The role of dietary antioxidants in exercise-induced oxidative stress and athletic performance

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A thesis submitted to the Auckland University of Technology in fulfilment of the degree of Doctor of Philosophy

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made.

Chapters 2 to 6 of this thesis represent five separate papers that have either been published or have been submitted to peer-reviewed journals for consideration for publication. My contribution and the contribution by the various co-authors to each of these papers are outlined at the beginning of each chapter. All co-authors have approved the inclusion of the joint work in this doctoral thesis.

Andrea Braakhuis

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CANDIDATE CONTRIBUTIONS TO CO-AUTHORED PAPERS

Chapter publication reference	Author %
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Ethical approval

Ethical approval for this research was granted by the Auckland University of Technology Ethics Committee (AUTEC). The AUTEC references were 06/230 and 08/103, with approval granted on 2nd March, 2007 and 22nd August, 2008 respectively.

CHAPTER 1

INTRODUCTION AND RATIONALISATION (PREFACE)

Personal Background

When I commenced this research I was employed by the New Zealand Academy of Sport as a consultant sport dietitian and was often requested to give advice on antioxidant supplement protocols for elite athletes. However, I also considered myself an evidence-based practitioner and felt there was unremarkable evidence to support the recommendation of such supplements. My initial conclusion that antioxidant supplements had nothing to offer athletes was only tentative. Certainly some indications from the work of Alessio (Alessio, 1993; Alessio, Goldfarb, & Cao, 1997) demonstrated antioxidants were capable of decreasing oxidative stress experienced by athletes during a period of overloaded training. Books by reputed scientists also suggested supplementation regimes for elite athletes (Ivy & Portman, 2004). The Australian Institute of Sport (AIS, 2009) has a fact sheet on antioxidants (Vitamins A, C and E) recommending intakes for a period of 1-2 weeks at onset of illness or during periods of increased training loads, such as training camps. If recommended at all, the antioxidant supplementation protocols are typically conservative and rarely include the addition of food alongside the supplements. Layered on top of this is the on-going concern competitive athletes have regarding taking any dietary supplement and the risk of an inadvertent positive drug test as a result. The increased awareness of the possibility that a supplement may contain substances that result in a positive drug test has caused athletes to be rightfully fearful of supplements. This is all the more reason to ensure evidence-based practise with regards to supplement recommendations. Once satisfied of the efficacy, as a dietitian I can ensure sourcing supplements that have been batch tested and are free of substances that may result in a positive drug test.

To determine the efficacy of antioxidant supplements, it is imperative to determine current antioxidant practices of athletes and investigate whether supplementation is beneficial, harmful or neither to athletic performance. Given the complexity of antioxidants, both in measurement of dietary intake, physiological function and interpretation of biomarkers, the aim of this research was to develop practical recommendations I can make to athletes.

Theoretical Background

Exercise increases the production of reactive species, and if these exceed the antioxidant capacity of an individual, muscle and immune function can be impaired (Verhagen, Buijsse, Jansen, & Bueno-de-Mesquita, 2006). Reactive species are chemically reactive molecules produced as a by-product of normal metabolism, which increases during exercise. The body has antioxidant defence mechanisms to deal with reactive species when these are

overwhelmed, oxidative stress ensues. The term oxidative stress can thus be defined as the imbalance between the generation of reactive species and the activity of the antioxidant defences (White, et al., 2000). Excess oxidative stress leads to the damage of lipids, proteins, and nucleic acids, which in turn may contribute to muscle damage, impaired immune function, fatigue and poor recovery from exercise (Verhagen, et al., 2006). The antioxidant defence system includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic substances such as ascorbic acid (AA), uric acid (UA), which act in synergy (Aguila, et al., 2004; G. Davison, Gleeson, & Phillips, 2007; Goldfarb, Patrick, Bryer, & You, 2005). Similarly, dietary antioxidant supplements including vitamin C, vitamin E and complex nutrient mixtures such as fruit extracts, may assist in preventing oxidative damage by scavenging reactive species, but research to date suggests minimal effect on performance (Fischer, et al., 2004; Thompson, et al., 2003). Based on current research, dietary antioxidants do reduce oxidative stress, but do not appear to improve performance. Having said this, few studies of each kind of antioxidant have been designed with a performance outcome as the primary aim, with more research we may be able to conclude that antioxidants are of benefit or harm, with greater confidence.

The previously accepted paradigm of damaging oxidative stress mopped up by protective antioxidants is now regarded as too simplistic. Reactive species are a physiological necessity that may serve to up-regulate antioxidant systems and signal training adaptations (Gomez-Cabrera, et al., 2008). Therefore, whether or not antioxidant nutrients are beneficial to athletic performance is not clear. The advice to increase the consumption of fruits and vegetables due to their positive effect on health remains secure, but evidence is lacking for a benefit of the specific antioxidant constituents.

Key Question Investigated

While acknowledging the general concerns that many have about the antioxidant status of the general population, I have focused on the relevance of dietary antioxidants in athletes. The two broad themes of the thesis are: the effect of usual dietary intake on antioxidant status in athletes, and the impact of dietary antioxidants on athletic performance.

The overall aim of this thesis was threefold:

1) To determine the antioxidant intake of athletes
2) To investigate the impact dietary antioxidant intake has on antioxidant status
3) To assess the impact of dietary antioxidants on training and performance

Structure

This thesis comprises seven chapters (Table 1), beginning with this introduction and ending with an overall conclusion. The thesis comprises 7 chapters separated into in 2 thematic sections, each introduced with a literature review (Table 1) and followed with 1-2 experimental studies.

Table 1: Overview of doctoral thesis chapter flow

Chapter 1: Introduction and Rationalisation (Preface)
Thematic section: dietary intake and antioxidant status in athletes
Chapter 2: Literature Review: Dietary intake, metabolism and action of vitamin C and other dietary antioxidants in active individuals
Chapter 3: Development and validation of a food frequency questionnaire to assess short term antioxidant intake in athletes
Chapter 4: Effect of dietary antioxidants and exercise on antioxidant status in elite rowers
Thematic section: dietary antioxidants and athletic performance
Chapter 5: Literature Review: Impact of Dietary Antioxidants on Sport Performance
Chapter 6: Effects of dietary antioxidants on training and performance in female runners
Chapter 7: Conclusion
Appendices

Chapter 2 has summarized current knowledge of reactive species, oxidative damage and antioxidant defenses, with the aim to review the mechanisms of how dietary antioxidants and supplements affect health in active individuals.

One of the first aims of the research was to determine antioxidant intake in athletes, where the current protocol is to assess the intake of single antioxidant micronutrients such as vitamin C, E, A, selenium and retinol. However we wanted to develop a questionnaire that was a better indicator of total antioxidant intake, including the content of plant-based antioxidants. Chapter 3 of the thesis involved developing a food-frequency questionnaire to determine total antioxidant intake using a previously published antioxidant database. The validity and reproducibility of the food-frequency questionnaire-based estimate were assessed with endurance athletes against a food record and an antioxidant biomarker. The food-frequency questionnaire represents a valid estimate of total antioxidant intake that may be used by researchers to assess antioxidant intake from foods, without labour intensive food diaries. This questionnaire was later used in the study described in Chapter 6.

Chapter 4 was a cross-sectional study on competitive rowers, which investigated the correlation between antioxidant biomarkers and dietary intake of antioxidant status before and after exercise. The aim of the study was to determine current dietary and training practices, and investigate the relationship to antioxidant status. The blood markers of antioxidant status included ascorbic acid, uric acid, total antioxidant capacity and erythrocyte antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase). Interestingly, antioxidant status was associated more strongly with training and performance than diet.

The overarching aim of the second thematic section of the thesis was to investigate and discuss the impact dietary antioxidants have on athletic performance. Chapter 5 reviews the literature on dietary antioxidants and athletic performance. The conclusion of the review was vitamin C appears to impair athletic performance while N-acetylecysteine and polyphenols, including quercetin and beetroot juice, may improve it.

Chapter 6 describes a placebo-controlled cross-over design intervention to investigate the effect over three weeks of the daily ingestion of supplemental drinks on training efficacy and performance in female runners. The supplemental drinks included daily ingestion of a mega-dose of a single antioxidant (vitamin C) versus a blackcurrant-based drink. The implication of supplying the antioxidants in a food form versus straight supplemental form was of interest.

The appendices include additional written material on the chemistry of antioxidants, oxidative stress and training, additional data not presented in the chapters, copies of ethical approvals, sample consent and information forms.

CHAPTER 2

DIETARY INTAKE, METABOLISM AND ACTION OF VITAMIN C AND OTHER ANTIOXIDANTS IN ACTIVE INDIVIDUALS

This chapter comprises the following paper to be submitted to Nutrition Reviews.

Braakhuis AJ, Rush, EC. Dietary intake, metabolism and action of vitamin C and other antioxidants in active individuals, October 2010.

(Author contribution percentages: AB 90%: ER: 10%).

ABSTRACT

Highly active individuals have a higher mortality than their sedentary counterparts; possibly a response to the chronic production of reactive species produced during prolonged moderate to vigorous activity. Habitual consumption of antioxidant-rich foods is associated with reduced risk of disease; however the contribution of dietary antioxidants to the health and performance of active individuals is still debated. Health benefits of fruit and vegetables have been attributed to their antioxidant content. This review looks at balance between reactive species and the maintenance of redox homeostasis by antioxidants. For active individuals' dietary sources of antioxidants, their absorption and metabolism are discussed with recognition of the limitations in the methodology of determining oxidative stress and antioxidant status. Although evidence is not convincing, there is some indication that active individuals require higher amounts of antioxidants than sedentary people, and therefore a higher dietary intake. Recommendations cannot be made at this stage, however whole foods provide a range of antioxidant and matrix combinations not available in a single antioxidant nutrient supplement.

Reactive Species, Exercise and Oxidative Stress

Physical activity and oxidative stress

Prolonged moderate to vigorous physical activity is known to have many beneficial effects; however it also increases the production of reactive species. The impact of the reactive species on the health and performance of athletes is still very much debated, and for many years the general consensus was that reactive species contribute to muscle damage, immune dysfunction and fatigue. This indoctrinated view may under rate the important role reactive species play in athletic adaptation to training.

Research demonstrates a link between disease risk and regular participation in large volumes of intense exercise for men (Paffenbarger, Hyde, Wing & Hsieh, 1986). During 12 to 16 years of follow-up in the Harvard Alumini Health study death rates declined as energy expended on activity increased from less than 500 to 3500 kcal per week, beyond which rates increased

slightly. Mortality was one quarter to one third lower among alumni expending 2000 or more kcal during exercise per week than among less active men (Paffenbarger, et al., 1986). Ten years later, in the same cohort, there was an increase in the age-standardised mortality rate in participants reporting physical activity greater than 14,700kJ per week (Lee, et al., 1995). The increase in mortality occurred when energy expenditure in vigorous activities exceeded 12,600 kJ per week. A plausible link between the increase in mortality and high-volume physical activity implicates oxidative stress (Knez, Jenkins, & Coombes, 2007), malignant cancer (Polednak, 1976) atherosclerosis and myocardial damage (Corrado, et al., 2006). Direct evidence that exercise increases oxidative stress was first demonstrated by Bailey (2003) who detected free radicals released from exercising muscle. Exercise increased the serum concentration of free radicals and lipid hydroperoxides in 28 healthy males (Donato, Uberoi, Bailey, Wray and Richardson, 2010) It is possible that supplementation and fortification of foods with antioxidants could prove to be an important mitigating factor in reducing oxidative stress which may contribute to poor recovery, immune dysfunction and fatigue, just as it may contribute to cardiovascular disease and increased mortality long-term. Prior to a discussion of the interaction between dietary intake and antioxidant status within active individuals, this review will briefly consider the antioxidant defence system. Whilst not intended as a complete review some understanding is necessary to appreciate the role of diet and exercise on antioxidant status of the body.

Antioxidant defences against reactive oxygen species

Reactive species include oxygen and nitrogen derived free radicals, but this review will focus on reactive oxygen species (ROS). ROS are defined as molecules that contain oxygen with an unpaired electron. The presence of an unpaired electron makes the atom or molecule more reactive and able to donate an electron to other molecules; therefore behaving as oxidants (Jackson, 2000). The source of ROS is an incomplete reduction of O_2 to water in a variety of physiologic as well as potentially pathologic processes (Finaud, Lac, & Filaire, 2006). This process occurs in the mitochondrial electron transport chain and is catalysed by cytochrome oxidase. However, because there is a constant “leak” of electrons and partially reduced, toxic, intermediates, such as ROS, are produced (Jackson, 2000). Although ROS generated in our body are part of normal cell metabolism and play important roles in both immune-mediated defence and cell-signalling, at high concentrations ROS can modify and damage DNA, lipids, and proteins (Jackson, 2000; White, et al., 2000).

Antioxidants provide electrons that stabilize radical molecules or regulate reactions that convert ROS into less destructive forms (Alessio, et al., 1997; Finaud, et al., 2006) and maintain redox homeostasis. In healthy individuals antioxidants are derived exogenously from the diet and endogenously via antioxidants and enzymes, produced to defend tissue against ROS attack (Table 2). Exogenous dietary antioxidants include vitamins E and C and diverse phytochemicals. Vitamin E functions as a chain-breaking antioxidant preventing the propagation of free radical reactions in all cell membranes in the human body. Vitamin C is also part of the

antioxidant protection due to its ability to provide electrons and neutralise free radicals.. Other non-enzymatic antioxidants include carotenoids, flavonoids, other polyphenols and α-lipoic acid (Devasagayam, et al., 2004).

Endogenous protective reactions against the harmful effects of free radicals are dependent on the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and the non-enzymatic antioxidants uric acid, albumin and ferritin. Together the quantity and activity of the exogenous and endogenous antioxidants determine antioxidant capacity of an individual.

Table 2: Location and derivation of the various reactive oxygen species

Location	Antioxidant	Derivation
Mitochondria	SOD	Endogenous
	GPx	Endogenous
Cell membrane	Vitamin E	Diet
Cell cytosol	Vitamin C	Diet
	CAT	Endogenous
	GPx	Endogenous
	SOD	Endogenous
Blood/extracellular fluid	Uric acid	Diet & endogenous
	Vitamin C	Diet
	Vitamin E	Diet

Abbreviations: SOD: Superoxide dismutase. GPx: Glutathione peroxidase. CAT: Catalase.

Antioxidants are especially important during exercise because ROS production increases with intensity and duration modulated by the training status of the population in question (Bailey, Davies, Young et. Al, 2003; Donato, Uberoi, Bailey, Wray, Richardson, 2010). Thus, in the case of athletes consuming low antioxidant diets and completing a heavy training load, the accumulation of ROS and therefore increased oxidative stress may contribute to poor recovery, fatigue and muscle damage (Powers & Jackson, 2008). Redox homeostasis is a complex process but an understanding of the reactions is essential to better understand both how to measure biomarkers of oxidative stress and evaluate improvement or deterioration in athletic performance with training programs and understand better implications for long term health. No one measure is explanatory, therefore current understanding is based on a number of measures and their interdependence. Further discussion on the antioxidant defences of the body was thought to be outside the scope of this review and can be found in Appendix 1.

Methods to Assess oxidative Stress and Antioxidant activity in Biological Systems

Analysis of the dynamics between antioxidant status and diet is a complex issue, firstly because of the number of compounds involved but also because of the variety of ways in which they act and the different food matrices they are present in (Frankel & Meyer, 2000). Generalisation about the antioxidant capacity of a diet is not possible because there are many times more antioxidant components and interactions with their matrix than there are nutrients currently

quantified in food composition tables. Similarly, the total antioxidant capacity of plasma is not a simple sum of the activity of single antioxidants. Each antioxidant works in cooperation and synergy to provide greater protection against oxidative stress. There is no “gold standard” method capable of measuring the total antioxidant activity of plasma (Koracevic, Koracevic, Djordjevic, Andrejevic, & Cosic, 2001); therefore variable and comparable methods must be used to understand better antioxidant activity in plasma.

There is much debate in the literature as to which technique suits what outcome or purpose and the recommendation is that a combination of at least two assessment measures should be employed when investigating antioxidant concentrations and capacity (Gutteridge, 1986).
Specific antioxidants that can be measured directly include vitamin C, uric acid, glutathione, or alternatively total antioxidant capacity of biological samples (Jiankang, Yeo, Overvik-Douki, et. al., 2000). Total antioxidant capacity assays determine the degree to which a biological sample copes with a fixed, *in vitro*, addition of ROS. Activity of enzymes responsible for the detoxification of ROS can also be measured. Specific antioxidant enzyme assays include superoxide dismutase, glutathione peroxidise and catalase (Powers & Jackson, 2008). Enzyme activity can be measured in samples of muscle, plasma, erythrocytes and platelets. Selected measures are discussed in more detail in the following paragraphs.

