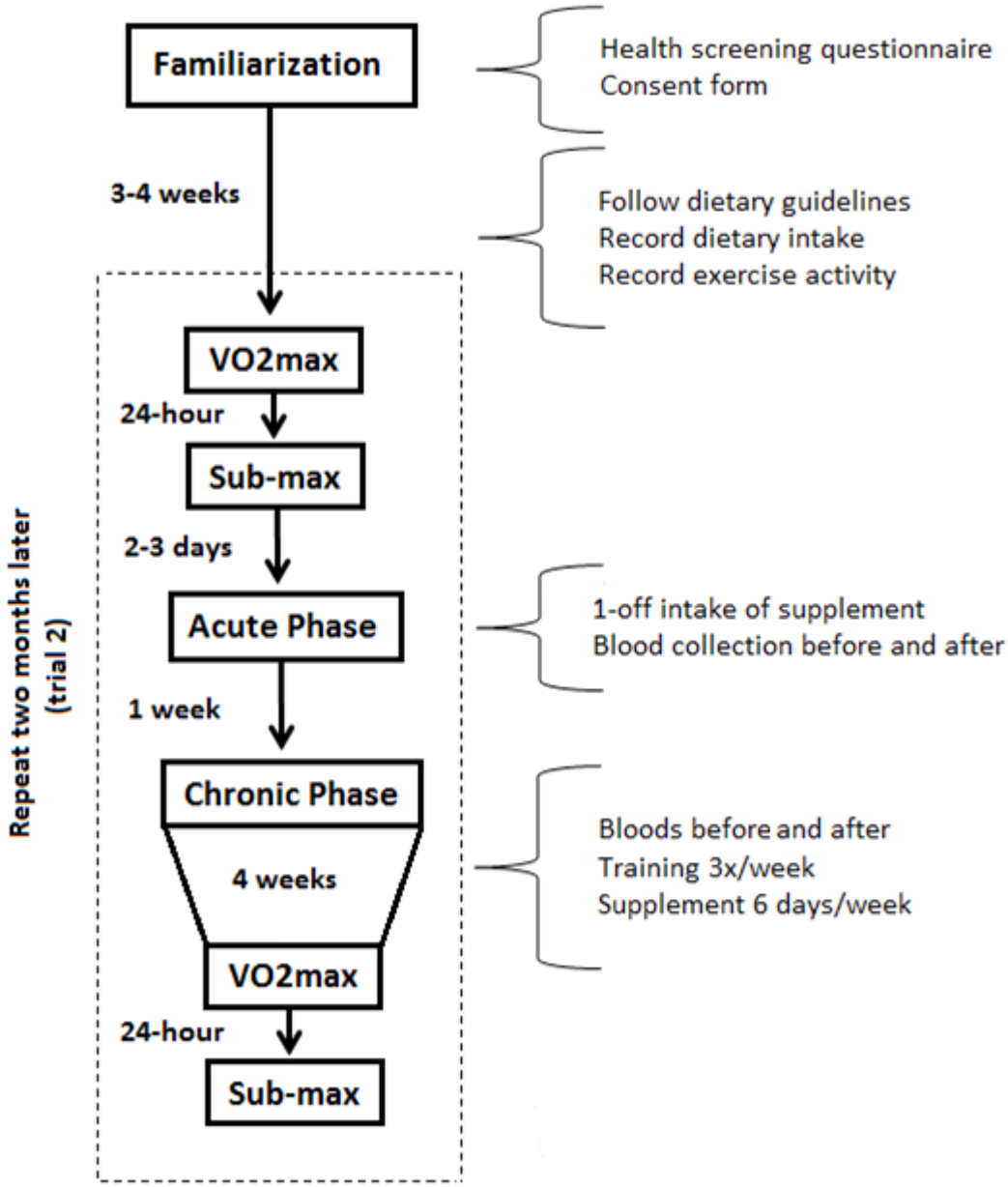

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Figure 6 Schematic diagram of 6-month protocol. Study is a blinded, randomized, cross-over design, with *n* = 15.

4.3.5.2 Submaximal test

Twenty-four hours following VO_{2max} testing, participants completed a two-hour submaximal cycle on the Lode ergometer at a power output corresponding to 65% of their VO_{2max}. HR and RPE were recorded every 15 minutes to monitor how the participant was feeling and the relative stress on the cardiovascular system. Blood samples were collected prior to and following the test. The two-hour ride aimed to determine the effect of supplementation on redox status.

4.3.6 Physiological measures

4.3.6.1 Blood Measures

Fasted blood collection was carried out prior to and following both the acute and chronic phase. Blood samples were collected into 8ml heparin BD vacutainer tubes (Becton, Dickinson and Company, New Jersey) and centrifuged (Eppendorf centrifuge 5804R) at 2500rpm for 12 minutes at 4°C. 1ml of plasma was immediately pipette into Eppendorf tubes, and stored at -80°C for later analysis.

4.3.6.2 Body composition

Prior to and following the chronic phase, participant's body composition were determined using DEXA. Participants were scanned in a supine position for approximately three minutes and data on lean and fat mass was collected using DEXA software (APEX Software Version 4.5.3). The use of DEXA to analyse body composition in men has been previously determined as accurate and reliable (Glickman, Marn, Supiano, & Dengel, 2004).

4.3.7 Biochemical Analyses

4.3.7.1 Total thiol content

Plasma Total Thiol Content (TTC) was analysed using a Measure-iT™ Thiol Assay Kit (M30550, Molecular Probes Inc., Eugene, USA). Stock solution was prepared with Measure-iT™ thiol quantitation standard and deionized H_2O. From this, a working solution was prepared and diluted to a ratio of 1:100, Measure-iT™ to working solution. 10µL plasma was added to 100µL working solution into microplate wells from which fluorescence was measured using a microplate reader. Samples were analysed in duplicate, and thiol content was determined from the fluorescence standard curve according to manufacturer's instructions.

4.3.8 Statistical Analyses

Data were analysed using the Statistical Program for Social Sciences (SPSS) for Windows (IBM SPSS Statistics v.22, IMB, New York, NY, USA). A 2-way repeated-measures analysis of variance (ANOVA) was used to compare lean body mass, performance measures, and pre- and post-acute and chronic total thiol content between KER and CAS trials. A 3-way repeated measures ANOVA was used to compare total thiol content before and after sub-maximal exercise within and between treatment (KER, CAS) groups. Statistical significance was set at $p < .05$, and the results displayed as mean ± SE. Post-hoc analysis using the Holm-Bonferroni method was

carried out on any significant results. Pearson Correlation coefficient was used to determine any correlations between certain measures.

4.4 Results

4.4.1 Blood analyses

4.4.1.1 Total thiol content

4.4.1.1.1 Acute supplementation

No significant change in plasma TTC was observed following a one-off (acute) intake of KER or CAS (p = .135). Further, no difference was observed between treatments (p = .871), however, a significant time x treatment interaction was found (p = .028), suggesting KER and CAS affect plasma TTC differently over time. Relative (%) changes show an increase in TTC with KER over time, and decreased TTC with CAS (Figure 7). Post-hoc analysis found this difference to be significant for the KER group (p = .006), but not CAS (p = .612).

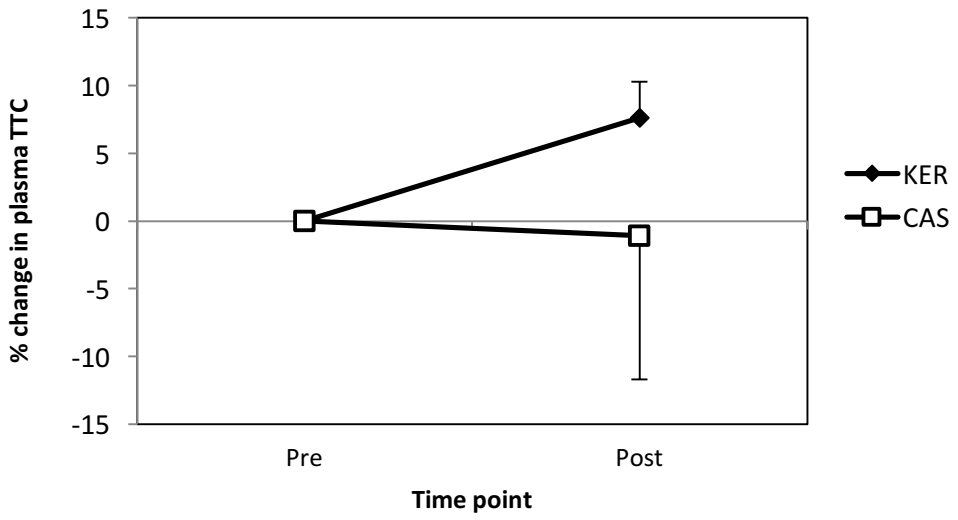

Figure 7 Percentage (%) change (mean ± SE) in plasma TTC after a one-off (acute; pre = day 1, post = day 2) supplementation with either keratin (KER) or casein (CAS), n = 15. Whilst plasma concentrations appear to decrease with CAS and increase with KER, differences between treatments were non-significant.

4.4.1.1.2 Chronic supplementation

Four weeks of either KER or CAS supplementation had no significant effect on plasma TTC (p = .478). Further, no significant difference between treatments was observed (p = .368), nor was there a time x treatment effect (p = .299) (Figure 8)

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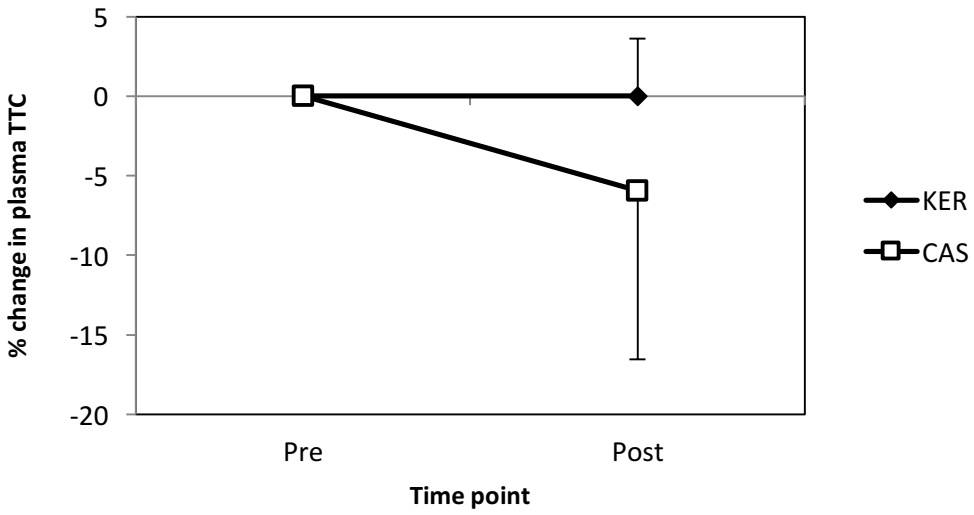

Figure 8 Percentage (%) change (mean ± SE) in plasma TTC after four weeks (chronic; pre = week 0, post = week 4) supplementation with either keratin (KER) or casein (CAS), *n* = 15. Whilst plasma concentrations appear to decrease with CAS compared to KER, differences were non-significant.

4.4.1.1.3 Submaximal testing

Plasma TTC content significantly decreased following a two hour submaximal ride ($p < .01$). This was observed for all submaximal tests regardless of treatment. Indeed, no significant treatment, time, or interaction effects (time x treatment, time x measure, treatment x measure, time x treatment x measure) were observed.

4.4.2 Performance measures

VO_{2max} did not significantly increase from pre-values over time ($p = .073$) with chronic supplementation. No significant difference in VO_{2max} changes were seen between KER and CAS trials ($p = .156$), nor was any treatment x time interaction observed ($p = .935$).

Maximal power output, MPO, in watts (W) significantly increased from pre-values over time ($p = .012$), with post-hoc analysis showing the greatest change to be with KER ($p = .027$) compared to CAS ($p = .043$). No significant difference in MPO was seen between KER and CAS trials ($p = .487$), nor was there a significant treatment x time interaction ($p = .317$). A positive correlation existed between MPO and VO_{2max} for KER ($p = .016$), but not for CAS ($p = .414$).

No significant order effect existed for either VO_{2max} or MPO.