Biomarkers of Oxidative Stress

Given that ROS are transitory, more stable biomarkers of the effects of ROS activity i.e. oxidative stress, are measured instead. A good biomarker will generally; be chemically unique, alter during times of oxidative stress and not be affected by cellular processes that are distinct from oxidative stress. End point alterations to proteins (e.g. protein carbonyl) or lipids (e.g malondialdehyde), are biomarkers of oxidative stress.

The protein carbonyl assay looks at the oxidative modification of proteins that can occur in a variety of physiologic processes, such as athletic training (Morabito, Tomaino, Cristani, et. al, 2005). Carbonyl groups may be introduced into proteins by reactions with ROS, or proteins may be modified in association with oxidation of other molecules. However, the incorporation of carbonyl groups into proteins is not specific for oxidative modification. For example, glycation seen in hyperglycaemia, may result in protein carbonylation. However, if interpreted with caution, the assay of carbonyl groups in protein provides a convenient technique for detecting and quantifying oxidative stress.

The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), one of the secondary products derived from peroxidation of polyunsaturated fatty acids, is the method most commonly used to measure lipid peroxidation. This TBA assay method is based on the calorimetric or fluorometric assessment of the reaction of TBA with MDA. However, TBA reacts not only with MDA but with carbohydrates, sialic acid and some prostaglandins reducing the specificity of the TBA reaction in both tissue and plasma (Pilz, Meineke, & Gleiter, 2000).

Recently, more sensitive and specific methods have been developed and adapted for biological materials. These allow direct measurement of MDA by high performance liquid chromatography (HPLC) (Pilz, Meineke, & Gleiter, 2000).

Measurement of specific antioxidants

A decrease in the plasma concentration of specific antioxidants is frequently used as a marker of oxidative stress (Adams & Best, 2002; Bryant, Ryder, Martino, Kim & Craig, 2003). However, changes in plasma do not necessarily reflect whole body stores or specific tissue concentrations and should be interpreted with caution. Factors such as changes in cellular regulation, storage, diet or shifts of antioxidants between extra-cellular to intracellular locations will modulate plasma concentration. For this reason, exercise may acutely increase plasma antioxidant concentration whilst chronic exercise may decrease antioxidant capacity (Senturk, Gunduz, Kuru et. al. 2005). One approach taken to get around this is the more functional measurement of the redox ratio (reduced/oxidized) of specific non-enzymatic antioxidants such as the ascorbate/ dehydroascorbate and glutathione/glutathione disulfide ratios in plasma or other biological fluid at different stages of exercise (Sen, Atalay & Hanninen, 1994) . Ascorbic acid, glutathione and total antioxidant capacity as examples of measures of non-enzymatic antioxidant status are discussed below. The physiology of vitamin C in relation to athletes is discussed in a later section.

Ascorbic acid

Ascorbic acid has been recognised as the most effective antioxidant in protecting against oxidative stress in plasma (Frei, England, & Ames, 1989). Ascorbic acid concentration in the plasma is tightly controlled by mediated tissue transport determined by dietary intake, absorption and excretion. The concentration of ascorbic acid in human plasma ranges between 10 and 100 μM and has been shown to increase slightly following intensive exercise (Davison & Gleeson, 2007; Davison, et al., 2007; Wannamethee, Lowe, Rumley, Bruckdorfer, & Whincup, 2006). Furthermore the increase in plasma ascorbic acid concentration following exercise has been shown to be greater in those supplemented with dietary vitamin C, suggesting increased storage and therefore a greater capacity for dealing with oxidative stress (Thompson, et al., 2003). The sequence of chemical reactions involved is provided below.

When ascorbate, an effective chain breaking antioxidant, encounters free radicals, semi-hydroascorbate, a stable radical, is produced.

2 semi-hydroascorbate $\rightarrow$ ascorbate + dehydroascorbate

The enzyme dehydroascorbate reductase requires glutathione (GSH) to further reduce dehydroascorbate to ascorbate. Therefore the ascorbate/ dehydroascorbate redox ratio has been used as another marker of oxidative stress.

White blood cells, such as lymphocytes and neutrophils, contain 1-4 mM concentration of ascorbate and saturate at a daily vitamin C intake of between 100- 200 mg. Lymphocytes are reported to attain saturation when plasma concentrations are > 50 μM. When plasma ascorbate approaches maximal saturation, additional ascorbate is lost in urine (Tur-Mari, Sureda, & Pons, 2006). For this reason white blood cell ascorbic acid concentration may represent a better marker of true ascorbate status. Jacobs and colleagues (Jacob, Skala, & Omaye, 1987) studied eleven subjects in the controlled condition of a metabolic ward. After two weeks of receiving 65 mg vitamin C each day, ascorbate stores were depleted by allowing only 5 mg of vitamin C a day for 4 weeks. Plasma ascorbic acid concentrations fell to 19% of baseline whereas white blood cell concentration fell to 55% of baseline. When ascorbic acid were replete with a dose of 605 mg a day for 3 weeks, plasma concentration increased more than fourfold above baseline while white blood cell concentrations increased to only 50% of baseline. The authors concluded that white blood cell ascorbic acid concentrations were the most reliable marker of vitamin C intake and that white blood cell ascorbic acid concentrations reflect long term status, rather than the rapid changes seen in plasma.

Glutathione

Glutathione, a peptide, is important in the circumvention of cellular stress as it serves multiple roles as an antioxidant defence. First, glutathione interacts with a variety of radicals including hydroxyl and carbon radicals, by donating a hydrogen atom (Yu, 1994). Secondly, glutathione removes hydrogen and organic peroxides (lipid peroxides, for example) producing water and alcohol. As a major by-product of enzymatic and non-enzymatic antioxidant reactions, glutathione is transformed to its oxidised form, glutathione disulfide. Intracellular glutathione disulfide is cytotoxic and the capacity to do revert to glutathione is finite with excess glutathione disulfide transported to the extracellular compartment. Therefore, oxidative stress in tissues may be reflected as an elevated plasma glutathione disulfide concentration (Atalay, Larson, Khanna, Hanninen, & Sen, 1996).

Glutathione is primarily synthesized in the liver and transported to tissues via the circulation. As a peptide it is digested in the small intestine, suggesting that cellular levels of glutathione are not dependent on dietary sources. Although, dietary selenium is a cofactor for the enzyme glutathione peroxidise (Sen, Atalay, & Hanninenn, 1994). This is an example of how other dietary factors may modulate antioxidant defences.

Measurement of total antioxidant capacity

Several methods have been developed to assess the total antioxidant capacity of human plasma because of the difficulty in measuring each antioxidant component separately and the interaction among different components in the serum or plasma (Cao & Prior, 1998). Oxygen radical absorbing capacity (ORAC) assay and Trolox equivalent antioxidant capacity (TEAC) assays measure, amongst other things, serum protein antioxidant such as albumin (Guohua

Cao & Prior, 1998). Ferric reducing ability of plasma (FRAP) assay measures the combined antioxidative effect of the non-enzymatic defences in biological fluids such as ascorbic acid, α tocoferol, uric acid, bilirubin and albumin (Benzie & Strain, 1996; MacDonald-Wicks, Wood, & Garg, 2006), thiobarbituric acid reactive substances assay (TBARS) measures lipid peroxidation (Koracevic, et al., 2001). The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS). This method has produced useful information regarding the antioxidant activity of specific phytochemicals as many phenolic compounds which have low redox potentials and thus react with ABTS. Limitations are that the TEAC assay may not give the same result for a slow reaction and it may take a long time to reach an endpoint (Prior, Wu, & Schaich, 2005). Van den Berg, et. al., (1999) concluded that "quantitative evaluation of antioxidant capacity using the TEAC can be troublesome or even impossible, but it can be used to provide a ranking order of antioxidants".

An alternative option, titled the FRAP assay, is simple and inexpensive but does not measure the SH-group-containing antioxidants such as cysteine, glutathione, peptides or proteins containing cysteine and thiol compounds (Cao & Prior, 1998). The relevance of the FRAP assay to biological systems has been questioned, as the reaction conditions use a ferric ion (ferric 2,4,6-tripyridyl-*s*-triazine) and are conducted at an acidic pH of 3.6, which may not reflect the conditions in vivo (Prior, et al., 2005). However the ease of conducting the FRAP assay has led many researchers to include it alongside another assay as a convenient way of measuring total antioxidant capacity. According to Cao & Prior, (1998) the ability of FRAP to detect ascorbic acid and uric acid (of which contributes 10.1 and 61.7% respectively, to total FRAP antioxidant score) makes it an ideal candidate for research investigating important non-enzymatic antioxidants.

Measures of Enzymatic Antioxidant Activity

Investigating the concentration of non-enzymatic antioxidants is important, as is the activity of the enzymatic antioxidants. As discussed earlier, enzymes act as a first line of defence against ROS and investigating the activity of these enzymes provides valuable information (Dekkers, Van Doornen, Kemper, 1996). Together the non- and enzymatic antioxidants give a balanced view of antioxidant status. The three major enzyme systems, superoxide dismutase, glutathione peroxidase and catalase, are considered next.

Superoxide dismutase

Numerous techniques to determine SOD activity in biological samples have been developed. However, the direct determination of SOD is difficult because both the enzyme and ROS are unstable products. Therefore a common approach to assay SOD activity is to employ a system for the production of superoxide in the presence of a biological sample containing SOD. The

reduction of an optically sensitive compound, such as nitroblue tetrazolium (Kakkar, Das, & Viswanathan, 1984).

Glutathione Peroxidase

Glutathione peroxidase (GPx) is commonly assayed by using GSH and H_2O_2 as substrates, with a coupled reaction involving both GPx and glutathione reductase. Glutathione reductase uses NADPH to regenerate glutathione from glutathione disulfide. The rate of change in NADPH oxidation is monitored spectrophotometrically at a wavelength of 340nm.

Catalase

Catalase (CAT) activity is commonly assayed via spectrophotometric techniques using H_20_2 as a substrate (Li Li & Hollander, 2000). Results presented in the literature show that the range in CAT activity varies widely between studies. This wide variance in CAT activity probably results from the fact that the turnover rate varies with both the amount of active CAT protein present and the concentration of H_20_2 in the reaction medium. Therefore, CAT activity in the tissues can only be compared between studies in which the conditions of the assay are carefully defined.

Is there an ideal method?

Although there appears to be a plethora of biomarkers to quantify oxidative insult, the measurement in biological systems has proven to be a difficult task. Some of these difficulties in quantifying oxidative stress include the short life time of ROS, and the lack of specific and sensitive assays for bio-molecules. For this reason, careful treatment of samples prior to and during analysis is paramount to achieving accurate and precise repeated measurements. In addition to treating samples carefully it should be noted that although a general pattern for oxidative stress exists, this pattern will be confounded by dietary and training history, exercise regime and athletic code, fluid homeostasis and environmental stress such as heat. Furthermore because there are numerous cellular sources of ROS and many different bio-molecules with which the ROS may react, one method cannot be relied on as an assessment of oxidative stress and studies should use a combination of assays.

Dietary Intake, Absorption and Metabolism of Vitamin C in active individuals

Absorption of Vitamin C

The bioavailability of water soluble vitamin C from both food and supplements are similar (S. Padayatty & Levine, 2001); however, the amount of vitamin C from foods varies as it is easily removed or degraded with cooking, processing, or the addition of preservatives (e.g., sodium bicarbonate). Approximately 70–90% of the usual dietary intake of vitamin C (30–180 mg/day) is absorbed, although absorption falls to less than 50% with doses above 1 $g.d^{-1}$ (Padayatty & Levine, 2001).

Vitamin C is absorbed along the entire length of the intestinal tract and to a greater degree in the condition of hypovitaminosis, suggesting that absorption rate is carrier-dependent (Karasov, Darken, & Bottum, 1991). However, the mechanisms for absorption ascorbic acid and dehydroascorbic acid (DHAA, oxidized vitamin C) differ, whilst DHAA is absorbed via facilitated diffusion, ascorbate is absorbed via sodium dependant glucose co-transporters (SGLT1) (Padayatty & Levine, 2001; Wilson, 2005). Once inside the enterocyte DHAA is reduced to ascorbate, ascorbate leaves the enterocyte basolateral membrane via a protein transporter (see Figure 1).

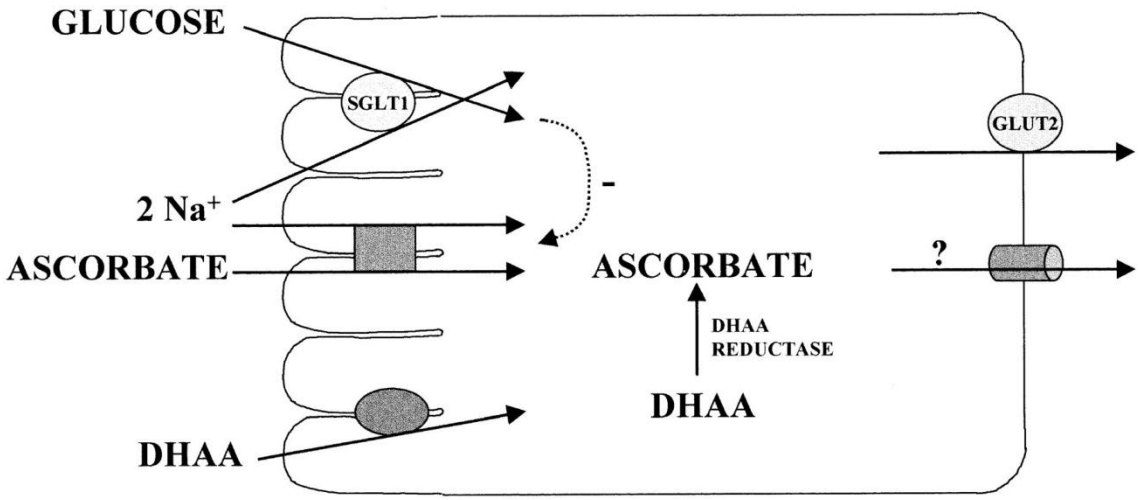

Figure 1: A model of vitamin C transport in enterocytes

The apical membrane contains a Na+-ascorbate cotransporter involving a minimum of two 2 Na+ ions (square) and a facilitated diffusion pathway for L-dehydroascorbic acid (DHAA) (oval). The Na+-glucose transporter, SGLT1, allows rapid entrance of glucose, which is then released into blood by crossing the basolateral membrane via GLUT2. An increase of glucose concentration in the enterocyte inhibits the ascorbate carrier in the apical membrane. Inside the enterocyte, DHAA is reduced to ascorbate, which may leave the cell at the basal membrane by an unknown mechanism (Malo & Wilson, 2000). Reproduced with permission from the AMERICAN SOCIETY FOR NUTRITION in the format Journal via Copyright Clearance Center (see Appendix 2).

Transport and Storage

While most of the ascorbic acid travels free in the blood stream, some is associated with albumin and less than 5% in the DHAA form. Intracellular concentration of ascorbic acid is higher than plasma (by about 3- to 10-fold) indicating an energy driven transport. When ascorbate acts as an antioxidant it becomes oxidized to DHAA, which if left unchecked will irreversibly be hydrolysed. To prevent this unfavourable event, DHAA is efficiently reduced back to ascorbic acid intracellularly, by erythrocytes, hepatocytes and other cells. Therefore, recycling plays a key role in preserving the cells antioxidant function (Lykkesfeldt, 2002; Lykkesfeldt, Viscovichand, & Poulsen, 2003). In healthy humans, the plasma concentration of DHAA is close to nil, indicating the high efficiency of the recycling process. However, under certain stress situations the ratio of ascorbic acid to DHAA may reduce as more of the DHAA

fails to be quickly reverted back to ascorbic acid. Certainly this situation has been implicated in smokers (Lykkesfeldt, et al., 2003), but may also be a factor associated with stressful training sessions in athletes.