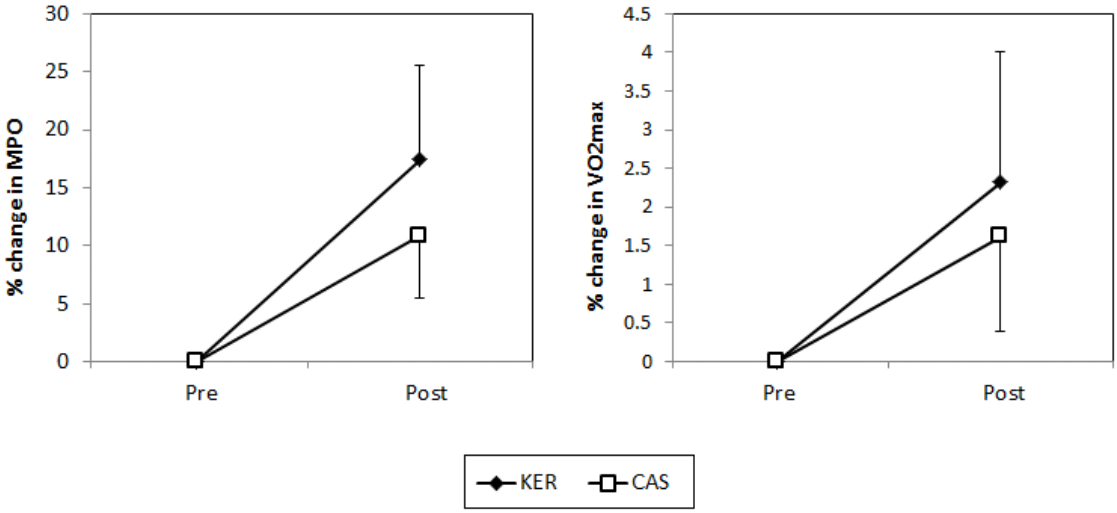

Figure 9 Percent (%) change (mean ± SE) in maximal oxygen uptake (VO_{2max}) and maximal power output (MPO) after four weeks of supplementation with keratin (KER) or casein (CA).

4.4.3 Lean Body Mass

Since initial LBM of participants covered a wide range, analysis was carried out using absolute change (in kilograms, kg) from pre- measures. Whilst neither treatment increased LBM significantly from pre- values (p = .092), a significant change in LMB was observed between treatments (p = .033). Indeed, within four weeks, the average increase in LBM with KER was 0.94 kg (p = .021), compared to CAS at 0.06 kg (p = .663).

Whilst not part of our original measurements, using Pearson Correlation coefficient, we determined that fat mass (FM), information also derived from DEXA, negatively correlated with LMB for KER. Although this correlation was non-significant (r = -.499, p = .058), this suggests KER beneficially affects overall body composition by increasing LBM and decreasing FM. In contrast, no correlation was observed between LBM and FM with CAS (r = .435, p = .105).

4.5 Discussion

Poultry feather-derived keratin protein has previously been used in animal feed, however its insoluble cysteine di-sulphide bonds render it essentially useless as a source of nutrition (Marshall, Orwin, & Gillespie, 1991). This is the first study of its kind, since the development of keratin hydrolysis methods, to investigate long-term keratin supplementation in humans. Further, its effect on exercise performance and antioxidant status has never been researched. Whilst casein protein has been used by athletes for decades as a source of protein, keratin may be of greater benefit due to its higher % of thiol amino acids (see Appendix F).

Thiols are considered to make up the majority of total body antioxidants, therefore total thiol content can be used as an indication of the antioxidant/oxidative state of an individual. TTC includes any molecule with a sulfhydryl (-SH) group (i.e. cysteine, methionine, taurine, GSH, albumin) and in humans is usually measured within serum or plasma. Results from our study found that a one-off intake of keratin protein, at 0.8 g.protein.kg^{-1} BW in male cyclists, affected plasma TTC differently from casein over 24 h. Whilst KER increased TTC by approximately 7% above pre- values, CAS resulted in a decline below pre- values. Several studies have found acute increases in plasma or serum thiols with thiol amino acid administration; often as a result of up-regulated generation of taurine or GSH (Ditscheid et al., 2005; Helms et al., 1999; Sadegh Soltan-Sharifi et al., 2007). Because hydrolysed keratin is high in cysteic acid, it is likely that the rise in TTC seen with KER was predominantly a result of metabolism to taurine via the cysteine sulfinic acid pathway. Certainly, plasma taurine has been shown to vary rapidly in response to supplementation with methionine (Obeid, Johnston, & Emery, 2004) or taurine (Jacobsen & Smith, 1968), thus a similar effect may have occurred in the current study; although without directly measuring taurine or GSH, we cannot confirm this. No significant difference between treatments was observed however, which is likely due to the large between-subject variance. Taking blood samples within two hours following consumption of either treatment may also have shown a greater treatment effect as suggested by variations observed by Micahilidis *et al.* (2007) in peak plasma concentrations of antioxidants. In their study, Micahilidis showed that while total antioxidant content (TAC) peaked two hours following exhaustive exercise, GSH peaked at 1.7 hours post- and catalase (CAT) activity was at its highest 30 minutes post. This suggests 24 h to be too long to observe acute effects in TTC between treatments.

Endurance training has been shown to increase TTC in skeletal muscle (Silva et al., 2009), however, four weeks of KER supplementation had no significant effect on plasma TTC

compared to CAS, nor did either significantly increase TTC over time. The human body continuously attempts to maintain homeostasis in the majority of body systems. Thiol regulation is no exception, and numerous studies have concluded the importance of maintaining GSH homeostasis for redox signalling pathways (Jones, 2002; Laaksonen et al., 1999; Ray, Huang, & Tsuji, 2012), cysteine regulation to avoid the detrimental effects of toxicity (Lehmann, 1987; Lehmann, Hagberg, Orwar, & Sandberg, 1993; Stipanuk, Dominy, Lee, & Coloso, 2006), and tight taurine control for its role in various physiological functions (De Luca, Pierno, & Camerino, 2015). Thus whilst a one off intake of KER may acutely raise TTC compared to CAS, long-term regulation of thiols may prevent a significant change in plasma levels. Indeed, in order to regulate body concentrations, studies have reported greater taurine urinary excretion when taurine intake is high (Chesney et al., 1985; Dawson Jr et al., 1999), and increased plasma cysteine and GSH, along with GSH urinary excretion with high doses of GSH (Aebi, Assereto, & Lauterburg, 1991; Fukagawa, Ajami, & Young, 1996). Additionally, the effect of endurance training to a similar intensity across both trials may have limited the effect of treatment. Indeed, data in Figure 8 suggests over the four-week training period, neither treatment showed any rise in plasma TTC from baseline. Whilst improved antioxidant defence has been shown to occur with endurance training, a result of moderate increases in ROS production and subsequent signalling (Groussard et al., 2003; Miyazaki et al., 2001), paradoxically the frequency and intensity of training athletes often undergo has been shown to markedly reduce circulating thiol antioxidants (Tong et al., 2015). This is likely due to chronically high levels of ROS (oxidative stress) and the potential for rate-limiting factors not being able to meet synthesis demands (Aguiló et al., 2005; Santos-Silva et al., 2001). This reinforces the importance of redox balance for optimal performance (Dröge, 2002). Amino acid analyses of keratin and casein suggest keratin to have a composition that is 7.92% higher in thiol amino acids than casein (McMeekin, Groves, & Hipp, 1949; Saravanan, 2012). This may explain why during endurance training KER appeared to maintain plasma TTC whilst CAS could not (Figure 8). However, since results are non-significant, more research in this area is needed to support this. Alternatively, the use of muscle biopsies to assess muscle TTC may have given a better indication of the effect of treatment.

It is well known that prolonged exercise at moderate to high intensity can push redox balance toward the oxidized state. This can be shown by increased plasma markers of oxidative stress, and decreased thiol antioxidants and antioxidant enzymes (Gohil et al., 1988; Inayama, Kumagai, Sakane, Saito, & Matsuda, 1996; Liu et al., 1999; Medved et al., 2003). Supporting these studies, our results show a significant decline in plasma TTC from pre- values following a

two-hour submaximal ride at 65% $VO_{2max.}$ However, results did not support our hypotheses; no differences in TTC decline were noted between KER and CAS, nor did submaximal exercise-induced decline in TTC respond to exercise training. This is likely due to setting post-treatment submaximal load to match 65% of post- treatment VO_{2max}. Instead, the same load from the first submaximal test of each particular trial should have been used. This would have determined whether training and/or treatment improved response to exercise-induced oxidative stress.

VO_{2max} is defined as the highest rate at which oxygen can be taken up and utilized by the body during intense exercise. VO_{2max} can give an indication of the fitness level of an athlete, with a greater VO_{2max} reflective of a greater ability for muscle to take up and utilize oxygen to produce work. Thus, it is commonly used as a measure of exercise performance. Endurance athletes have a much higher VO_{2max} than the general population, although there is a physiological upper limit to how much oxygen the body can consume (Hill, Long, & Lupton, 1924). Four weeks of KER or CAS alongside endurance training did not significantly improve VO_{2max} from pre- values. Neither was any difference observed between treatments. It is possible that this was less due to the treatment used, and more due to using trained cyclists, as highly trained athletes often show little improvement of VO_{2max} despite undergoing rigorous training regimes (Daniels, Yarbrough, & Foster, 1978; Fairbarn, Coutts, Pardy, & McKenzie, 1991; Gore et al., 1998). This may be due to VO_{2max} being dependent on and limited by various factors including cardiac output, maximal heart rate, blood volume, and blood oxygen carrying capacity (Bassett Jr & Howley, 2000; Hill et al., 1924); most of which are at their peak in trained endurance athletes (Warburton, Gledhill, Jamnik, Krip, & Card, 1999). Indeed, studies using untrained participants often show improvements in VO_{2max} with endurance training (a result of exercise-induced adaptation) (Davidson & McNaughton, 2000; Kohrt et al., 1991; Milanović, Sporiš, & Weston, 2015) which can also plateau after a certain period of time (Weltman et al., 1992). It is likely that we may have seen a change over time had we used untrained participants; however, it is hard to determine whether a treatment effect would have existed. Furthermore, we could not precisely determine habitual training workloads for each participant, thus it is likely that while some participants were on a greater training load than normal, others may have been receiving their normal training stimulus which would result in less VO_{2max} improvement.

MPO, noted at the cessation of VO_{2max} test, significantly increased from pre- values in both treatments, but no significant differences in MPO were observed between treatments. Oxygen consumption increases with exercise intensity, however as mentioned earlier, VO_{2max} is

limited by several physiological systems, and at this point, exercise intensity can continue to increase without the associated rise in oxygen consumption. This is made possible by the anaerobic energy systems, although as blood lactate increases, fatigue ensues (Jacobs, 1986). A number of studies have indicated the importance of MPO as a predictor of endurance performance (Hawley & Williams, 1991; Noakes, 1988; Noakes, Myburgh, & Schall, 1989; Scrimgeour, Noakes, Adams, & Myburgh, 1986), and some even suggest it to be a better indicator of endurance performance than VO_{2max} (Hawley & Williams, 1991; Noakes et al., 1989; Sharp, Troup, & Costill, 1982). In line with previous studies (Hawley & Noakes, 1992; Storer, Davis, & Caiozzo, 1990) our results showed a significant relationship between MPO and VO_{2max}, however, this was only observed with KER.

Historically, it was assumed aerobic training had little to no impact on skeletal muscle mass, shifting the focus to resistance training studies. However, over the past few decades, researchers have found high exercise volume with low external load, such as that seen with intense cycling, can stimulate muscle hypertrophy (Harber et al., 2009; Harber et al., 2012; Konopka et al., 2010). The difference in LBM gains between KER and CAS, which were balanced for total protein content, suggest in our case that gains were not wholly due to endurance training. Rather, KER appears to be a superior protein in this respect. This suggests it may be keratin's amino acid profile that has such an effect on muscle protein synthesis (see Appendix G). Keratin is high in cysteic acid, which is predominantly metabolised to taurine (Griffith, 1987). It could be proposed that subsequent metabolism to taurine is the reason behind this, with several studies reporting taurine's cytoprotective role in skeletal muscle (Dawson et al., 2002; Uozumi et al., 2006; Warskulat et al., 2004). Moreover, limited research suggests taurine is involved in skeletal muscle cell proliferation and regeneration via activation of the myogenic regulatory gene MyoD (McIntosh, Garrett, Megeney, Rudnicki, & Anderson, 1998; Uozumi et al., 2006). Others suggest taurine indirectly improves muscle protein synthesis by regulating osmotic stress that further modulates several protein kinases and their signalling cascades (Yamazaki, Komuro, & Yazaki, 1998). In support of this study, Wolber *et al.* (2016) also observed increased liver taurine as well as LBM in rats supplemented with keratin. Thus it could be proposed that taurine played a significant role in improving LBM. However, without having taken muscle biopsies or even analysed for plasma taurine, we cannot say this with complete confidence. Additionally, since greater LBM tends to produce greater power output, the significant improvement in MPO from pre- values with KER could be explained by a greater increase in lean mass, although no correlation existed between the two ($r = .353$, $p = .197$).