Due to homeostatic regulation, the biological half-life of ascorbate varies widely from 8–40 days and is inversely related to body stores. Total body stores are approximately 1.5 g of ascorbic acid, with a daily turnover of 30–45 mg. Scurvy symptoms are associated with total body stores of < 300 mg and 3–5 months of deficient vitamin C intake. (Gladys Block, et al., 1999) High levels of ascorbate are maintained in the pituitary and adrenal glands, leukocytes, eye tissues and the brain. (Jacob, et al., 1987).

Leucocytes can store vitamin C in higher concentrations than in plasma, as do the adrenal glands. Recent evidence shows that humans are capable of releasing vitamin C from the adrenal gland in response to stress (Padayatty, et al., 2007). Adrenocorticotrophic hormone (ACTH) secreted from the pituitary gland stimulates vitamin C loss from the adrenal cortex, along with cortisol secretion. The adrenal cortex is rich in vitamin C, with concentrations as high as 10 mmol/L (Padayatty, et al., 2007). The function of released vitamin C in response to stress is unknown, but may be protective against oxidative stress, or assist with the production of cortisol (Padayatty, et al., 2007). This needs to be explored further.

Dietary Intake of Athletes

Many studies have investigated the vitamin C intake of athletes, male and female, in association with various sports (Nieman, Butler, Pollett, Dietrich, & Lutz, 1989; Benson, Geiger, Eiserman, & Wardlaw, 1989; Braakhuis, Meredith, Cox, Hopkins, & Burke, 2003; Cupisti, D'Alessandro, Castrogiovanni, Barale, & Morelli, 2002; DeBolt, et al., 1988; Farajian, Kavouras, Yannakoulia, & Sidossis, 2004; Guilland, et al., 1989; Hickson, et al., 1987; Hinton, Sanford, Davisdson, Yakushko, & Beck, 2004; Papadopoulou, Papadopoulou, & Gallos, 2002; Peters, 1993). These studies are summarised in Table 3. The mean intake of vitamin C among athletes was above recommended daily intakes (RDI). The mean vitamin C intake for males ranged from 94 to 600 mg per day, while female athletes had 55 to 847 mg per day (RDI=45 mg/day) with higher intakes coming from supplements. Therefore, it appears in general that athletes consume adequate vitamin C to cover their requirements under most circumstances.

However, while the mean intakes for athletes are generally adequate, several studies report that a small proportion of athletes consumed suboptimal amounts of vitamin C. In male athletes, Hickson et. al. (1987) reported 10-20% of American football players consumed diets below the RDI for vitamin C. Guilland et. al. (1989) reported 25% of young athletes consumed low intakes, DeBolt et. al. (1988) reported that in 267 Navy Seals consuming an average intake of 353 mg of vitamin C, still 10% of the subjects reported inadequate consumption. Female athletes report a similar pattern (Keith, 2006).

According to the New Zealand National Nutrition Survey 1997 (Ministry of Health, 2003) the usual daily median intake of vitamin C is 102 mg (males 111 mg, females 95 mg). Males and females aged 15 to 18 years reported the highest intakes. The overall estimated prevalence of inadequate intakes in the total population of New Zealand was less than one percent. The major dietary sources of vitamin C were fruits and vegetables with a very minor contribution from animal organs and milk. Fruit and vegetables may contribute vitamin C, other antioxidants and phyto-nutrients that confer health benefits.

In a study completed on 3258 healthy males, plasma vitamin C, fruit intake, and dietary vitamin C intake were significantly and inversely associated with mean concentrations of C-reactive protein, an acute phase reactant, and tissue plasminogen activator (t-PA) antigen, a marker of endothelial dysfunction, even after adjustment for confounders. (Wannamethee, et al., 2006). While vitamin C is a good marker of fruit intake, many fruits and vegetables contain very little vitamin C but are an important source of antioxidants (Szeto, Tomlinson, & Benzie, 2002).

In a randomized control trial, an increase in consumption of vegetables and fruits from a regular intake of three servings per day to seven servings per day led to a substantial increase in plasma concentration of vitamin C: 63 mg (control group) compared with 275 mg (intervention group) (Zino, Skeaff, & Mann, 1999). This suggests that the current recommended intakes of vitamin C (45 mg in Australia and New Zealand and 75 mg in the United States) may need to be higher, if we assume that higher, food-derived plasma concentrations of ascorbic acid confer health benefits, especially to athletes. An American study by Levine *et. al.* (Levine, Rumsey, Daruwala, Park, & Wang, 1999) reported the adequate intake of vitamin C estimated to be either 200 $mg.d^{-1}$ from 5 servings of fruits and vegetables or 100 $mg.d^{-1}$ of vitamin C to prevent deficiency with a margin of safety. The study also stated that the tolerable upper intake level, which is the highest daily level of nutrient intake that does not pose risk or adverse health effects to almost all individuals in the population should be less than one gram of vitamin C daily.

Table 3: Details of studies investigating vitamin C consumption in athletes (Nieman, Butler, Pollett, CDietrich, & Lutz, 1989; Benson, Geiger, Eiserman, & Wardlaw, 1989; Braakhuis, Meredith, Cox, Hopkins, & Burke, 2003; Cupisti, D'Alessandro, Castrogiovanni, Barale, & Morelli, 2002; DeBolt, et al., 1988; Farajian, Kavouras, Yannakoulia, & Sidossis, 2004; Guilland, et al., 1989; Hickson, et al., 1987; Hinton, Sanford, Davisdson, Yakushko, & Beck, 2004; Papadopoulou, Papadopoulou, & Gallos, 2002; Peters, 1993)

Athlete Group	Sample size	Vitamin C intake ($mg.d^{-1}$) Mean ±SD	Days of food diary record	Reference
Males				
Marathon runners	291	147	3	Nieman (1989)
American football	134	180	1	Hickson (1987)
Various athletes	55	95		Guilland (1989)
Ultra-marathoners	41	494	1	Peters (1993)
Navy SEALS	267	353	1	DeBolt (1988)
Swimmers	31	178 ± 175	1	Farajian (2004)
Various	110	182 ± 94	Q	Hinton (2004)
Females				

Marathon runners	56	115	3	Nieman (1989)
Ballerinas	92	148	Q	Benson (1989)
Volleyball	65	93	3	Papadopoulou (2002)
Swimmers	27	132 ± 121	1	Farajian (2004)
Various	142	171 ± 84	Q	Hinton (2004)
Both genders				
Various elite	56	210 ± 274	7	Braakhuis (2003)
Various elite	60	111 ± 74	3	Cupisti (2002)

Abbreviations: Q: Questionnaire

Thus, while group means for vitamin C intake appear to be acceptable, around 10% of an athletic group may be consuming levels of the vitamin below that recommended for the general population (Keith, 2006). Previous studies to date have reported normal mean vitamin C concentrations in the plasma of athletes and physically active individuals (Fishbaine & Butterfield, 1984; Gleeson, Robertson, & Maughan, 1987 ; DiCheng, et al., 1989) however at least one study (Telford, Catchpole, Deakin, McLeay, & Plank, 1992) report 12% of its subjects had low plasma vitamin C concentrations, while another at the low end of the normal range (Duthrie, Robertson, Maughan, & Morrice, 1990). Interestingly, both studies reporting lower than normal blood ascorbic acid concentrations were runners, suggesting either a physiological burden associated with running on ascorbic acid status within the body, or that runners typically eat a diet poor in vitamin C. It is likely that different sports and activities display different stress loads on the ascorbic acid levels in the blood and warrants further investigation. For these athletes to ensure optimal antioxidant status, immune function and physical performance increased intake of vitamin C which could include an increase in fruit and vegetable intake is recommended.

In order to minimize the variability of nutrient intake by athletes, or the variability in coding food diaries, completing a 7-day food diary has been shown to be 2-3 times more reliable than a 1-day food record (Braakhuis, et al., 2003). Also, nutrients such as vitamin C, vitamin A and cholesterol require a larger sampling period than energy or carbohydrate as their intake is around 3 times as variable. This would suggest that a 1-day food diary is an inadequate time period to accurately assess true vitamin C or antioxidant consumption. Reviewing the studies in table 3, most have not adopted the practice of using food diaries for more than 3-days. However, only studies with subject numbers greater than 30 were included in this review which may have biased the studies included to those with shorter food diary recall periods (Braakhuis, et al., 2003).

Dietary Vitamin C Requirements for Active Individuals

Independent of its function as an antioxidant vitamin C has a number of known and very important functions in relation to exercise. The vitamin has long been known to be necessary for normal collagen synthesis and the formation of the vitamin like compound carnitine, necessary for the transport of long-chain fatty acids into the mitochondria. The neurotransmitters,

norepinephrine and epinepherine also require vitamin C for their synthesis. Ascorbic acid assists in the transport of non-heam iron and the reduction of folic acid intermediates. The vitamin exerts antioxidant functions in plasma and probably interfaces at the lipid membrane level with vitamin E to regenerate vitamin E from the vitamin E radical (Peake, 2003; Keith, 2006; Janse de Jonge, 2003).

Through these various functions, vitamin C can interface with exercise at several levels. For example, poor development of connective tissue could result in increased numbers of ligaments and tendon injuries and poor healing of these injuries. Inadequate production of carnitine could decrease an athlete's ability to utilise fatty acids as an energy source. This would force the use of stored glycogen, exhausting stores earlier in exercise causing fatigue and decreased performance. With decreased production of norepinephrine and epinephrine, an athlete might not be able to adequately stimulate the neural and metabolic systems necessary for optimal performance. Poor iron and folate metabolism would result in anaemia, impairing the transport of oxygen to tissues. Lack of oxygen transport within the body is highly likely to hinder performance or recovery from a range of physical activities.

At rest, plasma concentrations range from 23-84 µmol/L, with numbers below 23 µmol/L considered inadequate (Peake, 2003). However, athletes, excluding runners, typically have higher resting values in the range of 56- 83 µmol/L (Keith, 2006). However, care should be taken when comparing ascorbic acid concentrations between a sedentary and athletic population as recent physical activity can elevate levels for up to 24 hours (Fishbaine & Butterfield, 1984; Duthrie, et al., 1990). Another method of examining vitamin C requirements for athletes is to study the relationship between white blood cell ascorbic acid concentrations and exercise. Supplementation with vitamin C for a short period of time may increase lymphocyte or neutrophil concentration of the vitamin, which is only seen following a physiological stress such as exercise. For example, a study by Sureda and colleagues (2006) completed on deep sea divers found that following 1 gram of vitamin C daily for 7 days caused no significant difference in resting plasma levels of ascorbic acid, but 1 and 4 hours following exercise the supplemented group displayed elevated levels within the lymphocytes. Antioxidant enzymes were elevated in the placebo group but not the supplemented group and the authors concluded that the supplemental vitamin C allowed the divers to store additional vitamin C in the lymphocytes detected once the body was under stress. The higher lymphocyte vitamin C concentration may have assisted the fight against post exercise free radical damage thereby limiting the need for the antioxidant enzymes. Additional research on Olympic cyclists report leukocyte ascorbic acid concentrations over a four year period and found that the lymphocyte and neutrophil concentrations declined over the length of the study. The authors further report that the lymphocyte and neutrophil ascorbic acid levels declined from a pre-Olympic to post-Olympic games event period (Ferrandez, Mayner, & De la Fuente, 1996). However, it is also possible that lymphocytes change their morphology during exercise to dilute the vitamin C within the cell rather than lose content. More evidence is needed to interpret these results.

The requirement for vitamin C may be higher for athletes than the sedentary population, but the evidence for this is far from clear, and certainly not strong enough to alter current recommended amounts. However, those athletes with inadequate diets and runners may benefit from increased intakes, best obtained by increasing the fruit and vegetable intake.

Antioxidant Intake, Food versus Supplements

The focus on health benefits of an increased intake of antioxidants has led to a demand for information about which components of food confer particular antioxidant benefits and the subsequent development of supplements that contain isolated antioxidant nutrients. Researchers looking at epidemiology or nutrition data require detailed information on the antioxidant composition and efficacy of foods when attempting to describe relationships between particular foods, or food components, and health benefits. Difficulties arise when comparing the effect of a whole food on sport performance or health indices to a supplement, which may contain a limited array of antioxidant nutrients and cofactors.

Identifying the antioxidant nutrients in food is very difficult as it is not possible to generalize about antioxidants in the same way as core nutrients. For example, most fruit and vegetables contain some carbohydrate, a small amount of protein and a range of twenty vitamins and minerals. What differs between different fruit and vegetables are not the nutrients themselves but the amounts of these nutrients. Antioxidants have a much larger possible spectrum of candidate molecules and differing amounts, but there is also much less commonality with each fruit or vegetable exhibiting its own unique array of antioxidants. Preparation of the food and loss of antioxidant activity with processing and storage should also be considered.

Whole foods invariably contain a multitude of nutrients (and anti-nutrients) that may act to enhance the health benefits of the individual nutrient (Cheynier, 2005). Block, Norkus, Hudes, Mandel & Heizisouer (Block, Norkus, Hudes, Mandel, & Helzlsouer, 2001) examined the correlation between fruit and vegetable intake and plasma levels of antioxidants in 116 males who were non-smokers and non-supplement users. Of the antioxidants tested (ascorbic acid, β carotene, β crytoxanthin and α and δ tocopherol), the strongest association with fruit and vegetable intake was noted for ascorbic acid, and the investigators concluded that ascorbic acid is an important component of the antioxidant effect of fruit and vegetables. Moreover, Szeto, Tomlinson & Benzie (Szeto, et al., 2002) measured the total antioxidant capacity and the total ascorbic acid content in vitamin C rich fresh fruit and vegetables (oranges, grapefruit, kiwi, mango, cabbage, turnips and cauliflower) and determined that vitamin C accounted for 35-75% of the antioxidant power of the food.

Johnson, Dancho & Strong (Johnston, Dancho, & Strong, 2003) investigated the contribution of 70 mg of vitamin C daily from orange juice or supplemental vitamin C over a two week period. The aim was to compare the antioxidant potential of fruit and vegetable (orange juice) versus supplementary pill based vitamin C. The vitamin C concentration and TBARS (lipid peroxidation) levels in the blood were similar in both groups. However, extrapolating the results from orange juice as representative of the association of the intake fruit and vegetables with antioxidant status is questionable. As shown in the Table 4, some forms of fruit and vegetables contain very few antioxidants, other than vitamin C, whereas others may be good sources of vitamin C, flavonoids, lycopenes and anthocyanins. Therefore some fruit and vegetables may not be expected to react any differently to orange juice over a two week period and certainly orange juice may not be expected to act all that differently from vitamin C supplements.

Table 4**: Distribution of different antioxidants in some fruit and vegetables** (Lister, 2006). Reproduced with journal permission. The Nutrition Society of New Zealand (see Appendix 2).

	Carotenoids					Flavonoids						Other		
	Beta-carotene	Alpha-carotene	Lutein/ zeaaxanthin	Beta-cryptoxanthin	Lycopene	Anthocyanins	Flaonols	Flavones	Flavanones	Flavan-3-ols	Proanthocyanins	Glucosinolates	Lipoic acid
Apples	S					S	Y			Y	Y		Y
Asparagus													Y
Blackcurrants						Y	Y			Y	Y		
Broccoli	Y		Y				Y					Y	
Carrots	Y	Y											
Onions													
Oranges	Y		Y	Y					Y	Y			
Pumpkins	Y	Y		S									
Tomatoes	Y	Y	Y		Y		Y		Y				
Watercress	Y		Y									Y	

Y=Yes, the antioxidant is contained in the fruit/vegetable. S=in some varieties only.

Foods containing multiple antioxidants will affect antioxidant status in different ways. Flavonoids may improve antioxidant capacity by sparing vitamin C and E from oxidative decomposition (Miller & Ruiz-Larrea, 2002), presenting an alternate potential source of protective effect. Keppler and Humpf (Keppler & Humpf, 2005) observed that intestinal mircoflora hydrolysed anthocyanins from berry products, within 20 to 120 minutes, which could be further degraded to more stable phenolic acids. The antioxidant effects of anthocyanin ingestion are potentially due to the effects of anthocyanin metabolites and phenolic acids rather than the intact glycocoside.

Antioxidant Action of Uric Acid

Uric acid has long been reported as an effective antioxidant and was first reported as a scavenger of hydroxyl radicals in 1960 (Howell & Wyngaarden, 1960). Since then other reports of urate acting as a significant intracellular and extracellular antioxidant have surfaced (Ames, Cathcart, Schwiers, & Hochstein, 1981; Davies, Sevanian, Muakkasshah-Kelly, & Hochstein, 1986). *In vitro* studies have shown urate attenuates damage to cells subjected to free radical formation (Ames, et al., 1981). Other investigators have suggested that urate is the main antioxidant in human plasma (Wagner, Burton, Ingold, Barclay, & Locke, 1987). The precise mechanism of how urate acts as an antioxidant is still to be determined but it has been postulated that uric acid may preserve vitamin C by chelating transition metals such as iron (Davies, 1986).