Throughout the study, diet and exercise were controlled where possible. In addition to being given a list of food items and supplements they could not consume (i.e. any protein powders), participants also recorded, daily, their food intake into MyFitnessPal software program. Diets across both trials were analysed for total protein, fat, and carbohydrate content and calculated totals were similar (all $p > 0.05$, see Appendix E). While reported dietary intake is suggested to be unreliable in a cross-over study (Jeacocke & Burke, 2010), due to budget constraints, we were unable to provide participants with all of their meals for the full length of the study. Exercise was controlled for as best as possible, with participants attending the laboratory three times per week for set training on a cycle ergometer or wind-trainer. Whatever participants did outside of this (i.e. a race) in the first trial, they were requested to repeat in the second trial in order to keep exercise consistent.

4.5.1 Conclusion

The results from this study suggest keratin protein is both safe to consume and a potentially beneficial supplement for endurance athletes at an intake of 0.8 g.protein.kg^{-1} BW.day^{-1}. Four weeks of KER intake alongside endurance training may increase LBM, and suggests the ability to maintain plasma TTC and improve aspects of endurance performance. This is the first study to look at the effect of KER on endurance performance in humans, thus it would be interesting to investigate how it may benefit resistance training. Furthermore, the increase in LBM seen in this study suggests that KER may have potential benefits in populations such as the elderly or ill, where maintaining or improving LBM is vital.

Chapter 5

The effect of cysteic acid supplementation (as keratin) vs. exogenous taurine supplementation on serum total thiol content in healthy humans

5.1 Abstract

Taurine, a thiol-amino acid derived from cysteine and/or cysteic acid, is found abundantly within skeletal muscle tissue and plays several important exercise-related physiological roles, including improved endogenous thiol defence, muscle protein synthesis, and even mood. Feather-derived keratin protein is high in cysteic acid and thus has the ability to improve endogenous taurine production. Additionally, keratin has been shown to maintain glutathione levels and improve lean body mass over a short time period. It is currently unknown to what extent the cysteic acid in keratin improves endogenous thiols compared to exogenous (i.e. taurine) supplementation. Thus, this study aimed to determine whether cysteic acid (as keratin) supplementation would increase serum total thiol content (TTC) to the same, if not greater extent than an equal amount of exogenous taurine would. Eight healthy mixed gender participants (age 25 ± 6.7 years, height 173 ± 7.6 cm, weight 72 ± 9.6 kg) were recruited for this randomized, blinded, three-armed, crossover study. Participants were asked to consume pea protein (PP), low in sulphur amino acids, taurine + pea protein (PP+T) or keratin (KER) twice daily at 0.8 g.protein.BW.day^{-1} for seven days followed by a one-week washout period. Each supplement was consumed in this way for seven days. All supplements were balanced for protein content, and taurine amount was matched to the amount of cysteic acid in KER. At the start and end of each week participants filled out a POMS questionnaire, and gave a blood sample for analysis of serum TTC. One week supplementation with KER, PP, or PP+T had no effect on serum TTC (p = .563) nor were any treatment (p = .540) or time x treatment effects observed (p = .670). Significant mood improvement occurred after one week of supplementation for PP+ T and PP (p = .014 and p =.02 respectively). No treatment or time x treatment effects were observed (all p > .05). Results from this study suggest supplementing with thiols in the form of cystic acid (KER) or taurine has no significant effect on serum TTC after one week. It also suggests pea protein with or without taurine may positively affect mood state.

5.2 Introduction

Taurine, a thiol-containing free amino acid, is well known for its various physiological roles; most notably its antioxidant activity. Adequate taurine concentrations within body tissues are crucial, and depleted levels have been shown to increase cell susceptibility to oxidative damage, compromising cell functionality (Heller-Stilb et al., 2002; Ito, Kimura, Uozumi, Takai, Muraoka, Matsuda, Ueki, Yoshiyama, Ikawa, Okabe, et al., 2008; Ito et al., 2010; Warskulat et al., 2004).

Taurine's involvement in several exercise-related pathways suggests a potential benefit to maintaining or increasing tissue concentrations during training and sporting events. In particular, taurine appears to play key roles in upregulating endogenous defence systems (Nandhini et al., 2002; Vohra & Hui, 2001), inhibition of free-radical generation and subsequent rise in inflammatory markers (Park & Imlay, 2003; Schuller-Levis & Park, 2004) , mitochondrial protein synthesis (Suzuki et al., 2002), membrane stabilization and subsequent muscle contraction (Pierno et al., 1998), and osmoregulation (Ito et al., 2004). Further, taurine may improve mood state through its interaction with GABA (Jia et al., 2008)), an important component in exercise training and performance (Beedie, Terry, & Lane, 2000). The inability to carry out these processes optimally during exercise can increase the rate of fatigue, negatively impacting upon performance. Additionally, it has been suggested that taurine may help maintain skeletal muscle mass (Ito et al., 2010) and assist in muscle regeneration (Uozumi et al., 2006); a potential advantage to athletes striving for a higher lean to fat mass ratio.

Taurine is generated endogenously from cysteine via the cysteine sulfinic acid pathway which converts cysteine to cysteic acid, and then into taurine. Cysteine can also generate glutathione (GSH), another powerful endogenous antioxidant that works to regulate redox balance. Indeed, studies suggest that cysteine is predominantly metabolized to GSH, with taurine being a secondary pathway when GSH levels are optimal (Stipanuk, Coloso, Garcia, & Banks, 1992). Cysteic acid on the other hand, is thought to directly metabolize to taurine. The consumption of cysteic acid may therefore benefit various population groups, due to taurine's important physiological roles. Research into the use of cysteic acid however is extremely scarce, as early studies observed significant liver tissue atrophy with direct supplementation (Earle, Smull, & Victor, 1942). Further, the development of a natural source of cysteic acid, hydrolyzed keratin protein has opened up the potential for a cysteic-acid based supplement. Naturally high in cysteine-cysteine bonds, keratin can be found in various structural tissues including skin, feathers, hair, and nails. The development of keratin hydrolysis, whereby cysteine is oxidized

to cysteic acid, results in a much more soluble protein. Indeed a recent study in rats (Wolber et al., 2016) found supplementation with hydrolyzed keratin for four weeks to increase liver taurine levels and maintain GSH compared to casein or pea protein. Further, keratin significantly increased lean body mass. In order to investigate any such effects in humans, in a blinded, crossover study, endurance athletes were supplemented with keratin for four weeks whilst following a cycling training protocol. At the end of the four weeks, those supplemented with keratin showed significantly increased lean body mass compared to casein (McLeay, Crum, Stannard, Barnes, & Starck, 2017). While taurine supplementation alone may have various physiological benefits, if keratin can up-regulate endogenous taurine production and in doing so increase TTC to the same extent as an equal amount of taurine, there may be a greater benefit to supplementing with keratin, than taurine alone.

This study therefore aimed to determine whether supplementing with cysteic acid, in the form of keratin, has a similar effect on serum TTC via endogenous taurine production, compared with an equivalent amount of exogenous supplemented taurine. Additionally, the study attempted to determine whether keratin and/or taurine supplementation significantly increased serum TTC, compared to that of pea protein (cysteine devoid).

5.3 Methods

5.3.1 Subjects

Eight healthy mixed gender participants (age 25 ± 6.7 years, height 173 ± 7.6 cm, weight 72 ± 9.6 kg) were recruited for this study. All participants were recreationally fit, engaging in exercise 2-3 times per week. Individuals were screened using a Health Screening Questionnaire designed to exclude those who could not take part due to physical, cultural, or religious reasons. Once deemed suitable, participants were asked to sign a consent form. Approval for this study was granted by the Massey University Human Ethics Committee, Southern A Application (approval number 15/59).

5.3.2 Pre-testing procedures

At least one week prior to their first trial, participants underwent a familiarization session involving an outline of the study protocol. Participant heights and weights were also recorded. Participants were advised to discontinue the consumption of any supplements, energy drinks, and alcohol, and were asked to record their food intake and exercise activity during the week

leading up to their first trial. Participants were also given a list of foods high in taurine and cysteine to avoid and/or limit throughout the study.

5.3.2 Overview

The study protocol was designed as a blinded, three-armed cross-over protocol, with treatment randomly allocated. Participants were supplemented with keratin, pea protein + taurine, or pea protein only, as a control, in powder form (mixed in water), and were asked to consume this twice daily for seven days. Each treatment week was followed with a wash-out week, whereby no supplements were taken, and food intake and exercise activity was replicated. This treatment/wash-out cycle was repeated three times, one for each supplement (see Figure 10). Prior to and following each treatment week, participants underwent fasted blood collection. They also completed a profile of mood states questionnaire (POMS) (Appendix D).

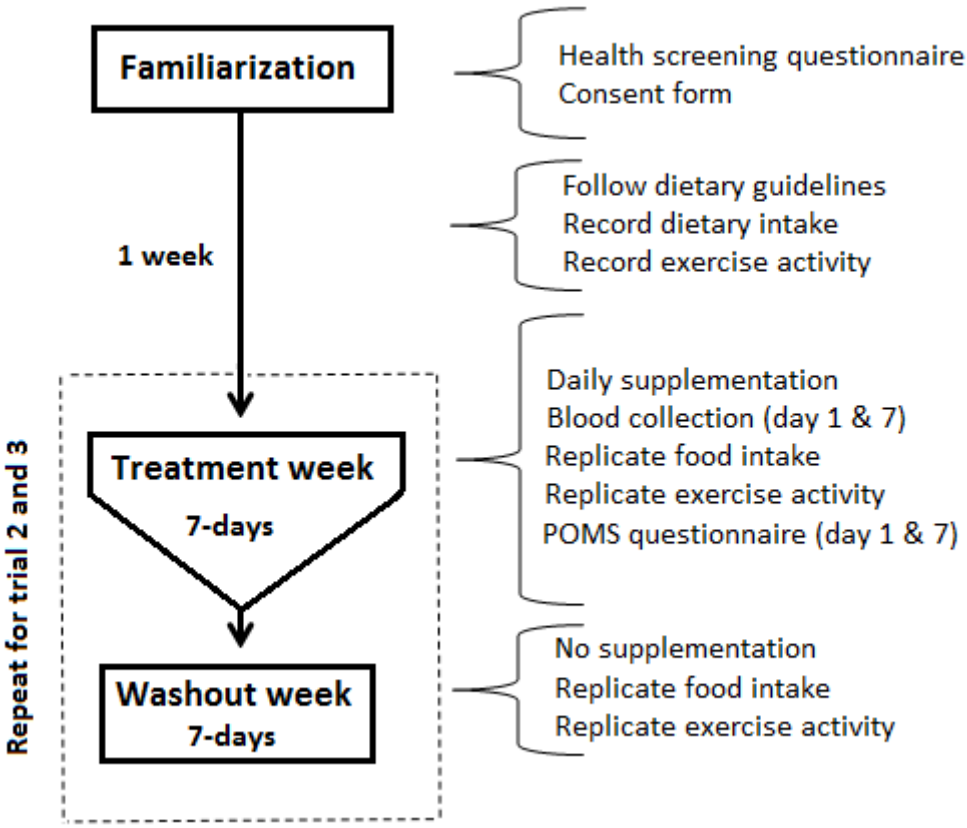

Figure 10 Schematic diagram of study protocol. This study is a blinded, randomized, cross-over design, totalling seven weeks in length.

5.3.3 Physiological measures

5.3.3.1 Blood collection

Fasted morning bloods were taken via venipuncture of the antecubital fossa vein at the beginning (day 1) and end (day 7) of each treatment week. A single 10 ml serum BD vacutainer tube (Becton, Dickson and Company, New Jersey) was used to analyze serum blood for taurine, glutathione, and total thiol content. Following collection, tubes were left at room temperature for 30 minutes to allow for clotting. They were then centrifuged (Eppendorf centrifuge S804R) at 2000rpm for 10 minutes at 4°C. Immediately after, separated serum was pipette into 1ml Eppendorf tubes and frozen at -80°C for later analysis.