It is known that urate levels in the blood increase with exercise and can be interpreted as a possible physiological mechanism to help cope with increased oxidative stress. The mechanism of this increase is thought to be the marked inhibition of renal clearance of urate by the lactate and β-hydroxybutyrate that accumulate during exercise or via increased purine metabolism with exercise (Ames, et al., 1981). Purines are nitrogenous bases that are part of the structure of DNA and RNA found in all living cells. Purines are broken down into uric acid when cells die. Normal plasma levels of uric acid are in the range of 250-350 µmol/L at rest in healthy individuals (Hellsten, Ahlborg, Jensen-Urstad, 1988).

CONCLUSION

Oxidative stress defined by an imbalance between ROS and the antioxidant capacity of the body increases with exercise and is thought to play a role in immune dysfunction, fatigue and poor recovery from exercise. The antioxidant defense system of the body is complex, consisting of various primary and secondary responses to ROS. Maintenance of optimal antioxidant capacity relies on the regular intake of exogenous antioxidants. The understanding of food sources and how the body absorbs stores and utilises vitamin C, anthocyanins and moderate fructose, is developing and evolving. We now know the body is capable of storing small amounts of vitamin C either in the adrenal gland or white blood cells, ready for utilization as an antioxidant during periods of stress. There is evidence that athletes utilize more antioxidants and thus may need to consume more than the average person. The requirement for dietary antioxidants by athletes is still debated. The form of dietary antioxidants may also be important; those provided in the matrix of whole foods, such as intact fruit, may be more beneficial than mega-dosing with supplemental forms of individual antioxidants.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A FOOD FREQUENCY QUESTIONNAIRE TO ASSESS SHORT TERM ANTIOXIDANT INTAKE IN ATHLETES

This chapter comprises the following paper that has been accepted for publication, as described.

Braakhuis AJ, Hopkins WG, Lowe TE, Rush, EC. Development and validation of a food frequency questionnaire to assess short term antioxidant intake in athletes, International Journal of Sport Nutrition and Exercise Metabolism, in press.

(Author contribution percentages: AB: 80%, WH: 8%, TL: 8%, ER: 4%).

ABSTRACT

A quantitative food-frequency questionnaire was developed to determine antioxidant intake in athletes. The questionnaire will be valuable for researchers wishing to standardise antioxidant intake, or simply document habitual intake during an intervention trial. 113 athletes participated in the validity study of which 96 completed the questionnaire and blood test, 81 completed the seven-day food diary and questionnaire, and 63 completed the seven-day food diary and blood test. Validity was investigated by comparing total and food-group antioxidant intakes from the questionnaire with those from a subsequent 7-d food diary. Measures of construct validity were determined by comparing a biomarker of antioxidant capacity (ferric reducing ability of plasma) in a blood sample with antioxidant intakes from the questionnaire and diary. The correlation between the diary and questionnaire energy-adjusted estimates of total antioxidant intake was modest (0.38; 90% confidence limits, ±0.14); the correlation was highest for antioxidants from cereals (0.55; ±0.11), which contributed the greatest proportion (31%) of the total antioxidant intake. Correlations were also high for coffee and tea (0.51; ±0.15) and moderate for vegetables (0.34, ±0.16) and fruit (0.31; ±0.16). The correlation of the plasma biomarker with the questionnaire estimate was small (0.28; ±0.15), but the correlation with the diary estimate was inconsequential (-0.03; ±0.15). One-week test-retest reliability of the questionnaire's estimates of antioxidant-intake in 20 participants was high (0.83; ±0.16). In conclusion, the food-frequency questionnaire is less labor intensive for participant and researcher than a 7-d diary and appears to be at least as trustworthy for estimating antioxidant intake.

INTRODUCTION

Common questions directed to dietitians, physiologists and coaches focus on whether high antioxidant diets are beneficial and if supplementation should be incorporated into the nutritional plan of athletes. To do this, a questionnaire that characterises an athlete's antioxidant intake is

important to develop. This questionnaire will be useful for researchers investigating the impact of dietary supplementation over an intervention and placebo period, or by sports dietitians' wishing to investigate the total antioxidant intake in athletes.

Many antioxidant supplementation studies are completed with little regard for dietary control of habitual antioxidant intake, other than to instruct subjects to continue with usual dietary intake or avoid micronutrient antioxidant supplements (Lafay, et al., 2009; Larcombe, et al., 2008; Nieman, et al., 2004; Umit Kemal Senturk, et al., 2005; Teixeira, Valente, Casal, Marques, & Moreira, 2009). The likely reason for this omission is that food diary completion and analysis is time consuming. It is estimated that food diaries take a minimum of 30-min for the subject and a further 45-min per day to analyse. Even the simple task of analyzing dietary intake for antioxidant consumption is difficult, with current protocols reporting single antioxidant micronutrient intake rather than attempting to characterise total antioxidant consumption. The complexities of antioxidant absorption, digestion and metabolism add further complication to the seemingly simple task of characterising an athlete's antioxidant intake. One method that may minimise the complexity of assessing antioxidant intake is to incorporate the total antioxidant capacity (TAC) calculation into food diary and questionnaire estimates.

TAC is a measure of how well a food product or biological sample can reduce an oxidant, and thus takes into account the synergies between antioxidants found in a single sample. The TAC measurement is defined as the moles (or millimoles) of radicals neutralised per gram of tested sample and therefore provides a measure of total antioxidant activity within the sample (Dragland, Senoo, Wake, Holte, & Blomhoff, 2003; Halvorsen, et al., 2006). Foods assessed for TAC can then be added up over a day or week to provide a total antioxidant intake over a period of time. At this stage, making recommendations to athletes regarding ideal total antioxidant intake is difficult, as no studies to date have characterised the antioxidant intake of athletes using TAC, however with this antioxidant questionnaire, researchers and dietitians will better able to obtain this information.

Assessing the dietary intake of simply the antioxidant vitamins could result in misguided conclusions about the effects of dietary antioxidant intake has on physiology, health and athletic performance. An example is blueberries, which contain vitamin C, but also less recognized antioxidant nutrients such as phenolic compounds such as anthocyanins and flavonols (Mason, Sun, Wang, Hider, & Bekhit, 2006). Therefore an assessment of vitamin C in blueberries will only estimate a portion of the true antioxidant capacity of blueberries.

The individual antioxidant nutrients that are commonly analyzed to characterize dietary antioxidant intake include vitamin C, vitamin E, vitamin A, selenium, fructose, phytochemicals and β-carotene. However, an alternate strategy is to analyze the total antioxidant capacity of the foods consumed by utilizing existing antioxidant databases of the total antioxidant capacity of foods (R. L. Prior, et al., 2003), thus allowing assessment of the antioxidant capacity from the

entire diet. The antioxidant capacities reported in the published database, are based on various assays, including FRAP (ferric reducing ability of plasma) (Halvorsen, et al., 2006), TRAP (total radical trapping antioxidant parameter) (Pellegrini, et al., 2006), TEAC (trolox equivalent antioxidant capacity), and ORAC (oxygen radical absorbance capacity) (R. L. Prior, et al., 2003). These same assays have been modified to assess total antioxidant capacity of plasma.

To date, a small number of questionnaires have been developed to assess antioxidant intake. Pellegrini and colleagues developed a short questionnaire to assess total antioxidant intake validated with a group of 285 older individuals (Pellegrini, Salvatore, Valtueña, Bedogni, & et al., 2007). The questionnaire assessment correlated moderately (Spearman; p=0.52) with that of the 3-d food diary, but there was only a weak correlation between the plasma biomarkers (TEAC and FRAP) and the questionnaire (p=0.13). The authors concluded that the blood (specifically plasma) biomarker was a poor reflection of dietary intake. Another antioxidant questionnaire validation study on 108 older women estimated antioxidant intake correlated moderately with ORAC and TRAP (Pearson r=0.24 and 0.23) but not FRAP (0.07) in plasma samples (Rautiainen, Serafini, Morgenstern, Prior, & Wolk, 2008). Both the studies mentioned, correlating dietary antioxidant intake and plasma biomarkers focused on older, less active individuals and did not fully adjust for energy intake. Therefore a questionnaire to assess antioxidant intake, validated on an active population, is necessary.

Dietary assessment to investigate the effect of antioxidant supplements or related dietary interventions requires accurate reports and control of all foods consumed that contain antioxidants. A likely reason for failure of such assessment is poor compliance with food diaries, which are time consuming for participants to complete and researchers to analyze (Bingham, et al., 1994). Food-frequency questionnaires could be more successful in this respect, because they require little effort to complete and are inexpensive to process. The objective of this study was therefore to develop a food-frequency questionnaire for assessing total dietary antioxidant intake in athletes, and to investigate its validity by comparing with a 7-day food diary. The questionnaire was also assessed for the ability to accurately determine the relative contribution of different antioxidant food groups to the total antioxidant intake. The construct validity of the questionnaire was determined by comparing with the FRAP in blood samples.

METHODS

Study Design

A total of 113 athletes (56 males, 57 females; age 17-36 y) from Rowing New Zealand and local rowing clubs volunteered to participate. There are approximately 200 rowers in the national or regional performance squads, thus we have recruited over half those available. An estimated 30 athletes declined the invitation to participate. The athletes completed the questionnaire,

provided a blood sample for analysis of plasma FRAP (the biomarker for antioxidant capacity) and received oral and written instructions regarding the accurate completion of the seven-day food diary. Athletes were instructed to weigh their food and were provided with food scales accurate to 2g (Salter digital food scales, model 1004SSDR, HoMedics Group Ltd, UK). When athletes ate meals away from home they provided estimates of intake based on household measures, as instructed. Athletes were asked to follow their usual eating pattern. On the eighth day food diaries were reviewed for completion and athletes asked by the dietitian to clarify any omissions and ambiguity. Questionnaires with more than three questions missing were excluded from the study and those with one to three missing questions were assigned as zero for those questions. According to Cade and colleagues (2002), correct validation of a dietary questionnaire must occur by having subjects complete the questionnaire at a separate time to the food diary. The purpose of this time separation is to minimize subject memory of what was recorded on each method and for this reason, the questionnaire was completed first, followed by the food diary. The authors recognize this as a potential source of error in the validation against the blood biomarker (taken at the time of the questionnaire) (Cade, Thompson, Burley, & Warm, 2002). Of the 113 athletes in this validity study, 96 completed the questionnaire and blood test, 81 completed the seven-day food diary and questionnaire, and 63 completed the seven-day food diary and blood test. Exclusion criteria included smokers and those reporting energy intake lower than the minimal calculated requirement, according to the Goldberg equation (Goldberg, et al., 1991). Only two athletes were excluded, both due to inadequate energy intake.

A further 20 athletes of similar age to those completing the validation study volunteered for the reliability study by completing the antioxidant questionnaire on two occasions, one week apart.
Exercise performance data were obtained using a 30-minute rowing ergometer effort on a wind resistance braked rowing ergometer (Concept IIc, Nottingham, UK). To simulate training pressure and enhance motivation, rowers were tested in groups competing against each other.
All subjects provided written informed consent. The AUT University Ethics Committee approved the investigation (Number: 06/230). See Appendix 3, 4 and 5 for ethical approval, sample consent forms and information sheet.

Antioxidant Biomarker

Blood was collected from an antecubital vein into EDTA-containing evacuated tubes, by a registered nurse or phlebotomist. Collected blood was centrifuged at 2700 g at room temperature for 2 min, the plasma separated and stored at -80°C until analysis. The plasma total antioxidant capacity was analyzed with the FRAP assay, as described by Benzie & Strain (1999). The FRAP method was used by Halvorsen (2006) to determine the TAC of the foods in a large antioxidant database that was used as the basis of this questionnaire (Halvorsen, et al., 2006) The coefficient of variation between runs was 4.5%, which compares well to other studies that have measured FRAP in human plasma (CV range 1-5%) (Fernandez-Pachon, Villano,

Troncoso, & Garcia-Parrilla, 2005; Guohua & Prior, 1998). See Appendix 6 for detail on the laboratory methods.

Antioxidant Questionnaire

Development of the antioxidant questionnaire began with identification of the antioxidant content of foods using the database of redox-active compounds in food (Halvorsen, et al., 2006). Foods that contained >0.1 mmol FRAP activity per serving and those that were commonly consumed were included. All antioxidant food values from the antioxidant database were reported as absolute values in mmol of electrons/hydrogen atoms donated in the redox reaction for each food (Halvorsen, et al., 2006). Antioxidant content of foods tested by (Lister, Wilson, Sutton, & Morrison, 2002) and (Wu, et al., 2004), but not (Halvorsen, et al., 2006) were also included.

Commonly consumed foods of the subject group were identified by a registered dietitian familiar with the participants. Foods included on the questionnaire were those that, though relatively low in antioxidant content, may be eaten frequently and in a quantity that would contribute an important proportion of the total antioxidant intake. Identified foods were then grouped by food group and antioxidant content into individual questions. For example, one question asked, “How often in the last month did you consume plums and/or pineapple?”, because plums and pineapple are a similar food type and have a similar antioxidant content (Halvorsen, et al., 2006). The antioxidant content of vegetables was as raw, unless the vegetable is generally eaten in cooked form only, for example potatoes.

The 70 items covered by 45 questions in the food frequency questionnaire are listed in Table 5. Questions relating to antioxidant supplementation were also included in the questionnaire. The intake of multivitamin and mineral supplements was questioned in four categories, as suggested by Park and colleagues (2006).

Separate questions relating to supplement intake and meal pattern and portion size were included. The questionnaire invited respondents to report their intake over the previous month. The frequency section of each question consisted of nine categories: never; 1 time per month, 2 or 3 times per month; 1 or 2 times per week; 3 or 4 times per week; 5 or 6 times a week; 1 time per day; 2 or 3 times a day; 4 or 5 times a day; or 6+ times per day. Portion sizes included various descriptions, based on the food item; for example, less than 1 cup, 1 to 2 cups, 3-4 cups, more than 4 cups. The antioxidant content of each food was then multiplied by the weighted frequency and volume of consumption to provide antioxidant intake.

The questionnaire requested additional information related to physical activity and sporting competition. The physical activity questions asked the average hours spent a week doing on-water, resistance and additional aerobic training. The competition questions asked the number of years the athlete has been competing. The answers were given as a frequency tick box. The questionnaire was trialled on a group of ten participants with similar age, activity and eating

habits as the main study group. Techniques of cognitive testing and in particular retrospective probing were applied (Willis, 1994). Questions that lacked clarity were reworded prior to the start of the study. Refer to Appendix 7 for a copy of the questionnaire.

Food Diaries

Nutrient intake from the food diaries was determined by a registered dietitian using the New Zealand Food Database on Foodworks™ nutritional software (Version 5, Xyris Software, Brisbane, Australia). For antioxidant intake, each food item in the New Zealand Food Composition tables was assigned an antioxidant value based on the same databases (Bingham, et al., 1994; Lister, et al., 2002; Park, et al., 2006) used to develop the questionnaire. The final antioxidant database generated from the Foodworks™ programme was checked by an independent registered nutritionist to ensure certain assumptions were acceptable. Data for each participant was imported into Microsoft Excel from Foodworks™ and used to calculate the consumption of antioxidants from all foods and by category: cereals, fruits and berries, fruit juice and drinks, vegetables, coffee, tea, wine, beer, chocolate items and vitamin C supplements.

Statistical Analysis

General characteristics of the analysis are presented as mean ± SD. To evaluate validity and reliability between the diary and questionnaire, we calculated Pearson correlation coefficients and 90% confidence limits using a published spreadsheet (Hopkins, 2000). All data were log transformed before analysis to improve uniformity. The correlations were adjusted for energy intake using the residual methods as described by Willet and Stampfer (1998). Correlation coefficients are described by the following descriptors: 0.0-0.1 insubstantial; 0.1-0.3 low; 0.3-0.5 moderate, 0.5-0.7 high; 0.7-0.9 very high; 0.9-1 nearly perfect (Cohen, 1988; Hopkins, Marshall, Batterham, & Hanin, 2009). Bias in the questionnaire relative to the diary was analyzed by linear regression (Hopkins, 2000). Intra-class correlation and 90% confidence limits were calculated to assess the reliability of the questionnaire retest.