5.3.4 Other measures

Prior to and following each supplement week, participants were asked to fill out a modified version of Lorr and McNair's (1982) original Profile of Mood States (POMS) questionnaire. This modified version, consisting of 40 adjectives that measure tension, depression, fatigue, vigour, confusion, anger, and self-esteem, has previously been shown to have acceptable psychometric properties for use in a sport setting (Grove & Prapavessis, 1992) and can predict performance outcome (Beedie et al., 2000). Total mood disturbance (TMD) was calculated by subtracting vigour scores from the other measures. Differences in TMD were compared from the beginning to end of each week within treatments, and between treatments.

5.3.5 Treatment

Participants were given either cysteic acid as keratin protein (KER) (Kerapro, patent), pea protein + taurine (PP+T) (NOW foods, Bloomingdale, IL, USA), or pea-protein only (PP) (NOW foods, Bloomingdale, IL, USA) at 0.8 grams of protein per kilogram of bodyweight per day ($g.protein.kg^{-1}BW.day^{-1}$) for seven days. All supplements were in powder form and were given in sealed plastic bags. Powder amounts were based on protein content of the powder. Pea protein was 73% protein in powder, while keratin was 87% protein in powder, thus daily powder amounts were calculated as follows:

Pea protein: (participant weight in kg x 0.8g)/0.73

Keratin: (participant weight in kg x 0.8g)/0.87

Taurine in the PP+T trial was calculated at an amount equivalent to cysteic acid in the keratin based on molecular weight (cysteic acid = 169.16 g) and the assumption that 100% of cysteic acid would metabolize to taurine. The actual amount that metabolizes to taurine has yet to be

researched. The keratin treatment contained 9.28 g of cysteic acid per 100 g (9.28%). The amount of cysteic acid given in this form of keratin was 0.074 grams per kilogram of bodyweight per day ($g.kg^{-1}.bw.day^{-1}$). This was based on a supplemented protein intake of 0.8 $g.kg^{-1}.bw.day^{-1}$ (9.28 g/100 = 0.0928 * 0.8). PP and PP+T treatments were balanced for protein content of the keratin/cysteic acid treatment using pea protein. Raw powders had cocoa (Sunvalley Foods, AKL, NZ) and stevia (Natvia, Prahran, VIC, Melbourne) added for flavor.

Participants were advised to take half the powder in the morning, and half in the afternoon, mixed in water. These amounts (morning and afternoon) were pre-measured into individual sealed plastic bags. Treatment allocation was randomized and blinded, and each supplement was taken on alternate weeks over a six-week period.

5.3.6 Biochemical analyses

5.3.6.1 Total thiol content

Serum TTC was analysed using a Measure-iT™ Thiol Assay Kit (M30550, Molecular Probes Inc., Eugene, USA). Stock solution was prepared with Measure-iT™ thiol quantitation standard and deionized H_2O. From this, a working solution was prepared and diluted to a ratio of 1:100, Measure-iT™ to working solution. 10µL serum was added to 100µL working solution into microplate wells from which fluorescence was measured using a microplate reader. Samples were analysed in duplicate, and thiol content was determined from the fluorescence standard curve according to manufacturer's instructions. Due to blood collection issues with several participants, n = 6 for TTC.

5.3.7 Statistical analyses

Data were analysed using the Statistical Program for Social Sciences (SPSS) for Windows (IBM SPSS Statistics v.22, IMB, New York, NY, USA). A repeated-measures analysis of variance (ANOVA) was used to compare treatments over time for total thiol content. Statistical significance was set at $p < .05$, and the results displayed as mean ± SD. Post-hoc analysis using the Holm-Bonferroni method was carried out on any significant results.

5.4 Results

5.4.1 Serum Total thiol content

One week supplementation with KER, PP, or PP+T had no effect on serum TTC (p = .563). No significant differences in TTC between treatments were observed (p = .540), nor was any time x treatment interaction evident (p = .670).

5.4.3 Profile of Mood States Questionnaire (POMS)

A significant improvement in mood was observed after one week of supplementation (p = 0.033), with post-hoc analysis revealing the greatest difference to be with PP + T (p = 0.014), followed by PP (p = 0.020). No significant difference was seen with KER (p = 0.846). No treatment or time x treatment effects were observed (p > 0.05).

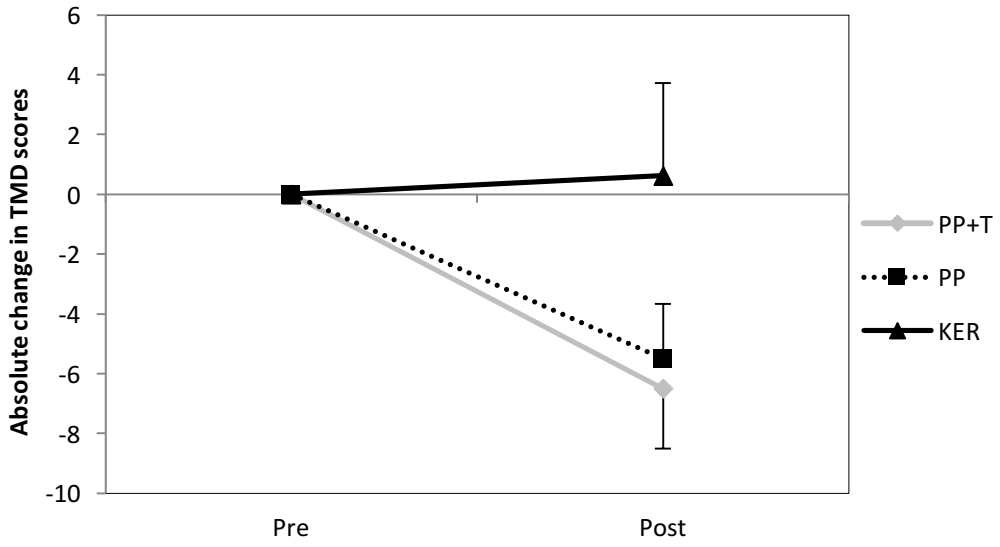

Figure 11 Absolute change in total mood disturbance (TMD) scores (mean ± SE) after one week of supplementation with keratin (KER), pea protein (PP), pea protein + taurine (PP + T). Higher TMD disturbance scores indicate greater mood disturbance, thus a negative change indicates improved mood. PP + T and PP groups significantly improved over time, whilst KER did not. No significant differences were observed between treatments, n = 8.

5.5 Discussion

This study aimed to determine whether cysteic acid (as dietary keratin) increased TTC to the same extent as a matched amount of exogenous taurine in healthy participants, and whether both of these treatments improved serum TTC more than a thiol-devoid pea protein. Our results found that contrary to our hypotheses, one week of KER, PP + T, or PP at 0.8

g.protein.kg^{-1}BW.day^{-1} had no effect on serum TTC from pre- values. Furthermore, no difference was observed between the high thiol treatments (KER, PP + T) and low-thiol PP. It is difficult to compare results with previous studies, since limited research on this topic exists. While the majority of taurine supplementation studies appear to analyse taurine changes in plasma/serum (Dawson et al., 2002; Rosa, Freitas, Deminice, Jordao, & Marchini, 2014), hepatic tissue (Eppler & Dawsome, 2001), or skeletal muscle (Goodman et al., 2009; Yatabe et al., 2003), limited studies have assessed effects of taurine on TTC. Moreover, of the few that have, not only do these studies use non-human subjects (thus doses are often far greater per kg body weight), but the focus appears to be on skeletal muscle TTC rather than plasma or serum (Silva et al., 2011; Terrrill, Pinniger, Graves, Grounds, & Arthur, 2016). Our results may be due to the fact that adequate taurine intake may have been met by participants' normal diets, and that anything over and above this was tightly regulated by the kidneys and excreted within urine (Colombo et al., 1988; De Luca et al., 2015; Odle, Glass, Czarnecki-Maulden, & Baker, 1992).

Research has suggested taurine may play a role in improving mood state through activation of the gamma-aminobutyric acid (GABA) neurotransmitter (Jia et al., 2008). Low levels of GABA have previously been associated with depression, inability to focus, anxiety, and panic (Brambilla, Perez, Barale, Schettini, & Soares, 2003). Increasing GABA levels may be beneficial for athletes, as mood state has been found to be one of the predictors of athletic performance (Beedie et al., 2000). However, the effect of taurine on mood state and subsequent sport performance has not been tested in humans, and results from animal studies are contradictory. Whilst some show oral taurine to improve mood state (El Idrissi et al., 2009; Kong et al., 2006), others have found no effect (Whirley & Einat, 2008). Our study appears to be the first to investigate the effect of taurine supplementation on mood state, and our results suggest taurine supplementation for one week does indeed improve mood. Mood improved from pre- values with both PP and, to a slightly greater extent, PP + T. This suggests that PP, despite being significantly lower in thiol amino acids, may still benefit mood. Indeed, several amino acids found in pea protein are well known for their role in mood enhancement such as tryptophan (Young & Leyton, 2002) and glutamate (Sanacora, Treccani, & Popoli, 2012) and it appears that adding taurine (PP + T) can further improve mood and subsequent TMD score.

Several limitations existed with this study. Firstly, PP + T and KER were matched for (potential) taurine levels, with the assumption that all cysteic acid would metabolise to taurine. This left no margin of error for any that perhaps did not. For example, to form taurine, cysteic acid is firstly metabolised to hypotaurine which plays a role in neurotransmission (Kontro, Marnela, &

Oja, 1983). Thus is it possible that metabolism of KER-derived cysteic acid was varied in each participant, resulting in different rates of taurine production. Secondly, several participants experienced issues with having their blood taken; one had very small veins, making it extremely difficult to take a sample, and the other felt very faint. For this reason, both of them were excluded from blood collection, reducing the sample size. And lastly, participants were asked to record dietary intake during the week leading up to the first trial, which they then were asked to replicate in all three trials in order to maintain a similar dietary intake. This method of dietary reporting however has been considered unreliable (Jeacocke & Burke, 2010), and it is likely that diet was not exact across all three trials which may have affected blood results. Further, whilst told to replicate any physical activity across the trials, this was not monitored, so it is possible activity level was not kept consistent between treatments.

5.5.1 Conclusion

The main aim of this study was to determine whether the cysteic acid in keratin would have an equal or greater effect on serum TTC compared to the exogenous thiol antioxidant taurine. As predicted, one week of PP did not affect serum TTC; however, neither did one week of KER or PP + T, which did not support our hypothesis. On the other hand, PP and PP + T improved TMD, suggesting the amino acid composition of both may enhance mood. Based on this study, future research could look at the amount of cysteic acid within KER that is metabolised to measurable taurine. This may determine whether supplementing with keratin, which has other physiological benefits in addition to its conversion to taurine, is more advantageous than taurine alone.

Chapter 6

The effect of taurine on recovery from eccentric-exercise induced muscle damage in males

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6.1 Abstract

Eccentric exercise is known to bring about microstructural damage to muscle, initiating an inflammatory cascade involving various reactive oxygen species. This, in turn, can significantly impair physical performance over subsequent days. Taurine, a powerful endogenous antioxidant, has previously been shown to have a beneficial effect on muscle damage markers and recovery when taken prior to eccentric exercise. However, to date no studies have looked at post-exercise supplementation and performance recovery. Thus this study aimed to determine whether supplementing with taurine over three days (72 h) following eccentric exercise can prevent the rise in serum creatine kinase and improve performance recovery in males. In a blinded, randomized, crossover design, ten recreationally fit male participants completed 60 eccentric contractions of the biceps brachii muscle at maximal effort. Following this, participants consumed 0.1 $g.kg^{-1}$ $BW.day^{-1}$ of either taurine or rice flour (as placebo) in capsules. Over the next three mornings participants underwent blood tests for the analysis of the muscle damage marker creatine kinase, and total thiol content, and carried out performance measures on the isokinetic dynamometer. They also continued to consume the capsules morning and evening. The entire protocol was repeated two weeks later on the alternate arm and supplement. Significant decreases were seen in all performance measures from pre- to 24-h post eccentric exercise ($p < .001$) for both taurine and placebo, indicating the attainment of muscle damage. Significant treatment effects were observed only for peak eccentric torque with taurine ($p < .05$). No significant time x treatment effects were observed (all $p > .05$). Total thiol content did not significantly change over time for either treatment, nor was there any difference between treatments ($p > .05$). No time x treatment effect was observed. Serum creatine kinase levels did not significantly differ over time for either treatment, nor between treatments ($p > .05$). The treatment effect seen with taurine on recovery of eccentric torque suggests that taurine supplementation taken twice daily for 72 h

following eccentric exercise-induced muscle damage may help improve eccentric performance recovery of the biceps brachii.