RESULTS

The food diary and questionnaire were completed by 113 athletes (age 22 ± 3 y, body mass 78 ± 11 kg, and training period was 4.5 ± 2.2 y). Reported weekly durations of training were: on-water, 9.6 ± 2.7 h; resistance or weights, 3.5 ± 1.7 h; and biking, running or other aerobic training, 4.5 ± 5.3 h. Performance data conducted (30-min maximal ergometer time trial) saw the females covered a distance of (mean ±SD) 7300 ±260m, males 8080 ± 320m. This compares to the current world record of 8275m for females (USA, 2004) and 9063m for males (Great Britain, 2009) (Concept2, 2010). Immediate follow-up of participants who failed to complete the questionnaire adequately resulted in no complete exclusions due to missing data, but nine questionnaires still had 1-3 missing responses for frequency of intake of antioxidant food items; these missing values were set to zero.

The daily total energy intake of the group assessed by food diaries was 14.5 ± 5.8 MJ (mean ± SD; range 7.5-25.9 MJ), whilst the average percentage energy from carbohydrate, protein and fat intakes was 53.5 ± 6.8 %, 17.9 ± 5.8 % and 28.0 ± 6.2 % respectively. The average number of fruit servings consumed per day was 2.5 ± 1.3. Twenty-eight percent of participants reported consuming antioxidant supplements (vitamin C, E, A or selenium) more than once weekly. Dietary intakes of the macronutrients and antioxidant micronutrients are presented in Table 6.

The total antioxidant intakes from the various food groups for the diary and questionnaire are presented in Table 6. Cereal, fruit and berries, and vegetables contributed 31%, 9.8% and 9.6% of the total antioxidant intake of the food diary and 19%, 21% and 21% of the questionnaire, respectively. The remaining food groups showed only small percentage differences between the food diary and questionnaire antioxidant intakes.

Using linear regression modeling, the mean bias in the questionnaire compared with the criterion measure of food diaries was 4.8%, a trivial difference. However, for participants at low antioxidant intakes (20 $mmol.day^{-1}$) the questionnaire overestimated by 42% and at high intakes (120 $mmol.day^{-1}$) it underestimated 73%, both moderate-large differences. With the exception of beer and wine, the other antioxidant food groups displayed a greater mean bias, vegetables being the highest at 140%, a moderate difference.

The food groups with a high correlation between diary and questionnaire included cereals, alcoholic drinks (beer and wine) and vitamin C supplements, whilst coffee and tea consumption had moderate correlations (see Table 6). Food groups with a low correlation were chocolate items, fruit juice and fruit drinks. The overall correlation between questionnaire and diary for antioxidant intake was moderate.

The correlations between FRAP in the plasma sample and the questionnaire estimate of antioxidant intake were small, but clear (r = 0.25; energy adjusted r=0.28, 90% confidence limits, ±0.20). Correlations between the food diary and the biomarker were insubstantial (r= 0.15, energy adjusted r=-0.03, ±0.15).

The retest correlations for total antioxidant, cereal and vitamin C supplementation intake from the questionnaire were very high. Retest correlations fruit and berries were high. Retest correlations for the antioxidant intakes of the other food groups were moderate for fruit drinks, vegetables, coffee and tea, wine and beer and insubstantial for chocolate. The retest correlations between questionnaires are presented in Table 7.

Table 5: Antioxidant containing foods included in the food frequency questionnaire

Cereals	Fruit & Berries	Fruit juice & fruit drinks	Vegetables	Coffee & tea	Wine & beer	Chocolate	Other
bran flakes	blackberries	blueberry fruit drink	artichokes	coffee	red wine	unsweetened cooking chocolate	pecans
wholegrain breakfast cereal (Weet-bix, VitaBrits)	blackcurrants	blackcurrant fruit drink	artichoke heart	black tea	beer	sugar free dark chocolate	walnuts
All-Bran™	dried fruit	orange juice	cabbage	green tea		milk chocolate	canned spaghetti
Sultana-Bran™	strawberries	apple juice	potatoes	oolong tea		dark chocolate	meat lasagna
cocoa crisp breakfast cereals	blueberries	pineapple juice	spinach	iced tea		chocolate cake	canned tomato soup
corn flakes	boysenberries	grape juice	capsicum			chocolate chip cookies	milk
rice crispies	raspberries	blackberry fruit drink	broccoli			chocolate ice-cream	flavored milk
Grinners™	cranberries	strawberry fruit drink	tomato juice				yoghurt
	cherries	cranberry fruit drink	vegetable juice				cinnamon
	plums	raspberry fruit drink					cloves
	pineapple						oregano leaf
	pears						ginger
	oranges						mustard seeds
	kiwifruit						turmeric
							canned baked beans
							canned kidney beans

Table 6: Antioxidant intakes (in FRAP units) and Pearson correlation coefficients between log-transformed values for 63 athletes who completed diaries and questionnaires

	Intake (mean ± SD, mmol.wk^{-1})		Correlation[a]	
	Food diary	Questionnaire	Raw	Adjusted
Total intake	57 ± 30	61 ± 31	0.41	0.38
Cereals	18 ± 16	12 ± 15	0.63	0.55
Fruit & berries	5.6 ± 5.0	13 ± 12	0.33	0.31
Fruit juice & drinks	7 ± 2	9.0 ± 11	0.29	0.21
Vegetables	5.5 ± 3.4	12.9 ± 6.9	0.36	0.34
Coffee & tea	2 ± 5	0.7 ± 2.1	0.57	0.30
Beer & wine	1.2 ± 3.5	1.3 ± 2.0	0.44	0.51
Chocolate items	2.0 ± 2.2	3.5 ± 4.6	0.21	0.06
Vitamin C supplements	0.7 ± 1.1	1.7 ± 2.3	0.74	0.73
Other	15 ± 10	7.6 ± 6.3	0.55	0.51

[a]90% confidence limits are approximately ±0.11 for the highest correlation through ±0.18 for the lowest. Adjusted for energy.

Table 7: Intra-class correlation coefficient between log-transformed values for 20 athletes who completed the questionnaire one week apart.

Total antioxidant intake	0.83
Cereals	0.76
Fruit & berries	0.81
Fruit juice & drinks	0.56
Vegetables	0.34
Coffee & tea	0.46
Beer & wine	0.62
Chocolate items	0.08
Vitamin-C supplements	0.81

90% confidence limits are approximately ±0.14 for the highest correlation through ±0.30 for the lowest.

DISCUSSION

A new questionnaire designed to assess total dietary antioxidant intake in athletes has been validated. This targeted questionnaire offers a user-friendly, convenient and quick method to obtain limited dietary antioxidant intake useful for researchers wishing to characterize the antioxidant intake of athletes. Examples of when researchers may wish to use this questionnaire include acute or chronic nutrition intervention studies where results may be influenced by habitual antioxidant intake and when funds preclude full dietary control. The use of the questionnaire will provide information on differences in antioxidant intake between two groups, or if the same subject has altered their antioxidant intake between trials or during a wash-out period. This questionnaire is at least as reliable as a 7-day food diary.

Previous studies using a questionnaire to determine total antioxidant intake (Andersen, Bere, Kolbjornsen, & Klepp, 2004; Kristjansdottir, Andersen, Haraldsdottir, de Almeida, & Thorsdottir, 2006; Pellegrini, et al., 2007; Rautiainen, et al., 2008) were validated on older, less active subjects. The correlations were not corrected for energy intake in any of these studies; thus

previous associations may be erroneously high. The present study used an extensive database of antioxidant-containing foods and has validated the questionnaire with an athletic group. In addition to the development and validation of the questionnaire, this study provides a valuable insight into the antioxidant intakes of this population According to Silvaas (1994) a group of sedentary participants had a total antioxidant intake of 17.3 ± 9.43 mmol.d^{-1}, cereal 0.8 ± 0.3 mmol.d^{-1}, fruit and berries 1.8 ± 1.2 mmol.d^{-1}, coffee and tea 13 ± 11 mmol.d^{-1}. The antioxidant intake was higher in the sedentary group than the athletes tested in this study, however this can be explained by the higher tea and coffee intake alone. Interesting to note that a greater amount of antioxidant intake came from cereals than vegetables (Svilaas, et al., 2004).

Fruit and vegetables are high in antioxidants (Cao, Booth, Sadowski, & Prior, 1998), but other food items with low antioxidant activity, such as cereals, might be consumed in sufficient quantity to contribute a large proportion of dietary antioxidants. Grains and cereal products are known to contain antioxidants, but their potential contribution to health through the diet has essentially been ignored (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000). We found grains and cereals to be a major contributor to the total antioxidant intake in both the food diary and questionnaire (between 19-31%), higher than that consumed by a sedentary population (Svilaas, et al., 2004) . Examples of the types of foods included in the cereal group were bagels, dry biscuits, bread crumbs, tortillas, bread rolls, egg noodles, English muffins, oatmeal, French bread, wheat bread, rice and pasta. The quantities of cereals commonly consumed suggest this food group make an important contribution to antioxidant consumption. Grain products contain many kinds of antioxidant not found in fruits and berries, including avenathramides and avenalumic acid (Miller at. al., 2000). Fat-soluble mono-esters of caffeic and ferulic acids are also commonly found in grain products and are equal to vitamin E in antioxidant activity (Miller, et al., 2000). Cereals also had the highest correlation between antioxidant values calculated from the questionnaire and food diary. It is possible that including an antioxidant questionnaire in future dietary research will demonstrate that cereals are an important contributor to total antioxidant intake in other populations.

The use of a blood biomarker has been suggested as a good method to validate dietary questionnaires, owing to problems with the self-reports of dietary intake, bioavailability, variation in the antioxidant content of foods, and analytical errors (Svilaas, et al., 2004). According to a review on validating questionnaires, only 19% of studies used a biomarker (Cade, et al., 2002). Relying on blood biomarkers to validate nutritional questionnaire data is problematic, and finding an adequate marker of dietary antioxidant intake is no exception. Whilst there are biological markers for energy, nitrogen and sodium (Cade, et al., 2002) intake, there is no single criterion marker for antioxidant intake. According to Chow and colleagues (2007), the use of a questionnaire and biomarker as a validation tool for antioxidant intake is fraught with problems arising from the way antioxidants in food are digested and absorbed. The authors suggest that antioxidants are complex nutrients that may be altered in the gastrointestinal tract prior to absorption, or excreted altogether. Despite the concerns with using a blood biomarker to

validate antioxidant intake, it is still considered the gold standard and therefore important to include (Cade, et al., 2002). Questions do remain as to whether a biomarker is appropriate when assessing antioxidant intake. We found agreement between the food-frequency questionnaire and blood biomarker antioxidant estimates not seen in previous studies using the same blood tests (Andersen, et al., 2004; Kristjansdottir, et al., 2006; Pellegrini, et al., 2007; Rautiainen, et al., 2008). However, we did not find substantial agreement between the blood biomarker and the food diary antioxidant estimate, once adjusted for energy intake. The lack of correlation between the food diary and the blood test is likely due at least in part to the mismatch between the time the blood was taken and the food diary completed, whereas the questionnaire was completed at the same time of the blood test. The blood biomarker may represent an adequate tool for validation of antioxidant intake, if done concurrently. The activity of an antioxidant in food and plasma is dependent on a multitude of factors, including the reactive species present, the localization of antioxidants, and absorption of intact antioxidant function. The majority of antioxidant measurements of blood are one-dimensional and thus useful for food, but less reliable on plasma or other biological samples (Frankel & Meyer, 2000). So the antioxidant food database used in this study is a probably a true reflection of the antioxidant content in vitro, the issue is whether the antioxidant content in the food reaches the blood stream and thus available for an in vivo blood test.

The greatest food-group discrepancy between the diary and questionnaire was for fruit/ berries, chocolate items and vegetables, suggesting there are different patterns of under- or over-reporting for certain food groups. It is possible that as these food groups are considered either healthy or unhealthy, athletes may misreport their true consumption on the questionnaire. In a study that demonstrated the opinion of participants about healthy foods, 80% of respondents agreed that both fruit and vegetables are an important component of a healthy diet (Margetts, Martinez, Saba, Holm, & Kearney, 1997). In the present study, fruit/berries and vegetables were indicated to be consumed in greater amounts on the questionnaire than the food-diary. If we infer that fruit/berries and vegetables are considered more healthy participants may overestimate the consumption of these. The higher fruit and vegetable intake in the questionnaire compared with the food diary suggests an overestimation of intake for food items or supplements perceived to be beneficial. Whilst it is possible to overstate the consumption of healthy food in a food diary, it is probably easier to exaggerate in a tick-box questionnaire. Therefore using a questionnaire for information on the antioxidant dietary intake of isolated food groups, in particular fruit, vegetables and chocolate should be avoided until the questionnaire can be developed further.

A common question plaguing human dietary research is the concern regarding the appropriate length of time a subject should complete a food diary to provide meaningful data. At present, it is unclear how many days subjects would be required to complete a food diary to determine antioxidant intake with adequate precision. For the majority of macronutrients a period of 3-4 days may be an adequate time frame, as the variability from day to day is well regulated by

appetite. However, for some micronutrients found in a small range of food items in large quantities, the variability from day to day can be large (Braakhuis, et al., 2003). In order to minimize the variability of nutrient intake by participants, or the variability in coding food diaries, a 7-day food diary is 2-3 times less variable than a 1-day food record (Braakhuis, et al., 2003). Also, nutrients such as vitamin C, vitamin A and cholesterol require a longer sampling period than energy or carbohydrate, as their intake is around three times as variable (Braakhuis, et al., 2003). Previous studies have relied on a shorter time frame (anywhere from 1-3 days) to determine antioxidant intake from food diaries in comparison to the 7-day food diary used in this study.

In conclusion, we have developed a food-frequency questionnaire to estimate total antioxidant intake. The questionnaire also estimates the contribution of various food groups to the total antioxidant intake. The questionnaire provides a useful estimate of total antioxidant intake that can be used by researchers to assess antioxidant intake from foods, without the time-consuming completion and analysis of food diaries.

CHAPTER 4

EFFECT OF DIETARY ANTIOXIDANTS AND EXERCISE ON ANTIOXIDANT STATUS IN ELITE ROWERS

This chapter comprises the following paper to be submitted to Research Quarterly for Exercise and Sport.

Braakhuis AJ, Hopkins WG, Lowe TE, Rush, EC. Effect of dietary antioxidants and exercise on antioxidant status in elite rowers. October 2010

(Author contribution percentages: AB: 82%, WH: 6%, TL: 6%, ER: 6%).

ABSTRACT

Exercise increases the production of reactive species, which can impair muscle and immune function. However little is known about the effect of diet and training on the antioxidant status of athletes. In this study antioxidant blood biomarkers were assessed before and after a maximal rowing test in 28 males and 34 female rowers. Antioxidant blood biomarkers assessed were ascorbic acid, uric acid, total antioxidant capacity (TAC), erythrocyte- superoxide dismutase (SOD), glutathione peroxidise (GPx) and catalase (CAT). Rowers completed a 7-d food diary and an antioxidant intake questionnaire. Effects of diet, training and performance on resting biomarkers were assessed with Pearson's correlations and their effect on exercise-induced changes in blood biomarkers were assessed by a method of standardisation.

Dietary antioxidant micronutrient consumption was within recommended dietary intakes for all nutrients except vitamin D (38% of male and 51% of female requirements) and possibly vitamin E (93% of requirements for males). With the exception of GPx, there were small to moderate increases with exercise for all markers. Blood resting TAC had a small association with total antioxidant intake (Correlation 0.29; 90% confidence limits, ±0.27) and the exercise-induced change in TAC had a trivial/small association with dietary antioxidant intake from -vitamin C (Standardised effect 0.19; ±0.22), -vegetables (0.20; ±0.23) and vitamin A (0.25; ±0.27). Most other dietary intakes had trivial associations with antioxidant biomarkers. Years of training had a small inverse correlation with TAC (-0.32; ±0.19) and a small association with the exercise-induced change in TAC (0.27; ±0.24). In conclusion, biomarkers of antioxidant status have stronger positive associations with training than with dietary intake of antioxidants.

INTRODUCTION

Exercise increases the production of reactive oxidation species and if these exceed the antioxidant capacity of an individual, muscle and immune function can be impaired (Verhagen, et al., 2006). This has direct implications for athletic performance, yet the relationships between diet, regular training, in-vivo antioxidant status and athletic performance are virtually unknown.

To prevent exercise-induced oxidative stress, the athlete is well equipped with antioxidant defence systems including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), ascorbic acid (AA) and uric acid (UA); all acting in synergy (Aguila, et al., 2004; Davison, et al., 2007; Goldfarb, et al., 2005). Antioxidant status may influence how athletes cope with maximal exercise (Chevion, et al., 2003; Lafay, et al., 2009).

A difficulty in interpreting previous studies is that antioxidant status can be assessed by one or more of the following; 1) individual plasma concentrations of antioxidants, 2) total antioxidant capacity in plasma (TAC), or 3) activity of the enzymes responsible for scavenging reactive species. TAC is a measure of how well a food product or biological sample can reduce an oxidant, and thus takes into account the synergies among the antioxidants found in a single sample. The TAC measurement is defined as the moles (or millimoles) of radicals neutralised per gram of tested sample and therefore provides a measure of total antioxidant activity within the sample (Dragland, et al., 2003). Erythrocyte antioxidant enzyme activity (including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) has been used as an indicator of oxidative stress, with higher activity suggesting higher concentrations of reactive species present (Cazzola, Russo-Volpe, Cervato, & Cestaro, 2003).

In the untrained, positive associations have been found between fruit and vegetable consumption and antioxidant capacity of the blood (Talegawkar, et al., 2009). Lipid peroxidation (a marker of oxidative stress) has been shown to increase in rats fed an antioxidant deficient diet, but reduces to baseline with exercise training over 11 weeks (Teixeira, et al., 2009). Thus training may induce adaptive responses that ameliorate antioxidant inadequacies in the diet. Not only whole foods, but consumption of specific antioxidant nutrients may increase whole body content that may protect the athlete against oxidative stress. Exercise-induced oxidative stress is counteracted by mobilisation of intracellular ascorbic acid (AA) and production of uric acid (UA) (Halliwell & Gutteridge, 1990). Dietary vitamin C and fructose may increase AA and UA concentrations. The intertwined relationship between diet, training, antioxidant biomarkers and performance has not previously been investigated in competitive rowers.

There is evidence provided in current literature that demonstrates athletes experience less oxidative stress at rest than sedentary counterparts, however there is still an acute rise with intense training. (Atalay, et al., 1996; Bloomer, Ferebee, Fisher-Wellman, Quindry, & Schilling, 2009). The degree to which oxidative stress increases with acute exercise is likely related to fitness. Oztasan et. al.(2004) report resting activity of erythrocyte SOD decreased with chronic training whilst GPx increased. Furthermore a study on 15 athletes demonstrate reduced SOD activity and lipid peroxidation following acute exhaustive exercise, compared with pre-training values. Training may down-regulate activity of antioxidant enzymes (Knez, et al., 2007; Oztasan, et al., 2004), suggesting athletes have adapted to the demands of exercise and thus require less enzyme activity than those completing unaccustomed exercise. However antioxidant supplementation has been shown to increase antioxidant enzyme activities (Tauler,

Aguilo, et al., 2006). Thus, training and dietary antioxidants influence the activity of antioxidant enzymes differently (Tessier, Margaritis, Richard, Moynot, & Marconnet, 1995; Ohno, Yahata, Sato, Yamamura, & Taniguchi, 1988).

The aim of the study was to investigate the relationship between dietary antioxidants, antioxidant biomarkers, training and performance, which has not been investigated in athletes before. To state more generically, was diet or training a better predictor of antioxidant status of the blood or athletic performance? Do athletes with above average antioxidant status row better? Or does it simply come down to how much training the athlete did? A secondary aim was to investigate the change in antioxidant status with exercise, and how this change related to diet and training.

METHODS

Study Design

Sixty two elite rowers (28 males, 34 females) from the New Zealand national squad or regional performance centres, completed all measurements. There are approximately 200 rowers in the national or regional performance squads, therefore 58 represents a 29% of possible participants. The characteristics of the group are presented in Table 8. Rowers excluded from participating (n=18) were those who did not complete the 7-day food diary, under-reporters (according to the Goldberg equation (Goldberg, et al., 1991)) and those unable to complete the 30-minute rowing test.

A trained nurse obtained from the antecubital vein of the rower venous blood samples which were placed into an ethylenediaminetetraacetic acid (EDTA) containing vacutainer. Venous samples were obtained between 30 minutes and 1 hour before, and 50-60 minutes after the termination of a specific power based 30-minute rowing ergometer effort on a wind resistance-braked rowing ergometer (Concept IIc, Nottingham, UK). To simulate training pressure and enhance motivation, rowers were tested in groups competing against each other. Performance data for the specific power based 30 minute trial are presented in Table 8. The weight adjusted distance was calculated using the following formulae (Concept2, 2010):

Weight factor = ((body weight in kg))x 2.2/170)$^{0.222}$
Corrected distance = (Actual distance covered in m)/(Weight factor)

Athletes were asked to complete a 7-d food diary and this was analysed as described below. Plasma ascorbic acid, uric acid, total antioxidant capacity (assessed by the ferric reducing antioxidant power assay) (TAC), superoxide dismutase (SOD), glutathione peroxidise (GPx) and catalase (CAT) in erythrocytes were assessed as biomarkers of antioxidant status.

Food diaries

Refer to Chapter 3 methods on food diaries.

Antioxidant Food Frequency Questionnaire

Refer to Chapter 3 methods on the antioxidant food frequency questionnaire.

Antioxidant enzyme activities and plasma concentration

Collected blood was centrifuged at 900 g at room temperature for 2 min. A 200 µL sample of plasma was transferred to 250 µL of 10% *meta*-phosphoric acid in EDTA for later analysis of vitamin C. The remaining plasma was frozen and stored at -80^{o}C. The erythrocyte phase was washed three times with phosphate buffered saline and centrifuged as above. Ascorbic acid concentration was determined within a week and all other biochemical assays were performed in duplicate or triplicate within a month of sampling.

The erythrocytes were treated and assessed for superoxide dismutase (SOD) activity by the method described by Kakkar (Kakkar, Das, & Viswanathan, 1984). Glutathione peroxidise (GPx) was measured by the method described by Mannervik (Mannervik, 1985). Catalase (CAT) was measured using the method by Abei (Abei, 1984). The coefficient of variation (CV) for duplicate samples of SOD and CAT was 7.2% and 4.9% respectively, whilst triplicate samples of GPx had a CV of 2.5%. Haemoglobin concentration (mmol.L^{-1}) was assessed using the cyanmethemoglobin method using Drabkin's solution and maximal absorbance at 540nm, as described by Dacie & Lewis (1984). Ascorbic acid and uric acid were measured by HPLC with electrochemical detection as described by Lykkesfeldt, (J. Lykkesfeldt, 2002).
The plasma total antioxidant capacity (TAC) was analyzed with the FRAP assay, as described by Benzie & Strain (Benzie & Strain, 1996). The CV for the FRAP assay was 4.5%, as determined from duplicate samples.

Refer to Appendix 6 for further detail on the laboratory methods.

Statistical analysis

Subject characteristics are presented as mean ± SD. Analyses were performed with the Statistical Analysis System (version 9.2, SAS Institute, Cary, NC). All variables were log transformed before analysis to improve uniformity. Effects on measures of resting antioxidant status were expressed as Pearson correlations. The correlations with measures of dietary intake were adjusted for energy intake using the residual method (Willet, 1998). The magnitude/strength of correlation coefficients is described by the following descriptors: <0.10 insubstantial; 0.10-0.29 low; 0.30-0.49 moderate; ≥0.50 high (Cohen, 1988). Because of difficulties in interpreting magnitudes of correlations with change scores, we opted to express the relationships of exercise-induced change in biomarkers with the predictors (diet, training and performance) by regressing the change scores against predictor, then evaluating the difference in the change score associated with a difference of 2SD in the predictor (Hopkins, et al., 2009). The difference in the change score was standardized using the SD of the resting value of the

variable. The magnitudes of the standardized effects were interpreted with a modification (Hopkins, et al., 2009) of Cohen's scale (Cohen, 1988) for such effects: trivial <0.20; small 0.20-0.59; moderate 0.60-1.19; large ≥1.20. Uncertainty in effects was expressed as 90% confidence limits, which were estimated using 5000 bootstrap samples. Outcomes were interpreted using magnitude-based inferences (Hopkins, et al., 2009).

RESULTS

Table 8 contains anthropometric, dietary, blood antioxidant and physical characteristics of all rowers who participated. The heart rates achieved during the 30-min ergometer test correspond to 78% of age-predicted maximum, and average heart rate to 72% of age-predicted maximum. Weekly training (h) for the following activities, training time on water 9.2 ± 2.9 h, additional aerobic training 4.6 ± 3.2 h, additional resistance training 3.2 ± 1.4 h. The rowers represented a tight range of age, weight and training characteristics due to the elite nature of the group. Proportion of energy supplied by macronutrients was not different by gender with on average 55% of energy being derived from carbohydrate. The reported dietary intakes of antioxidant micronutrients were all within recommended dietary intakes (Ministry of Health, 2003), except for vitamin D and possibly in vitamin E for males. The other dietary feature was the high intake of vitamin C by athletes, four of whom where mega-dosing in the order of 1 g daily.

Table 8: Subject characteristics (28 males, 34 females) and dietary intake. For micronutrients, the intake as a percent of recommended dietary allowance is shown in brackets

	Males (mean ± SD)	Females (mean ± SD)
Age (yr)	20 ± 3	22 ± 1
Weight (kg)	84 ± 8	69 ± 8
Total training (yr)	4.4 ± 2.3	5.4 ± 2.5
Average training ($h.wk^{-1}$)	17 ± 5	16 ± 3
Maximum heart rate (min^{-1})	173 ± 17	186 ± 6
Average heart rate (min^{-1})	164 ± 13	177 ± 9
30-min ergometer test (m)[a]	8050 ± 360	7500 ± 310
Percent of world record[a]	93 ± 4	92 ± 3
Dietary intake.d^{-1}		
Energy (MJ)	15.6 ± 5.4	10.8 ± 3.8
Carbohydrate (g)	510 ± 190	370 ± 160
Protein (g)	170 ± 70	104 ± 44
Fat (g)	110 ± 45	91 ± 44
Total Antioxidant (mmol)	62 ± 29	51 ± 24
Fructose (g)	28 ± 14	32 ± 17
Vitamin C (mg)	210 ± 240 (700%)	350 ± 370 (1200%)
Vitamin E (mg)	14 ± 8 (93%)	18 ± 21 (120%)
Vitamin A (µg)	1450 ± 630 (160%)	1220 ± 810 (244%)
Vitamin D (µg)	3.8 ± 2.8 (38%)	3.6 ± 5.4 (51%)
Beta-carotene (mg)	4.9 ± 2.5[b]	5.2 ± 3.2
Selenium (µg)	77 ± 30 (110%)	61 ± 46 (100%)

[a] Weight adjusted data for 30-min ergometer test.
[b] No recommended dietary allowance exists.

Table 9 presents the blood markers of antioxidant status, at rest and post exercise. All antioxidant biomarkers increased with the exercise stress test, except GPx activity which had a possible small reduction.

Table 9: Resting blood antioxidant parameters and changes following a 30-min rowing ergometer test

	Rest (mean ± SD)	Change (%) (mean; ±90%CL)	Inference
TAC	590 ± 120 mmol.L^{-1}	12.4; ±2.1	Moderate ↑
AA	67 ± 17 µM	18.3; ±2.4	Moderate ↑
UA	440 ± 110 µM	22.8; ±2.4	Moderate ↑
CAT	250 ± 53 U.gHb^{-1}	4.0; ±4.0	Trivial/Small ↑
GPx	55 ± 8.3 U.gHb^{-1}	-1.1; ±0.7	Trivial/Small ↓
SOD	1.8 ± 0.42 U.gHb^{-1}	9.4; ±3.0	Small ↑

Abbreviations: 90%CL: 90% Confidence limits, TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid, CAT: erythrocyte catalase activity, GPx: glutathione peroxidase, U.gHb^{-1}: units of activity per gram of hemoglobin, SOD: superoxide dismutase.

The dietary antioxidants assessed included fructose, vitamin E, vitamin C, vitamin D, vitamin A, beta-carotene, selenium and fruit serves.day^{-1} and antioxidant content of cereal, fruit, drinks, vegetables, coffee/tea, beer/wine, chocolate. Associations between diet and antioxidant biomarkers are not presented in a table, as the majority were insubstantial, but unclear. The clear correlations included that of blood TAC with total antioxidant intake (0.29; 90% confidence limit, ±0.27) and antioxidant intake from drinks (0.23; ±0.21). Uric acid correlated with the antioxidant intake from cereals (0.29; ±0.22). Fructose intake had an inverse association with enzyme activities; CAT (-0.24; ±0.17) and GPx (-0.24; ±0.21) activity at rest. Antioxidant intake from beer and wine correlated negatively with SOD activity at rest (-0.22; ±0.18).

Also not shown in the tables are the relationships between diet and performance. A moderate positive correlation was found between the distance covered in the performance test and vitamin C intake from supplements (0.22; ±0.18). Average training hours each week correlated with antioxidant intake from drinks (0.31; ±0.21) and chocolate (0.23; ±0.19) and vitamin D consumption (0.28; ±0.19). The total years of training correlated with vitamin A intake (0.35; ±0.20) and antioxidant intake from chocolate (0.25; ±0.20). The data on the relationships between diet and antioxidant status is found in appendix 8.

Table 10 presents the associations between the antioxidant biomarker, training and performance. Antioxidant biomarkers were negatively associated with years of training. That is, athletes that have trained for greater years, completed more training hours a week, competed at a higher intensity and rowed further in 30-min had lower concentrations of antioxidant biomarkers in their plasma and red cells at rest

Table 10: Correlations of antioxidant resting blood biomarkers with chronic training and 30-min rowing ergometer performance

	TAC	AA	UA	CAT	GPx	SOD
Years of training	-0.32	0.21	-0.16	-0.05	-0.12	0.04
Training hours	0.25	0.10	0.10	-0.15	-0.05	-0.20
Performance test	0.41	-0.08	0.29	-0.17	-0.15	-0.23

Abbreviations: TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid, CAT: erythrocyte catalase activity, GPx: glutathione peroxidase, SOD: superoxide dismutase. Confidence limits for all correlations ~±0.20. Correlations of magnitude greater than 0.10 are clear and substantial. Interpretation of magnitude: <0.10 trivial; 0.10-0.29 small; 0.30-0.49 moderate.

The standardised effects on the exercise-induced changes in antioxidant blood biomarkers and the association with dietary intake were assessed, and presented in text. The change in TAC with exercise had a small association with dietary intake from vitamin C (0.19; ±0.22), vegetables (0.20; ±0.23) and vitamin A (0.25; ±0.27). The change in uric acid with exercise associated with dietary fructose (0.32; ±0.23) and vitamin C (0.28; ±0.24). The change in enzyme activity with exercise has a moderate association with vitamin C intake (ΔCAT -0.79; ±0.55; ΔGPx -0.58; ±0.30) and antioxidant intake from drinks associated with the change in GPx activity with exercise (0.33; ±0.33). Overall the change in TAC and UA was associated with diet, but other dietary factors assessed had insubstantial and unclear associations with the exercise-induced change in biomarkers

Associations of the exercise-induced change in antioxidant blood biomarkers with chronic training and 30-min rowing ergometer test are presented in Table 11. Total training years associate positively with the change in TAC of the blood with exercise; and negatively with change in CAT and GPx activity. Therefore a higher average training hours per week was associated with a greater ability to increase TAC with exercise and a less antioxidant enzyme activity. Average training hours per week correlated with change in uric acid with acute exercise, and distance covered correlated with change in CAT activity.

Table 11: Associations of the exercise-induced change in antioxidant blood biomarkers with chronic training and 30-min rowing ergometer test. Data shown are the effect of the difference of 2SD of the training or performance measure on the change in the biomarker, expressed in units standardized with the resting biomarker SD.

	ΔTAC	ΔAA	ΔUA	ΔCAT	ΔGPx	ΔSOD
Years of training	0.27	0.01	0.20	-0.56	-0.29	0.03
Training hours	-0.08	-0.16	-0.29	0.14	0.04	-0.15
Performance test	0.00	0.07	-0.10	0.44	-0.07	-0.06

Abbreviations: Δ: post - pre change in the performance test, TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid, CAT: erythrocyte catalase activity, GPx: glutathione peroxidase, SOD: superoxide dismutase. The magnitudes of the standardized effects were interpreted as: trivial <0.20; small 0.20-0.59; moderate 0.60-1.19; large 1.20.