6.2 Introduction

Eccentric actions, whereby muscle lengthens while under tension, can produce significant microstructural damage as a result of high force per fibre ratio. Not only can this directly reduce the ability of muscle to contact, it can also generate an inflammatory cascade, activating various ROS generating pathways including xanthine and NADPH oxidase, and phagocytic respiratory burst (Close, Ashton, Cable, Doran, & MacLaren, 2004). The resulting formation of ROS at high concentrations can surpass the ability of endogenous antioxidant defence, leading to oxidative stress. This in turn can elicit ROS-induced secondary tissue damage, including lipid peroxidation and protein oxidation, further reducing the ability of skeletal muscle to perform (Schaffer et al., 2009; Vissers & Winterbourn, 1991)

Dietary antioxidants have the ability to lower oxidative stress markers within the human body, and a number of studies have shown attenuation in exercise-induced ROS increases following supplementation (Cannon et al., 1990; Hartmann et al., 1995; Rokitzki, Logemann, Huber, et al., 1994; Satoshi et al., 1989). Additionally, certain antioxidants such as vitamin E appear to have membrane-protective effects, possibly reducing microstructural fibre damage (Itoh et al., 2000). As a result, antioxidant supplementation is common practice amongst athletes hoping that the reduction of ROS will lessen muscle tissue damage, diminishing any subsequent performance reductions. However, despite observed reductions in ROS following exercise with antioxidant supplementation, there appears to be no correlation to improved recovery and performance. Indeed, while few studies show dietary vitamin supplementation to have potential protective effects on muscle damage and subsequent force loss (Shafat, Butler, Jensen, & Donnelly, 2004; Thompson et al., 2004) the majority show little to no beneficial effect (Avery et al., 2003; Bryer & Goldfarb, 2006; Connolly, Lauzon, Agnew, Dunn, & Reed, 2006; Jakemanl & Maxwell, 1993). Some even suggest negative performance effects (Childs, Jacobs, Kaminski, Halliwell, & Leeuwenburgh, 2001; Close et al., 2006). Furthermore, due to their non-specific ROS scavenging action, long-term use of antioxidants has been shown to potentially blunt important muscle adaptations. This can have detrimental outcomes on training progression and ultimately, performance, and several studies have observed this (Makanae et al., 2013; Paulsen, Hamarsland, et al., 2014). In recreationally strength-trained men and women, ten weeks of high-dosed vitamin C+E supplementation was shown to significantly blunt post-exercise increases in MAPK and lessen strength gains compared to a placebo group (Paulsen, Hamarsland, et al., 2014). A similar blunting effect of vitamin C was

seen in overload-induced rats (Makanae et al., 2013), with significantly less muscle hypertrophy and lower MAPK than controls. In contrast, endogenous thiol antioxidants appear to have a more targeted approach to ROS (Powers & Jackson, 2008) suggesting that improved endogenous antioxidant defence may benefit exercise performance without hindering adaptations.

Taurine, a sulphonic acid, is produced endogenously from the amino acid cysteine. Containing a thiol group, it has high antioxidant activity and is found abundantly in skeletal muscle.
Taurine's cytoprotective effects, attributed to its role in osmoregulation, membrane stabilization, antioxidant action, conjugation, and regulation of Ca^{2+} (Schaffer, Azuma, Takahashi, & Mozaffari, 2003) have been reported to protect against various tissue diseases including those affecting the retina (Heller-Stilb et al., 2002), heart (Ito, Kimura, Uozumi, Takai, Muraoka, Matsuda, Ueki, Yoshiyama, Ikawa, Okabe, et al., 2008), hepatic (Miyazaki, Bouscarel, Ikegami, Honda, & Matsuzaki, 2009) and skeletal muscle (Camerino et al., 2004). Indeed, several studies have observed accelerated aging (Ito, Yoshikawa, Inui, et al., 2014) and incidence of muscular disorders (De Luca et al., 2015) in taurine-depleted skeletal muscle. However, to date few studies have looked directly at the potential of taurine to protect skeletal muscle against eccentric-exercise induced muscle damage and oxidative stress. A study in rats found one month of taurine supplementation significantly increased muscle taurine content and completely block post-eccentric exercise rises in the circulating muscle damage marker TBARS (Dawson et al., 2002). Similarly, in men, two weeks of taurine supplementation (50 mg.kg body $mass^{-1}.day^{-1}$) resulted in reduced plasma levels of the muscle damage marker creatine kinase, reduced muscle soreness, and increased strength levels following eccentric exercise (da Silva et al., 2013). Taurine supplementation did not affect post-exercise rises in inflammatory markers important for adaptive pathways, suggesting it to be a better alternative to common dietary antioxidants. In their study, Ra *et al.* (2013) observed reduced delayed onset muscle soreness (DOMS) following eccentric exercise of the elbow flexors in men supplemented with taurine for two weeks (2 $g.day^{-1}$, 3x daily) compared to their placebo counterparts. However, no significant difference in muscle damage markers were seen between treatments. While these studies have used taurine pre-supplementation, to date no research has looked solely at post-exercise taurine supplementation on recovery from eccentric-exercise induced muscle damage in humans. This study, therefore, aimed to determine the effects of post eccentric-exercise taurine supplementation on creatine kinase activity, serum total thiol content, and muscle performance recovery in the elbow flexors (biceps brachii).

6.3 Methods

6.3.1 Subjects

Ten healthy males (mean ± standard deviation (SD) age = 26.5 ± 6.5 years, height = 180 ± 9.2 cm, weight = 80 ± 11.5 kg) were recruited for this study. All participants were recreationally fit, engaging in a mix of cardio and strength training 2-3 times per week. Individuals were screened using a Health Screening Questionnaire designed to exclude those who could not take part due to physical, cultural, or religious reasons. Once deemed suitable, participants were asked to sign a consent form. Approval for this study was granted by the Massey University Human Ethics Committee, Southern A Application (approval number 16/33).

6.3.2 Pre-testing procedures

At least one week prior to their first trial, participants underwent a familiarization session involving an outline of the study protocol, and familiarization with the performance measures (isometric, concentric, eccentric) on the isokinetic dynamometer (Biodex Medical Systems, Inc., New York, NY, USA). Participants' height and weight were recorded, and dynamometer seat settings were determined and recorded for subsequent use. Participants were advised to discontinue any supplements, energy drinks, and alcohol consumption during the week leading up to their first trial. Three days prior to the first trial, and for three days (72 h) following eccentric exercise-induced damage, participants were asked to record their food intake and exercise activity, and were asked to abstain from any exercise aside from necessary walking. Food records from the first trial were replicated during the second trial to control for nutritional intake, as was exercise.

6.3.2 Overview

The study protocol was designed as a single-blinded, cross-over protocol, with exercised arm and treatment randomly allocated. Participants attended the laboratory, underwent pre-exercise blood collection, and then proceeded to perform six sets of ten eccentric repetitions of the biceps muscles on an isokinetic dynamometer. This protocol has been previously used (Cochrane, 2017; Lau, Blazevich, Newton, Wu, & Nosaka, 2015) to provoke significant levels of muscle damage observed by decreased muscular performance, and rises in circulating creatine kinase (CK) activity. At the completion of this protocol, participants were given either taurine or rice flour 'placebo' capsules to ingest immediately, and again in the evening, along with a standardized breakfast (CHO 44g, protein 8.4g, Fat 16.5g, 1450kJ). Participants returned to the lab, fasted, the following three mornings (24 h, 48 h, 72 h) for follow up muscular performance

measures of both the exercised and control arm and venous blood sampling. At the 24 h and 48 h visits, participants were given capsules to take that morning and in the evening. At least two weeks later, participants completed the same protocol on the other arm, and were given the other supplement to consume.

6.3.3 Blood measures

On the morning of their first trial, participants attended the lab, fasted, where they underwent the collection of a pre-exercise venous blood sample from the antecubital fossa into vacutainer tubes to determine serum levels of CK, and TTC. The tubes were inverted several times, left at room temperature for 30 minutes, and then centrifuged at 4°C for 10 minutes at 2000rpm (revolutions per minute) (Eppendorf centrifuge 5804R, Eppendorf, New York, NY, USA). Serum was then aspirated into 1ml aliquots and frozen at -80°C for later analysis. Blood samples were also collected at 24 h, 48 h and 72 h post-exercise.

6.3.4 Muscular performance

Following blood collection, participants carried out a two-minute warmup on an arm ergometer (Excalibur Sport, Lode, Netherlands). After completion of the warmup, participants were seated on the isokinetic dynamometer (Biodex Medical Systems, New York, NY, USA) and straps were fixed across the chest to isolate movement of the arm. Elbow joint range of motion was set and recorded for use in subsequent follow-up tests. Participants then performed separate sets of three maximal isometric (ISO), concentric (CON), and eccentric (ECC) contractions of the biceps muscles of both the exercising and non-exercising (control) arm, with two-minutes between each set. ISO tension was measured at an elbow angle of 75°, while CON and ECC torque was measured at an angular velocity of $30°.s^{-1}$ (degrees per second). Peak torque/tension over the three contractions was recorded. Muscular performance was measured again at 24 h, 48 h, and 72 h post-exercise.

6.3.5 Exercise protocol

Following the completion of pre-exercise performance measures, participants completed six sets of 10 eccentric contractions over a 110° range of motion at an angular velocity of $30°.s^{-1}$, using the biceps muscle of one arm (dominant vs. non-dominant randomly allocated to each trial). Each set was separated by two-minutes of passive recovery. Participants were encouraged to resist the downward action of the dynamometer arm with as much effort as possible, and could visually see their torque output on a screen during the protocol to help maintain maximal effort. Total work over the exercise protocol was recorded.

6.3.6 Treatment

Within 30 minutes of post-exercise blood collection, participants consumed either taurine powder (NOW foods) or rice-flour (Bob's Red Mill, Milwaukie, OR, USA) as a placebo in the form of vege-capsules. The amount of powder was set at 0.1 $g.kg^{-1}$.body $weight.day^{-1}$, based on several studies that found no adverse effects at levels of up to $10g.day^{-1}$ (Azuma et al., 1982; Durelli et al., 1983). No participant was at a body weight that resulted in consuming more than 10 $g.day^{-1}$ (i.e. all were below 100 kg). Powder was split evenly into 1 g capsules that were then divided into two bags. Participants were advised to take one bag with breakfast and one with dinner. Identical capsules were taken over the following two days. During the second trial, the contralateral arm was exercised, and the other supplement was consumed.

6.3.7 Biochemical analyses

6.3.7.1 Total thiol content

Serum Total Thiol Content (TTC) was analysed using a Measure-iT™ Thiol Assay Kit (M30550, Molecular Probes Inc., Eugene, USA). Stock solution was prepared with Measure-iT™ thiol quantitation standard and deionized H_2O. From this, a working solution was prepared and diluted to a ratio of 1:100, Measure-iT™ to working solution. 10µL serum was added to 100µL working solution into microplate wells from which fluorescence was measured using a microplate reader. Samples were analysed in duplicate, and thiol content was determined from the fluorescence standard curve according to the manufacturer's instructions.

6.3.7.1 Creatine kinase

Serum CK activity was determined by enzymatic method using reverse reaction. This assay was carried out using a Roche CK-NAC liquid assay kit (Roche Diagnostics GmbH, Manheim, Germany) according to the manufacturing instructions.

6.3.8 Statistical analyses

Data were analysed using the Statistical Program for Social Sciences (SPSS) for Windows (IBM SPSS Statistics v.22, IMB, New York, NY, USA). A repeated-measures analysis of variance (ANOVA) was used to compare treatments over time for performance measures, total thiol content, and CK. Statistical significance was set at $p < .05$, and the results displayed as mean ± SD. Post-hoc analysis using the Holm-Bonferroni method was carried out on any significant results to determine where that significance lay.

6.4 Results

6.4.1 Performance measures

Total work completed during the eccentric protocol did not significantly differ between trials (p = .742), suggesting a similar level of effort during both trials.

Completion of 60 eccentric muscular contractions of the biceps brachii resulted in significant decreases in ISO, CON and ECC peak and average peak torque over time in the exercising arm only (all p < .01, Table 5). The greatest declines in force output for all performance measures were seen from pre- to 24 h in the placebo (PLA) groups (all p < 0.05). Only peak ECC performance showed a significant treatment effect (p = .049), with a significantly greater force recovery with taurine at 48 h (p = .048). No treatment x time effects were observed for any performance measures (all p > .05).

Table 5 Changes in torque (Nm) following strenuous eccentric exercise (mean ± SD). ISO = isometric, CON = concentric, ECC = Eccentric, PLA = placebo, TAU = taurine. * Significantly different to pre-exercise value ($p < .01$), # significantly different to pre-exercise value ($p < 0.05$). † significantly different between trials ($p < .05$) $n = 10$

	Exercised				Control			
	Pre	24h	48h	72h	Pre	24h	48h	72h
Peak ISO								
PLA	74.1 ± 17	-21.3 ± 16.7*	-16.3 ± 13.6*	-10.9 ± 12.4	75.5 ± 19.5	-2.8 ± 4.9	-2.4 ± 6.7	-3.7 ± 7.4
TAU	75.4 ± 24.9	-18.2 ± 4.3*	-18.5 ± 6.6*	-9.7 ± 7.9*	75 ± 17.1	-1.7 ± 8.3	-4.5 ± 6.6	-5.3 ± 7.1
Peak CON								
PLA	56.6 ± 16.2	-19.7 ± 8.4*	-16.7 ± 11.4*	-12.6 ± 7.9*	56.5 ± 16	-3.7 ± 7.6	-4.7 ± 6.5	-4.9 ± 6.6
TAU	55.1 ± 17.9	-14.1 ± 3.9*	-11.4 ± 7*	-7.2 ± 9	57 ± 17.4	-5.1± 6.8	-5.6 ± 6.6	-6.5 ± 6.6
Peak ECC								
PLA	73.8 ± 16.5	-22.7 ± 13*	-25 ± 11.2*†	-15.5 ± 9.6*	72.5 ± 18.4	-0.4 ± 9.4	-0.7 ± 9.4	-4.9 ± 12.6
TAU	79 ± 24.3	-25.1 ± 14.9*	-18.6 ± 12.3*†	-18.4 ± 14.7*	73 ± 21	-4.8 ± 6.4	-5.4 ± 6.8	-6.6 ± 6.8
Average ISO								
PLA	71.4 ± 16.4	-20.5 ± 14.6*	-17.5 ± 13.6*	-12.5 ± 12.5	72.5 ± 18.4	-2.4 ± 5.1	-1.7 ± 9	-3.9 ± 6
TAU	71 ± 20.7	-17 ± 7.7*	-17.3 ± 6.6*	-9.2 ± 7.3*	70.7 ± 15.7	0 ± 8.1	-2.6 ± 7.5	-3.3 ± 6.5
Average CON								
PLA	53.5 ± 15.9	-19.1 ± 8.3*	-16.9 ± 11.5*	-11.8 ± 8.1*	52.1 ± 14.4	-4.2 ± 6.3	-3.5 ± 5.3	-5.2 ± 5.4
TAU	51 ± 15	-12.9 ± 4.9*	-10.8 ± 8.2*	-4.6 ± 8.2	55.1 ± 14.6	-4.9 ± 5.8	-5.3 ± 6	-5.5 ± 6.6
Average ECC								
PLA	69 ± 16.2	-21 ± 14.5*	-23.2 ± 11.6*	-13.9 ± 9.2*	67.3 ± 18.2	-0.6 ± 7.9	-0.6 ± 7.7	-3.3 ± 11.2
TAU	72.8 ± 23.2	-22.7 ± 12.7*	-17.6 ± 12.6*	-18 ± 14*	70.1 ± 20	-4.7 ± 6.5	-6.3 ± 6.2	-7.5 ± 6.2#

6.4.2 Serum total thiol content

Levels of serum TTC did not significantly change over time (p = .120). Furthermore, whilst data shown in Figure 12 suggests TAU is able to prevent a decline in TTC compared to PLA, differences between treatments were non-significant (p = .347). No time x treatment effect was observed (p = .607).

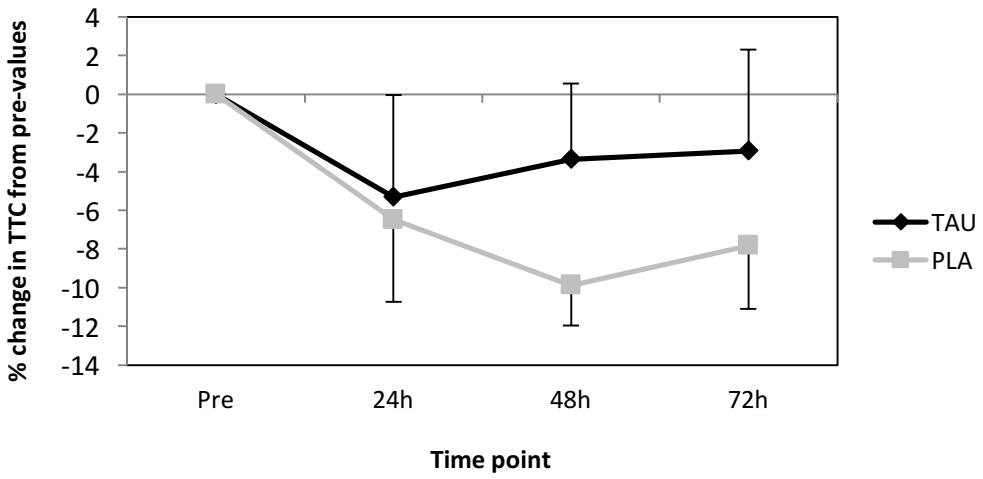

Figure 12 Percentage change (%) in mean serum total thiol content (TTC) from pre-eccentric exercise values (mean ± SE). n = 10.

6.4.3 Serum creatine kinase activity

Completion of 60 eccentric muscular contractions of the biceps brachii had no significant effect on serum CK activity from pre- to post- 72 h. Treatment effects were also statistically non-significant (p = .352). Furthermore, no time or time x treatment effects were observed, with p = .382 and p =.184, respectively.

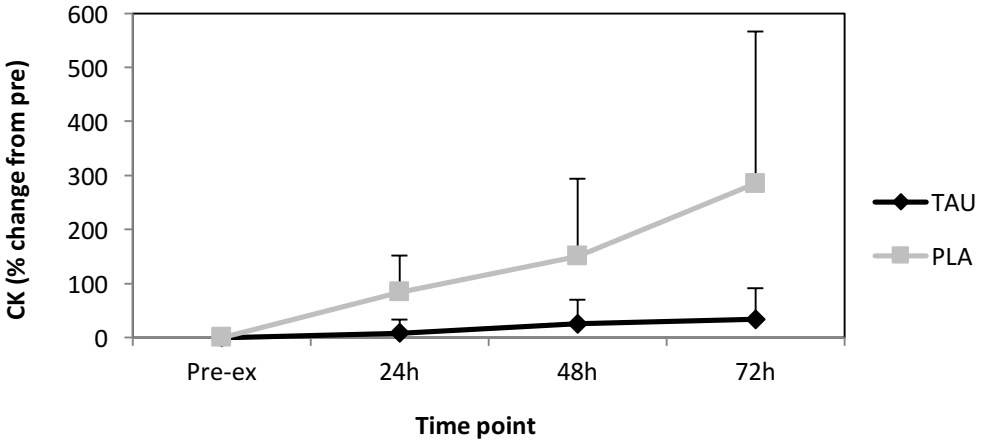

Figure 13 Percentage change in creatine kinase (CK) activity from baseline values following strenuous eccentric exercise (mean ± SE). TAU = taurine, PLA = placebo. *n* = 10.

6.5 Discussion

Research has shown that supplementing with taurine for several weeks prior to eccentric-based activity prevents exercise-induced rises in oxidative stress and muscle damage markers in rats (Dawson et al., 2002; Silva et al., 2011), and humans (Ra et al., 2013) and improves strength in young men (da Silva et al., 2013). Our study is the first that has looked solely at post-exercise taurine supplementation in humans, its effect on muscle damage markers, and subsequent performance recovery over 72 h.

Significant declines in all performance measures in the exercised arm at 24 h post exercise indicate the attainment of muscle damage. Treatment effects were only significant for peak eccentric torque, where force recovery significantly increased toward pre-values at 48 h with taurine compared to placebo. This suggests that taurine may expedite the recovery of eccentric force, potentially by mitigating inflammation-induced secondary damage. No such *in vivo* studies have looked at the effect of taurine supplementation on force recovery previously, however, Goodman *et al.* (2009) observed that isolated skeletal muscle removed from rats following two weeks of taurine supplementation showed a greater recovery of force post high-frequency *in vitro* stimulation compared to controls. In a similar manner, Hamilton *et al.* (2006) found force production in taurine depleted mouse skeletal muscle to be significantly lower than controls, whilst Bakker and Berg (2002) found taurine supplementation to increase force response in skinned rat skeletal muscle. It is clear that more *in vivo* studies are needed to determine if *in vitro* results can be replicated.

Research suggests eccentric-exercise induced performance reductions peak between 24-72 h post exercise, and return to full muscular force within seven days (Armstrong, 1984; Cheung, Hume, & Maxwell, 2003). In line with this, our results show that neither treatment group fully recovered force output by 72 h. Perhaps measuring performance up to seven days post-damage may have shown better potential differences in return to full force between treatments.

Serum CK activity is often used as a marker of exercise-induced skeletal muscle damage. Unlike several studies that have observed otherwise (da Silva et al., 2013; Dawson et al., 2002), we

did not find any significant changes in CK over time or between treatments. It is possible that the use of a smaller muscle group provided a lower relative release of CK into the circulation compared to that of a larger muscle group, such as the quadriceps, worked to the same intensity may have (Serrao et al., 2007). Furthermore, the significant difference between participant measures gave rise to large standard errors, negating any possible difference between treatments. Indeed, a study by Nosaka and Clarkson (1996) found a large inter-subject variability in CK response following eccentric exercise of the elbow flexors, largely due to the extent of muscular damage, which is dependent on a variety of factors. A larger number of participants or testing a larger muscle group may have reduced this error.

Several rodent studies have shown taurine supplementation to spare other thiols, including GSH, methionine and cysteine, thereby increasing overall endogenous antioxidant status controls (Anand et al., 2011; Oudit et al., 2004; Silva et al., 2011). TTC is often used to indicate overall endogenous antioxidant status, with measures being taken from plasma, serum, hepatic tissue, and skeletal muscle. Results from this study showed taurine to have no significant effect on serum TTC compared to placebo. While these results may have been affected by the large variation, another possible scenario is the ability of the human body to tightly regulate the various circulating thiols (Ookhtens & Kaplowitz, 1998; Stipanuk et al., 2006). Liver biopsies have been used in rodent studies to determine TTC (Jocelyn & Kamminga, 1974), however being an almost impossible (and inhumane) practice in humans, skeletal muscle biopsies would have been the only other way to assess TTC changes. Still, it appears even in skeletal muscle, thiols are tightly regulated (Galloway et al., 2008).

It may have been useful to measure serum taurine throughout the study to determine whether the dose given was effective. Some studies report increased serum taurine with supplementation in animals (Dawson Jr et al., 1999; Dirican, Tas, & Sarandol, 2007) and humans (Beyranvand et al., 2011). Additionally, measuring markers of oxidative stress may have indicated any protective effect of taurine against exercise-induced ROS. Overall, this study suggests that taurine, when taken twice daily (at 0.1 g.kg-1body weight.day-1) for 72 h following eccentric exercise may help accelerate eccentric performance recovery of the biceps brachii. Further human studies are needed to support this.

6.5.1 Conclusion

In this first of its kind study, the results suggests that supplementation with taurine twice daily for 72 h following eccentric exercise-induced muscle damage may improve eccentric performance recovery of the biceps brachii in healthy males. This may be a result of taurine's

antioxidant and cytoprotective roles within skeletal muscle. The implications for taurine as a recovery supplement for athletes competing in eccentric-based sports is intriguing, but requires further research.

Chapter 7

General Discussion and Conclusion

7.1 General discussion

The use and application of keratin protein has previously been limited to animal feed. Recently, hydrolysed keratin, derived from poultry feathers, was administered to rats for four weeks, from which several beneficial observations were reported. Firstly, keratin increased lean body mass even in the absence of exercise. Secondly, liver taurine was increased, and thirdly, liver GSH levels were maintained compared to pea protein. Whilst novel, these results could not be extrapolated to humans, thus, this is the first research to investigate the effects of a four-week keratin intake on various physiological parameters in humans.