DISCUSSION

To our knowledge this is the first study to investigate relationships between diet, exercise training, performance and antioxidant status in competitive athletes. The following four observations can be made from the present study. First, for the majority of nutrients the dietary antioxidant and micronutrient intake of the rowers were within recommended ranges of intake. Second, reported diet had predominantly small to trivial associations with antioxidant biomarkers, suggesting a limited impact of diet on antioxidant status. Third, acute rowing performance was positively associated with years and hours of training, and antioxidant status of the blood. Compared with less trained rowers, the most highly trained rowers had lower TAC at rest, and exhibited a greater rise in plasma TAC following acute exercise. Fourth, acute rowing performance, years and hours of training had very little relationship with dietary antioxidant intake. Thus training appears to have greater impact on antioxidant reserve/mobilization after exercise in elite athletes, when compared to diet. Diet of the athletes studied and the effects of acute exercise, training status and history on markers of oxidative stress will be discussed in turn.

Overall diet appeared to be balanced and sufficient in dietary antioxidants. The major dietary concern was the mean low intake of vitamin D, likely caused by the limited number of New Zealand foods that contain vitamin D. Exposure of the skin to the sun is the major source of vitamin D but particularly from May to October the latitude of New Zealand reduces the effect of sun exposure on vitamin D status as does increased use of sunblock. The implications of inadequate vitamin D intake may include increased stress fracture risk and other musculoskeletal issues (Larson-Meyer & Willis, 2010). However, the correlations between dietary vitamin D, training and performance were small to trivial, so our data do not support a negative impact of the low vitamin D intake on this group of rowers. Having said this vitamin D status is certainly worthy of further investigation in athletic populations including measures of blood vitamin D3 concentrations. The second dietary issue of note is the small number of rowers mega-dosing on vitamin C. The high intakes of vitamin C in the rowers is consistent with other research on athletes (Peters, 1993; Farajian, et al., 2004) and reflective of the fact that elite athletes are more likely to supplement than other populations (Sungot-Borgen, Bergland, & Torstveit, 2003). The primary source of vitamin C in rowers consuming greater than 500 mg daily came from supplements rather diet. A high intake of vitamin C coupled with intense exercise may be conducive to a pro-oxidant effect, leading to greater oxidative stress, not less (Bryant, 2003), While not a widely held belief, it is fair to suggest athletes should be advised on moderation with regards to vitamin C supplements and focus on obtaining the nutrient via fruit and vegetables.

The current literature indicates acute exercise which is the same as acute oxidative stress is associated with an increase in TAC (Margonis, et al., 2007; Miyazaki, et al., 2001). In agreement with this we show that TAC, AA and uric acid concentrations in blood all increased moderately (12-20%) after 30 minutes of exercise. The participants in the current study were

competitive rowers and thus likely to have a well-adapted antioxidant capacity as a result of regular training. The level of adaptation and variable response to oxidative stress markers was possibly related to the variation in habitual training load, dietary intake and stores. A positive correlation between training status (hours of training per week) and markers of oxidative damage has been reported by Orhan (2004) in a small study of 18 moderately trained males. The authors suggested the association between training status and oxidative damage to lipid, protein and DNA reflects a rapid repair response to oxidative damage, supported by greater excretion of oxidative stress biomarkers in the urine. Changes in plasma antioxidants were not measured. Weekly training volume has been shown to correlate highly ($r > 0.7$) with post-exercise isoprostanes (a marker of oxidative stress) and gluthione ratio; and moderately with protein carbonyls (a marker of oxidative stress), TAC and GPx (Margonis, et al., 2007). Furthermore our results show the increase in exercise-induced change in TAC was higher in those who had spent less years training. This suggests that while the better trained athletes have a lower blood TAC, they are better able to mobilise antioxidant defences following exercise.

We found a negative correlation between total training years and change in uric acid with exercise. Uric acid, as the final product of purine metabolism, has been considered an important plasma antioxidant, and contributes between 35-65% of the antioxidant activity detected in TAC (Benzie & Strain, 1996). Halliwell developed an antioxidant hierarchy of the effectiveness of individual antioxidants (Halliwell, Rafter, & Jenner, 2005). Halliwell reasoned that ascorbate and thiol groups of proteins are used initially, then bilirubin and uric acid, and then α-tocopherol. Uric acid did increase more than ascorbic acid and total antioxidant capacity with exercise in the rowers and appears to be a major contributor to the antioxidant response to exercise. Not only does the exercise-induced purine metabolism increase uric acid concentrations, but also diet may impact. We show a positive association between reported fructose intake and the change in uric acid with exercise. The associations between fructose intake with the biomarkers uric acid, CAT and GPx activity should be further investigated and could support recommendations for a high cereal intake in athletes.

Conversely low resting concentrations of antioxidant biomarkers and a blunted rise with acute exercise may be indicative of overreaching (characterized by intramuscular metabolic disturbances and a deficit in recovery relative to training load) (Ogonovszky, et al., 2005). This hypothesis is based on the exercise-induced production of reactive species that can damage intracellular structures by its oxidation, inactivating lipids, proteins and even DNA, resulting in altered cellular metabolism (Ogonovszky, et al., 2005). Palazetti et al. (2004) found that overreaching led to a decrease in total antioxidant capacity after a single bout of exercise. For participants in an overreached state, markers of oxidative stress are increased and may be associated with a decline in performance (Margonis, et al., 2007). The increased reserve capacity for TAC to increase with exercise in relation to years of training, suggests this group of rowers were not overreached but had adapted to the need for mobilisation of antioxidants with

exercise. There was no reason to suggest that any of the athletes in this study had over reached as all had high measures of performance.

The response of erythrocyte antioxidant enzyme activity to exercise and dietary supplementation may be measures of adaption to chronic exercise. Up regulation of the production and activity of enzymatic antioxidants occurs in response to an acute bout of exercise (Tauler, Aguilo, et al., 2006; Tauler, Sureda, et al., 2006). However the increase in antioxidant enzyme activity with exercise may be insufficient to prevent oxidative stress. Previous studies report GPx and SOD to be up-regulated in response to exercise, particularly high intensity and/or prolonged duration exercise; while the activity of CAT is less responsive (Li Li, 1993; Machefer, et al., 2004). The rowers demonstrated elevated erythrocyte CAT and SOD with acute exercise, while GPx had a small decrease. The change in activity of SOD with exercise was greater in those with less hours of training a weekas was the change in GPx with years of training, but any association with dietary antioxidant intake was unclear.

There are relationships of antioxidant status in elite athletes and with diet, training and performance and each factor contributes to short and long term optimal function. In this group of competitive athletes the influence of diet appeared to be less influence of years of training. This research has application to elite rowers with a tight range of age, weight and training characteristics. Future research should focus on larger subject numbers, better quantification of the oxidative stress load of the maximal performance test and athlete groups other than just rowers.

In conclusion, training years and training load appear to influence antioxidant status and reserve capacity to a greater degree than the dietary intake of antioxidants.

CHAPTER 5

IMPACT OF VITAMIN C AND OTHER DIETARY ANTIOXIDANTS ON SPORT PERFORMANCE

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ABSTRACT

Antioxidant supplementation has been proposed as a treatment to reduce the adverse effects of exercise-induced reactive species, such as muscle damage, immune dysfunction and fatigue. However, reactive species can mediate beneficial adaptations to training, and dietary antioxidants may therefore attenuate these adaptations. Here we review the performance effects of dietary antioxidants, including vitamin C, vitamin E, mixtures of these vitamins, polyphenols including quercetin and related plant-based supplements containing antioxidants, and *N*-acetylcysteine (NAC).

Vitamin C in doses >400 $mg.d^{-1}$ impaired athletic performance substantially in 5 of 7 studies, possibly by reducing mitochondrial biogenesis, altering fuel utilization via reduction in interleukin-6, or by acting as a pro-oxidant. Smaller doses of vitamin C may be sufficient to reduce oxidative stress without impairing training adaptations. Vitamin E in doses up to 700 $mg.d^{-1}$ had little effect on athletic performance, although effects of megadoses (300 $mg.kg^{-1}.d^{-1}$) on endurance performance in rodents were beneficial. Polyphenol supplementation improved endurance performance in all of 9 studies, although not all outcomes were clear. Polyphenols may enhance vascular function, while quercetin (0.6-1 $g.d^{-1}$) also stimulates mitochondrial biogenesis. Beetroot juice contains anthocyanins and has clear beneficial effects on cycling endurance performance in all of 3 studies, but its effects may be mediated mainly by the vasodilatory effects of the nitrate in beetroot. Acute NAC intake improved performance in 2 of 3 studies of endurance cycling and in 2 studies on muscle force decline, but it has toxic side effects. Acute antioxidant supplement studies (<1 week) are rare compared with the chronic.

In conclusion, performance effects of chronic consumption of antioxidants depend on the nature of the antioxidant, with vitamin C, vitamin E and polyphenols likely to have harmful, trivial and beneficial effects respectively. We recommend further research on the effects of dose of vitamin C, polyphenols and plant extracts taken chronically, and on the effects of all antioxidants taken acutely before exercise.

INTRODUCTION

Physical activity increases the production of reactive species of which an excess damage lipids, proteins and nucleic acids. In a review article, 30 of 40 studies had increases in markers of oxidative stress with an acute bout of exercise (Williams, Strobel, Lexis, & Coombes, 2006). Clearly acute exercise increases the production of reactive species leading to oxidative stress. Given the conventional wisdom that oxidative stress is harmful, it has been suggested that athletes should supplement their diet with antioxidants to protect against oxidative stress and thereby delay fatigue during exercise. While antioxidants appear to reduce oxidative stress (Goldfarb, et al., 2005; Bushell & Lehmann, 1980; Richardson & Allen, 1983; Fischer, et al., 2004; Keren & Epstein, 1980; Thompson, et al., 2003), is this sufficient physiological effect to induce a performance benefit?

Antioxidants stabilize radical species or regulate reactions that convert reactive species into less destructive forms (Alessio, et al., 1997; Finaud, et al., 2006). Dietary antioxidants include vitamin C, vitamin E, NAC, polyphenols, carotenoids, flavonoids and α-lipoic acid (Devasagayam, et al., 2004). Some dietary antioxidants, in particular carotenoids, flavonoids and α-lipoic acid, have not been investigated with respect to athletic performance; therefore this review will discuss vitamin C, vitamin E, NAC, polyphenols and mixtures of these.

Reactive species not only cause damage but instigate cell signalling for training adaptation (Gomez-Cabrera, et al., 2008; Ristow, et al., 2009) and glycogen resynthesis (Richardson, et al., 2007). Research shows antioxidant supplements block expression of proteins related to adaptations to exercise, including antioxidant enzymes and mitochondrial biogenesis (Ristow, et al., 2009). Dietary antioxidants may therefore attenuate these adaptations.

The focus of this review is athletic performance, thus while muscle damage following eccentric exercise can be reduced consuming antioxidant supplements (Avery, et al., 2003; Shafat, Butler, Jensen, & Donnelly, 2004), the relationship this has to athletic performance is unknown or poorly characterised (Powers & Jackson, 2008). A thorough discussion on the effects of antioxidants on recovery from eccentric exercise is beyond the scope of this review and can be found elsewhere (Dekkers, van Doornen, & Kemper, 1996; Powers & Jackson, 2008; Urso & Clarkson, 2003). Further discussion of the effect of exercise on oxidative stress, as distinct from performance outcomes, can be found in Appendix 9.

The studies included in this review were sourced via Google Scholar and Sport Discus, using the search terms *antioxidant* and *exercise*. Additional studies were sourced from reference lists in related articles and books on the topic. All studies were crossover or controlled trials and supplementation period ranged from acute to 5 months. To be included, studies had to report performance effects of antioxidants on high intensity exercise, more specifically maximal efforts. Several controlled trial studies were later excluded due to a lack of a pre-supplementation performance test (Davison, Hughes, & Bell, 2005; Mastaloudis, Traber, Carstensen, & Widric,

2006; Senturk, et al., 2005b; Utter, 2009; Watson, et al., 2005) and details of these studies can be found in Appendix 10. These studies were valuable for elucidating antioxidant status or the mechanistic role of supplements, but less useful for determining performance effects. For example, quercetin was shown to improve a single 160-km time trial by 4.2%; however the uncertainty with 90% confidence was 660%, with a reported p value of 0.992, clearly an uncertain outcome (Utter, 2009). This literature review includes the tabulation and discussion of 41 studies.

VITAMIN C

The first experiments on athletes involving vitamin C and aspects of performance, appeared promising (Hoogerwerf & Hoitink, 1963; Howald, Segesser, & Korner, 1975; Keys & Henschel, 1942), however later studies adopting better design do not support the notion that vitamin C is an ergogenic aid (see Table 1). The initial studies on vitamin C have not been discussed further as the performance data was not reported to a standard expected in modern literature. In recent years very few studies investigating performance and vitamin C have demonstrated an ergogenic effect, the balance being in favour of performance impairment (see Table 1). Those vitamin C studies reporting impaired performance supplemented for 2-8 weeks, while the two studies reporting improved performance were acute to one week. Clearly the length of vitamin C supplementation affects performance outcomes, suggesting chronic intake is ill advised. To further demonstrate the effect of vitamin C supplementation time, acute intake had no effect on exercise induced mitochondrial biogenesis in rats (Wadley & McConell, 2010), unlike that seen in chronic vitamin C supplementation (Gomez-Cabrera, et al., 2008). The duration and intensity of the exercise test are also factors likely to influence the efficacy of antioxidant supplementation. A shorter or less intense exercise test will not produce the same level of reactive species and as a result may not demonstrate beneficial effects of acute antioxidant supplements, although there are insufficient data to provide evidence to address this possibility.

Two of the studies listed in Table 12 presented plausible mechanisms for the impairment in performance. Gomez-Cabrera et. al. (2008) gave humans 1 g of vitamin C daily and found endurance capacity, while not significant, was impaired in the supplemented group and similar but significant results in rats. Vitamin C prevented expression of transcription factors involved in mitochondrial biogenesis the training induced increase in cytochrome C concentration (a marker of mitochondrial content) and mRNA expression of the antioxidant enzymes superoxide dismutase and glutathione peroxidase. Decreases in mRNA are consistent with reduced training-induced adaptations when taking vitamin C.

An alternative mechanism by which vitamin C may impair performance involves (interleukin-6) IL-6. Contracting muscle is a major contributor to the exercise induced increase in IL-6, which vitamin C and E attenuate (Fischer, et al., 2004). Plasma concentrations of muscle-derived (IL-6) increase up to 100-fold during exercise (Febbraio & Pedersen, 2002). Running increases IL-6 and peaks at 30 minutes. The rise in IL-6 appears prior to that of other cytokines. IL-6 may be

an important link between contracting skeletal muscle and the glucose release from liver and thus maintain glucose levels during long endurance exercise (Pedersen, Steensberg, & Schjerling, 2001). Fischer *et.al.* (2004) showed that supplementation with vitamins C and E attenuated the systemic IL-6 response to exercise, by inhibiting release from contracting muscle. The attenuation of IL-6 may negatively impact on performance of greater than 1.5 hours, via a disruption in glucose homeostasis (Febbraio & Pedersen, 2002). The studies reporting impaired performance include many well below 1.5 hours, thus the relative importance of this mechanism requires further research.

Vitamin C is an antioxidant, but can act as a pro-oxidant (Podmore, Griffiths, Herbert, Mistry, & Lunec, 1998; Stadtman, 1991) in large concentrations, following intense exercise. Evidence for a pro-oxidant effect of vitamin C was demonstrated by Khassaf and colleagues (Khassaf, et al., 2003) who provided vitamin C daily and reported elevated SOD, catalase (CAT) activity and heat-shock proteins in lymphocytes at rest. However, on exercising the supplemented group had a lower antioxidant response. The authors originally hypothesized that vitamin C would attenuate oxidant damage and result in a lower production of antioxidant heat shock proteins in response to exercise stress; but ultimately concluded that vitamin C had exerted pro-oxidant effects at baseline. Bryant (Bryant, Ryder, Martino, Kim, & Craig, 2003) investigated the impact of vitamin C on elite cyclists and suggested that moderate doses provided adequate oxidative protection but supplementing the diet with 1 g per day of vitamin C promoted cellular damage. Vitamin C mega-dosing may be a pro-oxidant and increase tissue damage or reduce force production within the muscle.