Endurance athlete's performance is primarily based on the body's ability to prolong time to fatigue, a process that, amongst other factors, can be affected by exercise-induced, ROS-elicited oxidative stress. While training can improve endogenous antioxidant defence, therefore enabling the body to withstand higher generation of ROS, this cannot prevent oxidative stress from occurring during periods of intense or very frequent activity. In an attempt to further improve this defence, dietary antioxidants, namely vitamins C and E have been highly studied as a way to reduce oxidative stress and subsequently improve performance. However, the majority of studies failed to note performance improvement, despite a reduction of oxidative stress markers. Further, it has been suggested dietary antioxidants may hinder important exercise adaptations. The purpose of **Chapter 4** therefore, was to determine if, due to its high concentration of cysteic acid, four-weeks of keratin protein at 0.8 g.protein.kg^{-1} BW.day^{-1} would increase plasma TTC, reducing oxidative stress in the process via non-specific ROS targeting, leading to improved performance. However, while a one-off intake of keratin protein appeared to increase plasma TTC, four weeks supplementation had no significant effect compared with casein. Additionally, no significant improvements in exercise performance were observed between treatments. It should be noted however, that there were no time-dependent performance improvements in VO_{2max}, which is in line with other studies that have used trained participants (Daniels et al., 1978; Gore et al., 1998). In contrast, keratin significantly increased LBM by almost 1 kg more than casein over the four week supplementation period. Being a novel area of research, this increase in LBM is largely due to reasons we don't fully understand. It is possible that keratins

metabolism to taurine is partially responsible for this effect (Uozumi et al., 2006), however, more research would be needed to definitively prove this.

Since keratins' supposed advantage over casein is its ability to increase endogenous thiols (specifically taurine), **Chapter 5** aimed to investigate if cysteic acid (as KER) would increase plasma TTC to the same extent as exogenous taurine (PP + T, matched to KER's cysteic acid content). Further, it aimed to determine whether KER and PP + T would increase TTC significantly more so than low-thiol PP. An additional aim was to define whether KER and PP + T had the ability to improve mood state, which taurine has been previously suggested to do (Jia et al., 2008; Kong et al., 2006). Similar to **Chapter 4** results, no significant difference in TTC was observed with or between any of the treatments after one week of supplementation with 0.8 g.protein.kg^{-1} BW.day^{-1}. This may be due to tight regulation of various thiols within plasma, as evidenced by several studies (Ookhtens & Kaplowitz, 1998; Stipanuk et al., 2006). However, other studies have found increased taurine or GSH with thiol supplementation (Beyranvand et al., 2011; Dawson Jr et al., 1999; Dirican et al., 2007), so perhaps measuring these directly would have been more reliable. Due to financial constraints we were unable to analyse these markers. These measures would also have been useful to analyse for **Chapter 5.**

While the benefit of taurine in endurance exercise performance has been investigated through several studies,, any advantage in resistance exercise is largely unknown. Several studies have alluded to taurine's potential role in muscle cell differentiation and growth (McIntosh et al., 1998), an important aspect in muscle hypertrophy and recovery. Current research into taurine and resistance performance/recovery has predominantly used animal subjects (Bakker & Berg, 2002; Goodman et al., 2009) and whilst results are promising, supplementation has only occurred prior to eccentric exercise. Thus **Chapter 6** aimed to determine whether taurine supplementation following eccentric exercise-induced muscle damage reduced secondary ROS-related muscle damage and improved recovery. No significant differences in serum CK were observed over time within or between treatments, which is likely due to using a small muscle group and a small sample size (large variation). Indeed, serum CK has been shown to markedly increase following eccentric-exercise induced muscle damage of the quadriceps femoris (Barnes, Mündel, & Stannard, 2010; Bryne, Eston, & Edwards, 2001; McLeay et al., 2012). No significant changes in serum TTC were observed over time within or between treatments, despite graphed results suggesting otherwise. This is also likely a result of large inter-subject variance. As with **Chapters 4** and **5**, it may have been better to directly measure taurine rather than TTC. In terms of performance recovery, return of the biceps ECC force to baseline values was significantly faster in participants supplemented with taurine compared to

placebo, suggesting taurine may in fact improve muscle force recovery following resistance training. Without further research, it is hard to confirm the exact mechanism by which taurine may achieve this. It could be a result of taurine's high antioxidant activity, whereby it may reduce secondary damage caused by the ROS-forming inflammation cascade that follows microstructural damage to muscle (Aruoma et al., 1988); taurine's cytoprotective properties that work to protect skeletal muscle cell membranes from ROS attack; taurine's proposed role in hypertrophy (Ito, Kimura, Uozumi, Takai, Muraoka, Matsuda, Ueki, Yoshiyama, Ikawa, Okabe, et al., 2008; Jones, Miller, Dowling, & Chesney, 1991; McIntosh et al., 1998; Schaffer, Takahashi, & Azuma, 2000; Uozumi et al., 2006), or a combination of all three. Regardless of the mechanism(s) responsible, this study lays a platform for further research into this area.

7.2 Limitations

Despite attempting to carry out these study protocols and collect data as accurately as possible, several limitations still exist.

Likely the biggest limitation is found in **Chapter 4**. At the beginning of each trial each participant carried out a VO_{2max} test from which work load at 65% of VO_{2max} was used to set the resistance for the 2-hour submaximal cycle. Following the 4-week supplementation period, participants carried out another VO_{2max} test from which resistance for their following submaximal ride was set. Instead of basing submaximal load on individual VO_{2max} tests, it would have been much more effective to have taken the first VO_{2max} results and used them for both pre- and post- submaximal rides. This would have given a better indication of how participants responded physiologically to the same workload following the supplementation and training period. As it stands, even if blood results were significant within and between treatments, we could not suggest this is due to improved tolerance to endurance exercise. Further studies may be able to determine this.

For all three studies, diet and exercise were recorded daily by participants in an attempt to monitor and replicate these across cross-over trials. Self-reporting however, has been suggested as being unreliable, and under- or over-reporting is common (Jeacocke & Burke, 2010). In **Chapter 4** participants were asked to record food intake and exercise in separate software programs. Whilst this is more accurate in terms of the amount of each food consumed and duration/intensity of exercise, it is possible that due to the reliance on technology, information was input late or skipped altogether. Indeed, several participants did

not report food intake on a number of days, thus it was hard to accurately monitor intake and keep consistent across the trials.

With regards to blood collection, for a number of measures, timing of collection is crucial. Since we could not keep participants in the lab for most of the day, the majority of post-exercise measures (**Chapters 4** & **6**) were taken within five minutes from the cessation of exercise. It is likely that in doing so, peak rises in several plasma and serum markers including TTC and CK, were missed. In **Chapter 4**, blood collection for analysis of TTC following acute KER supplementation was close to 24 h after ingestion. Considering plasma thiols tend to peak after 1-2 h (Michailidis et al., 2007), collecting blood the following day may have missed this peak rise. It has even been suggested that several markers of oxidative stress may not peak in blood for several days following intense exercise (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997), thus for **Chapter 4**, measuring TTC immediately following sub-maximal cycling may not have accurately represented what impact exercise has had on redox status.

Whilst treatment allocation was randomized and blinded in all studies, keratin was a hard flavour to mask (**Chapters 4** & **5**). For this reason, particularly in **Chapter 4**, participants were convinced they were on KER treatment when they were. This has the potential to affect performance measures as if they deemed KER to improve performance, they may have subconsciously pushed harder. This behaviour has been observed in 'placebo effect' studies (Beedie & Foad, 2009).

During statistical analysis, it was evident that two of the studies lacked power. One participant in **Chapter 5** was unable to give blood, bringing the sample size down to 7 for blood analyses. In **Chapter 6** it was difficult to collect blood from two participants at several points throughout the study due to small veins, bringing the sample size down to 8 for blood analyses. This would have had a marked effect on standard errors and subsequent significance.

7.3 Conclusions

The primary aim of this thesis was to investigate the use of keratin protein as a dietary supplement to increase antioxidant status and lean body mass in endurance athletes, and how this may subsequently improve performance. Secondly, we aimed to determine whether the cysteic acid content in keratin had a similar effect on TTC compared to matched intake of taurine. And thirdly, whether taurine could improve muscle performance recovery following eccentric-exercise induced muscle damage. The findings from this thesis showed that:

1) A one off intake of keratin protein at 0.8 g.protein.kg^{-1} BW.day^{-1} alongside endurance training increases plasma TTC compared to a casein equivalent.

2) Four weeks of keratin protein supplementation at 0.8 g.protein.kg^{-1} BW.day^{-1}alongside endurance training does not significantly improve plasma TTC compared to a casein equivalent.

3) Four weeks of keratin protein supplementation at 0.8 g.protein.kg^{-1} BW.day^{-1} alongside endurance training does not significantly improve exercise performance ($VO_{2max,}$ MPO) compared to a casein equivalent.

4) Four weeks of keratin protein supplementation 0.8 g.protein.kg^{-1} BW.day^{-1}alongside endurance training does not prevent the decline in TTC during sub-maximal exercise compared to a casein equivalent.

5) Four weeks of keratin protein supplementation at 0.8 g.protein.kg^{-1} BW.day^{-1}alongside endurance training significantly increases lean body mass compared to a casein equivalent.

6) Serum TTC does not significantly differ with keratin supplementation at 0.8 g.protein.kg^{-1} BW.day^{-1}after one week compared to an equivalent dose of taurine. Neither increase TTC compared to pea protein.

7) Taurine supplementation at 0.1 g^{-1}.kg^{-1}.day^{-1} taken for three days post eccentric-induced muscle damage does not increase serum TTC. However, it does improve eccentric performance recovery in the biceps brachii.

7.4 Future directions

Overall, these studies suggest dietary thiols may benefit aspects of endurance and resistance exercise. However, there are definite gaps in the literature, and even in these studies, that warrant further investigation.

Firstly, it may be important to determine what specific thiols keratin increases. Whilst we investigated effects of keratin on plasma overall thiol concentrations (TTC) in **Chapter 4** and **Chapter 5**, it may be wise to measure taurine and GSH as separate measures. This could determine whether keratin predominately upregulates one or the other, and what effect that may have on other thiols. Additionally, It would be interesting to determine the effects on thiol antioxidants such as GPx. Moreover, since thiols in humans tend to be tightly regulated, measuring skeletal muscle TTC or individual thiols may give a better indication of what is occurring endogenously in response to keratin intake.

Secondly, **Chapter 4** observed keratin to increase LBM significantly more than casein over a four week of period. If such results can be seen with endurance exercise, perhaps a similar or even greater response would be seen with resistance training. Thus, investigating the role of keratin combined with a resistance training program on strength gains and performance could be both novel, and potentially breakthrough research due to its potential contribution to the multi-million dollar sport-supplement industry. Furthermore, the results seen in this study may have implications for non-athletic populations. The ability of keratin to increase LBM at such a rate compared to casein suggests keratin may benefit those who need to maintain or gain LBM such as the sick or elderly. Additionally, it would be interesting to see whether keratin can increase LBM in the absence of exercise, which may be more applicable for aforementioned populations.

And lastly, as observed in **Chapter 6**, taurine supplementation improved recovery of ECC force in the biceps brachii. While this is an interesting observation, determining the effect of taurine on larger muscle groups i.e. quadriceps femoris, may suggest any benefit of taurine for recovery in a number of eccentric based sports such as running and rugby.

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Appendix A

Ethics Application Documentation

Chapter 4

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

17 December 2014

Yanita McLeay
18 Clifton Terrace
Fitzherbert
PALMERSTON NORTH

Dear Yanita

Re: HEC: Southern A Application – 14/79
The physiological effect of a novel high cysteine protein supplement in healthy male cyclists

Thank you for your letter dated 9 December 2014.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Brian Finch, Chair
Massey University Human Ethics Committee: Southern A

cc Prof Stephen Stannard
School of Sport & Exercise
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Chapter 5

MASSEY UNIVERSITY
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29 October 2015

Yanita McLeay
20a Norwich Place
Awapuni
PALMERSTON NORTH

Dear Yanita

Re: HEC: Southern A Application – 15/59
The effect of cysteic acid supplementation (as keratin) on endogenous taurine production compared with exogenous taurine supplementation in healthy humans

Thank you for your letter dated 28 October 2015.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Dr Lesley Batten, Acting Chair
Massey University Human Ethics Committee: Southern A

cc Prof Stephen Stannard
School of Sport & Exercise
PN621

Dr Matt Barnes
School of Sport & Exercise
PN621

Dr Andrew Foskett, Interim HoS
School of Sport & Exercise
ALBANY

Massey University Human Ethics Committee
Accredited by the Health Research Council
Research Ethics, Research and Enterprise
Massey University, Private Bag 11222, Palmerston North 4442, New Zealand T 06 951 6841; 06 951 6840
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Chapter 6

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Date: 03 August 2016

Dear Yanita McLeay

Re: Ethics Notification - **SOA 16/33 - Effect of taurine on recovery from eccentric exercise-induced muscle damage in males**

Thank you for the above application that was considered by the Massey University Human Ethics Committee: **Human Ethics Southern A Committee** at their meeting held on **Wednesday, 3 August, 2016**.
On behalf of the Committee I am pleased to advise you that the ethics of your application are approved.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

BJ Finch

Dr Brian Finch
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

Research Ethics Office, Research and Enterprise
Massey University, Private Bag 11 222, Palmerston North, 4442, New Zealand T 06 951 6841; 06 95106840
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Appendix B

Statements of Contribution

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Yanita D McLeay

Name/Title of Principal Supervisor: Prof. Stephen Stannard

Name of Published Research Output and full reference:

Dietary thiols in exercise: oxidative stress, exercise performance, and adaptation

In which Chapter is the Published Work: Review on thiols that form the base of all chapters

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
  and / or
- Describe the contribution that the candidate has made to the Published Work:
  05%

Candidate's Signature — 4.12.17 Date

Principal Supervisor's signature — 4th Dec 2017 Date

GRS Version 3– 16 September 2011

Chapter 6

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Yanita D McLeay

Name/Title of Principal Supervisor: Prof. Stephen Stannard

Name of Published Research Output and full reference:

The effect of taurine on the recovery from eccentric exercise-induced muscle damage in males

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
  and / or
- Describe the contribution that the candidate has made to the Published Work:
  85%

Candidate's Signature — 4.12.17 Date

Principal Supervisor's signature — 4th Dec 2017 Date

GRS Version 3– 16 September 2011

Appendix C

Publications

Review for all Chapters

McLeay et al. Journal of the International Society of Sports Nutrition (2017) 14:12
DOI 10.1186/s12970-017-0168-9

Journal of the International Society of Sports Nutrition

REVIEW Open Access

Dietary thiols in exercise: oxidative stress defence, exercise performance, and adaptation

Yanita McLeay[1*], Stephen Stannard[1], Stuart Houltham[1] and Carlene Starck[2]

Abstract

Endurance athletes are susceptible to cellular damage initiated by excessive levels of aerobic exercise-produced reactive oxygen species (ROS). Whilst ROS can contribute to the onset of fatigue, there is increasing evidence that they play a crucial role in exercise adaptations. The use of antioxidant supplements such as vitamin C and E in athletes is common; however, their ability to enhance performance and facilitate recovery is controversial, with many studies suggesting a blunting of training adaptations with supplementation. The up-regulation of endogenous antioxidant systems brought about by exercise training allows for greater tolerance to subsequent ROS, thus, athletes may benefit from increasing these systems through dietary thiol donors. Recent work has shown supplementation with a cysteine donor (N-acetylcysteine; NAC) improves antioxidant capacity by augmenting glutathione levels and reducing markers of oxidative stress, as well as ergogenic potential through association with delayed fatigue in numerous experimental models. However, the use of this, and other thiol donors may have adverse physiological effects. A recent discovery for the use of a thiol donor food source, keratin, to potentially enhance endogenous antioxidants may have important implications for endurance athletes hoping to enhance performance and recovery without blunting training adaptations.

Keywords: Endurance, Thiol, Antioxidant

Background

It is well-established that muscular work is associated with oxidative stress and that prolonged or high-intensity exercise results in oxidative damage to macromolecules in both blood and skeletal muscle [1–3]. This exercise-induced oxidative damage can impair physical performance via various mechanisms relating to compromised myocellular structure and function. Furthermore, chronic oxidative stress in athletes, often brought about by overtraining, has been linked to chronic fatigue [4], longer term performance decrements [5], muscle atrophy [6], and illness [7]. Exogenous and endogenous antioxidants reduce oxidative stress, thus it is not surprising that dietary antioxidants are a popular supplement for athletes in an attempt to enhance recovery, optimise performance, and reduce the oxidant load to maintain long-term health.

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The two groups of biologically active molecules that fall under the umbrella term 'free radicals' include reactive oxygen species (ROS) and reactive nitrogen species (RNS). During exercise, oxygen consumption can increase as much as 100-fold [8] and is associated with rapid increases in ROS production and accumulation. This constant production of free radicals by skeletal muscle requires the buffering capacity of an endogenous defense system, and a multitude of mechanisms have evolved to detect and respond to elevated oxidant production. The action of these systems determines the overall endogenous antioxidant capacity, and if this capacity is exceeded, oxidative stress ensues, potentially resulting in detrimental oxidation of cell membranes, functional proteins, and DNA [9–11].

Paradoxically, the redox activities of ROS and RNS play critical roles in cell signaling and exercise adaptation, a phenomenon summarized by the concept of hormesis, where low levels of stress promote adaptation to and thus protection from, subsequent stress [12]. Various exercise studies have observed improved endogenous defence in

Chapter 6

antioxidants

MDPI

Article

The Effect of Taurine on the Recovery from Eccentric Exercise-Induced Muscle Damage in Males

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Received: 19 September 2017; Accepted: 12 October 2017; Published: 17 October 2017

Abstract: Eccentric exercise is known to bring about microstructural damage to muscle, initiating an inflammatory cascade involving various reactive oxygen species. This, in turn, can significantly impair physical performance over subsequent days. Taurine, a powerful endogenous antioxidant, has previously been shown to have a beneficial effect on muscle damage markers and recovery when taken for a few days to several weeks prior to eccentric exercise. However, to date no studies have looked at the effects of supplementing over the days following eccentric exercise on performance recovery. Thus, this study aimed to determine whether supplementing with taurine over three days following eccentric exercise attenuated the rise in serum creatine kinase and improved performance recovery in males. In a blinded, randomized, crossover design, ten recreationally-fit male participants completed 60 eccentric contractions of the biceps brachii muscle at maximal effort. Following this, participants were supplemented with 0.1 g·kg^{-1} body weight·day^{-1} of either taurine or rice flour in capsules. Over the next three mornings participants underwent blood tests for the analysis of the muscle damage marker creatine kinase and carried out performance measures on the isokinetic dynamometer. They also continued to consume the capsules in the morning and evening. The entire protocol was repeated two weeks later on the alternate arm and supplement. Significant decreases were seen in all performance measures from pre- to 24-h post-eccentric exercise ($p < 0.001$) for both taurine and placebo, indicating the attainment of muscle damage. Significant treatment effects were observed only for peak eccentric torque ($p < 0.05$). No significant time × treatment effects were observed (all $p > 0.05$). Serum creatine kinase levels did not significantly differ over time for either treatments, nor between treatments ($p > 0.05$). These findings suggest that taurine supplementation taken twice daily for 72 h following eccentric exercise-induced muscle damage may help improve eccentric performance recovery of the biceps brachii.

Keywords: taurine; reactive oxygen species; eccentric exercise; performance recovery

1. Introduction

Eccentric actions, whereby muscle lengthens while under tension, can produce significant microstructural damage as a result of high force per fiber ratio. Not only can this directly reduce the ability of muscles to contract, it can also generate an inflammatory cascade, activating various ROS (reactive oxygen species) generating pathways including xanthine and nicotinamide adeneine dinucleotide phosphate (NADPH) oxidase, and phagocytic respiratory burst [1]. The resulting formation of ROS at high concentrations can surpass the ability of endogenous antioxidant defence, leading to oxidative stress. This, in turn, can elicit ROS-induced secondary tissue damage, including lipid peroxidation and protein oxidation, further reducing the ability of skeletal muscle to perform [2,3]. Dietary antioxidants have the ability to lower ROS within the human body, and a number of studies have shown attenuation of oxidative stress markers following supplementation [4–7]. Additionally,

Appendix D

POMS Questionnaire

CYSTEIC ACID vs. TAURINE SUPPLEMENTATION IN HEALTHY HUMANS - POMS

SUBJECT ID _____ TRIAL NUMBER _____

DATE _____ TIME _____ DAY _____

PROFILE OF MOOD STATES-SHORT FORM (POMS-40)

Refer to the definitions below. Consider how you are feeling right now, when CIRCLING the appropriate response beside each item. Please check to make sure you have responded to all the items.

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Nervous	0	1	2	3	4
Discouraged	0	1	2	3	4
Confused	0	1	2	3	4
Fatigued	0	1	2	3	4
Cheerful	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Peeved	0	1	2	3	4
Powerful	0	1	2	3	4
Resentful	0	1	2	3	4
Grouchy	0	1	2	3	4
Angry	0	1	2	3	4
Miserable	0	1	2	3	4
Annoyed	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Ashamed	0	1	2	3	4
Weary	0	1	2	3	4
Full of Pep	0	1	2	3	4
Sad	0	1	2	3	4
Energetic	0	1	2	3	4
Helpless	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Satisfied	0	1	2	3	4
Worn Out	0	1	2	3	4
Proud	0	1	2	3	4
Furious	0	1	2	3	4
Uneasy	0	1	2	3	4
Anxious	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Restless	0	1	2	3	4
Exhausted	0	1	2	3	4
Bitter	0	1	2	3	4
Competent	0	1	2	3	4
Active	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Bewildered	0	1	2	3	4
Forgetful	0	1	2	3	4
Bushed	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Uncertain about things	0	1	2	3	4

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Hopeless	0	1	2	3	4
Lively	0	1	2	3	4
On-edge	0	1	2	3	4
Worthless	0	1	2	3	4
Tense	0	1	2	3	4
Embarrassed	0	1	2	3	4

Appendix E

Average Dietary Intake

Chapter 4 – Chronic phase dietary intake over the 4-week treatment period (s)

Keratin								
Participant	Calories (kcal)	Carbs (g)	Fat (g)	Protein (g)	Cholest (mg)	Sodium (mg)	Sugars (g)	Fiber (g)
1	3475	266	187	172	696	3233	116	28
2	1814	210	65	71	223	1564	73	21
3	1563	105	45	100	179	1123	43	23
4	2616	370	87	102	110	2770	116	31
5	1596	197	52	80	127	1469	67	18
6	3083	330	116	138	449	2449	101	32
7	2517	318	75	110	304	2959	105	28
8	1649	202	58	73	157	3488	54	20
9	2741	356	76	104	121	2020	141	28
10	2042	241	61	71	110	2156	82	19
11	3000	369	96	132	91	4548	158	26
Casein								
1	3308	289	167	155	572	2973	120	29
2	1776	206	62	67	177	1845	61	19
3	1844	114	56	114	131	1359	46	21
4	1897	236	67	93	204	1873	72	22
5	1646	197	70	78	169	1587	65	18
6	3374	333	124	165	726	2683	127	33
7	2514	250	86	120	485	2291	100	27
8	1435	187	50	78	253	2461	41	16
9	2399	320	80	97	144	2291	135	26
10	2482	294	84	83	235	2510	91	18
11	2980	358	110	134	144	4067	113	20
Average Keratin	2372	270	83	105	233	2525	96	25
Average Casein	2332	253	87	108	295	2358	88	23

Note: Four participants did not complete input adequately, therefore *n* = 11 for dietary intake

Appendix F

Amino Acid Profiles of Protein

Amino Acid (mg/100g)	Sodium Caseinate (CAS)	Kerapro (KER)	NOW Pea Protein (PP)
Protein (%)	**92**	**87**	**73**
Alanine	2690	3640	2951
Arginine	3480	5510	6151
Aspartic acid	6410	6460	8551
Cysteic acid	0	7350	0
Cysteine	250	190	1085
Glutamic acid	19030	9970	12987
Glycine	1770	6380	2988
Histidine	2880	470	1788
Isoleucine	4950	4620	3315
Leucine	8360	6830	6087
Lysine	6910	1070	5448
Methionine	2340	410	639
Phenylalanine	4760	3830	4006
Proline	9380	8690	3157
Serine	4440	9410	3781
Threonine	3640	4210	2836
Tryptophan	1190	0	639
Tyrosine	510	1350	2712
Valine	6440	8230	3584

Amino acid profiles for sodium caseinate (CAS), 'kerapro' keratin (KER) and NOW pea protein (PP), adapted from Table 1 in Wolber et al. (2016).

Appendix G

Detailed Diagram of Thiol Metabolism