High plasma concentrations of vitamin C *in vitro* are harmless, unless combined with free iron (Duarte & Lunec, 2005; Levine, 1986). Endurance exercise has been reported to produce an acute phase response not dissimilar to surgical or trauma profile, including release of free iron (Fallon, 2001). The combination of high concentrations of vitamin C and free iron produces radical species, and pro-oxidant effects. The concentrations of vitamin C showing pro-oxidant effects (~ 1 mM) are those that could be achieved with supplementation of 1-2 g of vitamin C daily. As such, the wisdom of mega-dosing supplementation of vitamin C is questionable (Yu, 1994).

Table 12: Details of studies investigating effects of vitamin C on exercise performance

Author	Dietary Antioxidant	Supplement Duration	Subjects	Performance Protocol[a]	Performance Outcome[b]
Nieman, et al., 2002	Vitamin C 1 .5g.d^{-1}	1 week	28 Trained males & females	Time to complete 48-80km run test; Controlled trial	↑1.7%, p=0.820
Thompson, et al., 2001	Vitamin C 1 g	1 hour prior to shuttle run	9 Untrained males	Time to complete 90 min intermittent shuttle run; Crossover design	↑0.8%, p>0.05
Marshall, et al., 2002	Vitamin C 1 g.d^{-1}	4 weeks	5 trained Greyhounds	Time to complete 500m (32-33 s); Crossover design	↓0.7%, p<0.05
Thompson, et al., 2001	Vitamin C 0.4 g.d^{-1}	2 weeks	16 Untrained males	Right leg flexor muscle function during 90 min intermittent shuttle run; Controlled trial	↓8.9%, p>0.05
Gomez-Cabrera, et al., 2008	Vitamin C 1 g.d^{-1}	8 weeks	14 untrained males	V0^2 max during an incremental treadmill run; Controlled trial	↓9.3%; p>0.05
Gohil, Packer, Lumen, Brooks, Terblanche, 1986	Vitamin C 3 g.kg^{-1}.d^{-1}	8-10 weeks	32 rats	Run time to fatigue @ constant speed (70 min); Controlled trial	↓34%, p<0.05
Gomez-Cabrera, et al., 2008	Vitamin C 0.5 g.kg^{-1}.d^{-1}	8 weeks	36 rats	Run time to fatigue @ constant speed (100 min); Controlled trial	↓64%; p=0.014

[a] Max: maximum; Approximate exercise times have been included if reported
[b] ↑performance improvement versus control; ↓performance decrement versus control

Having discussed the potential mechanisms by which vitamin C reduces athletic performance, it is important to comment on the dose. According to various review papers (Dekkers, et al., 1996) (Williams, et al., 2006) antioxidant supplements decrease oxidative stress, with 20 of the 43 papers report reduced markers. The studies that did show decreased oxidative stress had supplemented with vitamin C between 200-1000 mg daily. Thus it is plausible that chronic vitamin C doses impair performance, as previously discussed, but short-term (<1 week) low doses offers health protection without detriment to performance. Examples of when a short-term lower dose may be useful for an athlete include travel, training camps, and the onset of illness or situations whereby a lower dose of vitamin C may benefit to an athlete.

VITAMIN E

The ergogenic potential of vitamin E was believed to be in its antioxidant properties, protecting lipids from oxidative damage and consequent loss of function. Only a few human studies have examined the effect of vitamin E on performance and the majority reported no significant effect (see Table 13). Vitamin E does benefit performance in the following situations; altitude (Kobayashi, 1974; Simon-Schnass & Pabst, 1988) and swim time to exhaustion in mice (Devi, Prathima, & Subramanyam, 2003; Novelli, Bracciotti, & Falsini, 1990) (see Table 13). While the

swim time to exhaustion in mice report a benefit with vitamin E, Coombes (2001) demonstrated reduced contractile force in activated muscle, by providing a massive dose of the antioxidant, intravenously. In low concentrations, reactive species can regulate cell signalling and alter muscle function and circulation (Richardson, et al., 2007), however mega-doses likely inhibit optimal muscle force production. Thus a dose response of vitamin E to performance requires further investigation.

A few studies performed at altitude have suggested that vitamin E is beneficial in this environment (Kobayashi, 1974; Nagawa, Kita, Aoki, Meashima, & Shiozawa, 1968; Simon-Schnass & Pabst, 1988). Simon-Schnass and Pabst found vitamin E supplementation (400 mg/day for 10 weeks) in mountain climbers prevented some of the decrease in cycling performance, as measured by the anaerobic threshold usually experienced at high altitude. Kobayashi (1974) investigated the effect of vitamin E on athletic performance at a high altitude and reported significantly improved aerobic work capacity. Improved performance at altitude is likely due to the ability for vitamin E to restore erythrocyte deformity, common to this environment. This in turn allows for better oxygen delivery as the erythrocyte can flow more easily through the arterial tree (Senturk, et al., 2005a).

Exercise near sea level has presented a lack of strong evidence supporting ergogenic benefits for vitamin E supplementation for humans. Studies to date suggest that eating a well-balanced diet will provide adequate amounts of vitamin E to meet needs of the athlete.

Table 13: Details of studies investigating effects of vitamin E on exercise performance

Author	Dietary Antioxidant	Supplement Duration	Subjects	Performance Protocol[a]	Performance Outcome[b]
Novelli, et al.,1990	Vitamin E 300 $mg.kg^{-1}.d^{-1}$	10 min prior to trial	92 male mice (24 on vitamin E)	Swim time to fatigue in pool (5 min); Controlled trial	↑139%, $p<0.0001$
Devi, et al.,2003	Vitamin E 45 $mg.kg^{-1}.d^{-1}$	60 days	46 male rats (including young, middle age and old)	Swim time to fatigue in 4-mth old rats in pool (70 min); Controlled trial	↑26%, $p>0.05$
Simon-Schnass, 1988	Vitamin E 363 $mg.d^{-1}$	4 weeks	12 highly trained climbers	Anaerobic threshold during cycle incremental test at 5100m; Controlled trial	↑18%, $p<0.01$
Kobayashi, 1974	Vitamin E 400 $mg.d^{-1}$	6 weeks	12 trained males	Anaerobic threshold during cycle incremental test at 1525m;Controlled trial	↑8.9% (@ 1525m) ↑14.2% (@ 4750m)

Nagawa et al., 1968	Vitamin E 300 $mg.d^{-1}$	6 weeks	20 endurance athletes	Cycle time to fatigue@ 1300$km.min^{-1}$ on 75rpm	Improvement. No data
Piercy, et al., 2001	None Pre-race plasma vitamin E measured	N/A	670 trained sled dogs	Likelihood of finishing 1159 mile time trial	1.9 times more likely to finish with high plasma vitamin E
Keong, et al., 2006	Vitamin E 60 $mg.d^{-1}$	6 weeks	18 trained males	Run time to fatigue @70% $V0_2$ max in the Heat (80 min); Crossover design	↑5.2%, p>0.05
Gaeini, Rahnama, Hamedinia, 2006	Vitamin E 400 $mg.d^{-1}$	8 weeks	20 trained males	Time taken to cycle to fatigue in an incremental test (15 min); Controlled trial	↑0.7%, p>0.05
Rokitzki, et al.,1994	Vitamin E 330 $mg.d^{-1}$	5 months	30 elite males cyclists	Watts.kg^{-1} at anaerobic threshold during an incremental cycle test (15 min); Controlled trial	↑0.3%, p<0.05
Nieman, et al., 2004	Vitamin E 727 $mg.d^{-1}$	2 months	38 elite athletes	Time taken to complete an Ironman distance triathlon (5 hrs); Controlled trial	↓0.3%, p=0.96
Lawrence, 1975	Vitamin E 300 $mg.d^{-1}$	6 months	43 trained swimmers	Time taken to complete swim repetitions (best of 3-d test), 100 yards x 10 with 10s recovery; Controlled trial	↓0.7%, p>0.05
Tiidus, et al.,1993	Vitamin E rich diet 35 $mg.kg^{-1}$	8 weeks	Vitamin E deprived mice (n=40) Vitamin E adequate mice (n=60)	Number of mice to finish 60 min at 28 $m.min^{-1}$ @15% gradient; Controlled trial	↓7% completed the trial on vitamin E p>0.05
Coombes, et al., 2001	Vitamin E 10,000 $mg.kg^{-1}.d^{-1}$	8 weeks	23 rats	Muscle twitch tension of isolated rat muscle (60min); Controlled trial	↓24%, p<0.05

[a] Max: maximum; Approximate exercise times have been included if reported
[b] ↑performance improvement versus control; ↓performance impairment versus control

N-ACETYLCYSTEINE

N-acetylcysteine (NAC) is the acetylated precursor of both the amino acid L-cysteine and reduced glutathione. The oral availability is between 6-10% and NAC is excreted rapidly (Borgstrom, Kagedal, & Paulsen, 1986); hence the preference for intravenous administration in research studies (see Table 3). NAC appears successful in delaying muscle fatigue during sub-maximal exercise in humans, particularly in trained subjects (see Table 14). The majority of studies using NAC report an improvement in performance, with a range from an impairment of

2.9% to an improvement of 32%. The antioxidant properties of NAC allow re-synthesis of glutathione, which serves multiple roles in the cellular antioxidant defence (Sen, et al., 1994). NAC assists in maintaining maximal activity of the Na^+ -K^+ ATP pump during exercise as it requires an optimal redox range to function (McKenna, et al., 2006). NAC is thought to reduce the exercise-induced increase in reactive species that contributes to Na^+ K^+ ATP pump activity fatigue.

Initial research on the toxicity of NAC demonstrated supplementing mice with large doses for 3 weeks damaged the heart and lungs (Palmer, et al., 2007). NAC was metabolized to S-nitroso-N-acetylcysteine (SNOAC), which increased blood pressure in the lungs and right ventricle of the heart (pulmonary artery hypertension). The effect was similar to that observed following a 3-week exposure to an oxygen-deprived environment (chronic hypoxia). The authors also found that SNOAC induced a hypoxia-like response in the expression of several important genes both in vitro and in vivo. The implications of these findings for long-term supplementation with NAC have not yet been investigated. The dose used by Palmer and colleagues (Palmer, et al., 2007) was dramatically higher than that used in humans; nonetheless, the drug's effects on the hypoxic ventilatory response have been observed previously in human subjects at more moderate doses (Palmer, et al., 2007). Reid (Reid, Stokić, Koch, Khawli, & Leis, 1994) reported other serious adverse reactions, including conjunctival irritation, dysphoria, vomiting, diarrhoea, nausea, and loss of coordination, thus precluding NAC infusion during voluntary exercise The requirement for mega-doses via intravenous administration and the potential toxicity of NAC make further recommendation difficult.

Table 14: Details of studies investigating effects of N-acetylcysteine (NAC) on exercise performance

Author	Dietary Antioxidant	Supplement Duration	Subjects	Performance Protocol[a]	Performance Outcome[b]
Matuszczak, et al., 2005	150 $mg.kg^{-1}$ iv	Before	Untrained male and females	Number of repetitive handgrip exercises (5 s every 30 s: ≈ 40); Controlled trial	↑32%, $p<0.01$
Medved, et al., 2004	Initial: 125 $mg.kg^{-1}.h^{-1}$ iv. During: 25 $mg.kg^{-1}.h^{-1}$ iv.	Before and during	7 trained males	Cycle time to fatigue @ constant speed (6min); Crossover design	↑26%, $p<0.05$
McKenna, et al., 2006	Initial: 125 $mg.kg^{-1}.h^{-1}$ iv. During: 25 $mg.kg^{-1}.h^{-1}$ iv.	Before and during	8 male endurance athletes	Cycle time to fatigue during a 45 min @ 71% VO2peak (6 min) following 92% VO2peak test; Crossover design	↑24%, $p<0.05$
Reid, et al., 1994	150 $mg.kg^{-1}$ iv.	Before	10 untrained males	% decline in force output during 10Hz stimuli every second for 30 min; Crossover design	15% lesser decline in force output $p<0.001$

Medved, et al., 2003	Initial: 125 $mg.kg^{-1}.h^{-1}$ During: 25 $mg.kg^{-1}.h^{-1}$	Before and during	8 untrained males	Cycle time to fatigue During 3x 45s, then to fatigue @ 130% $V0_2$ Crossover design	↓2.9%, p>0.05

[a] Max: maximum; Approximate exercise times have been included if reported
[b] ↑performance improvement versus control; ↓performance decrement versus control

POLYPHENOLS

Berry extracts and beverages provide a protective antioxidant effect for athletes exposed to exercise-induced oxidative stress (Pilaczynska-Szczesniak, Skarpanska-Steinborn, Deskur, Basta, & Horoszkiewicz-Hassan, 2005; Skarpanska-Stejnborn, Basta, & Pilaczynska-Szczesniak, 2006), however as discussed with vitamin C this action alone may not improve performance. Studies have indicated that polyphenols, independently of their antioxidant properties, can enhance the production of vasodilating factors (nitric oxide, endothelium-derived hyperpolarizing factor and preactive speciestacyclin) and inhibit the synthesis of the vasoconstrictor endothelin-1 (Schini-Kerth, Auger, Kim, Etienne-Selloum, & Chataigneau, 2010). Polyphenols have been shown to improve blood pressure (Prior & Gu, 2005) and forearm blood-flow (Matsumoto, et al., 2005). Recent studies into the ergogenic potential of beet-root juice did report altered nitric oxide concentrations, blood pressure and improved cycling performance (Bailey, et al., 2009). Thus performance improvements due to altered hemodynamics are possible (Ghosh & Scheepens, 2009; Stoclet, et al., 2004).

The major polyphenols shown to influence vascular function in humans are primarily from cocoa, wine, grape seed, berries, tea, tomatoes, soy and pomegranate (Ghosh & Scheepens, 2009). Grape seed extract has been shown to alter vascular function, endothelial function, improve flow-mediated dilatation of blood vessels and reduce oxidative damage in a randomized, controlled cross-over study (Clifton, 2004). Blackcurrant has been shown to alter peripheral circulation dynamics, thus improving blood flow, at rest or after typing work in 9 healthy males (Matsumoto, et al., 2005). In athletes provided ground blackcurrant fruit for 6 weeks, performance in a 2000m ergometer time trial did not change significantly, but parameters of blood flow did (Skarpanska-Stejnborn, et al., 2006).Therefore, providing antioxidants in the form of a complex food, with a range of nutrients, may provide acute benefits to sport performance and warrants further investigation.

Beetroot juice contains anthocyanins (a group of natural polyphenolic pigments responsible for the red to blue colour of a wide range of fruits and vegetables) and high concentrations of nitrates. The three studies investigating the effect of beetroot on performance demonstrated clear improvements. However, improvements with beetroot have been solely attributed to their nitrate content. Certainly sodium nitrate alone has shown reduced oxygen utilization with exercise, but not cycling performance (Larsen, Weitzberg, Lundberg & Ekblom, 2010). It is likely the effect of nitrate and polyphenols are distinct from each other, and consumed in combination in the form of beetroot juice, are additive.

Quercetin, a natural polyphenol present in plant based-foods such as fruit, vegetables and berries (Harwood, Danielewska-Nikiel, Borzelleca, Famm, Williams & Lines, 2007). Quercetin has been shown to influence athletic performance via an alternative mechanism to generic polyphenols, by modestly increasing mitochondrial DNA and messenger RNA levels of four genes related to mitochondrial biogenesis (Nieman, et al., 2010). Polyphenols, such as quercetin and resveratrol, increase intracellular signalling pathways which increase mitochondrial biogenesis, improve endurance and health in mice (Lagouge, et al., 2006; Narkar, Downes, Yu, Embler, & Wang, 2008; Rasbach & Schnellmann, 2008). As shown in Table 15, quercetin did improve 30-km cycling time trial performance (MacRae & Mefford, 2006) and 12-min run time following a 60-min steady state run (Nieman, et al., 2010). Daily doses of 600 mg and above for at least two weeks appear necessary for a performance impact on aerobic exercise.

Polyphenols appear to be beneficial to athletic performance; however the exact mechanisms are unknown. Each class of polyphenol is likely to have a different physiological effect, just as quercetin has been studied in isolation, others will also. However, unlike other antioxidants polyphenols have shown egogenic potential in as little as 6 days of supplementation. Beetroot juice has been shown to improve cycling performance, consuming $0.5L.d^{-1}$ for 6 days, however the polyphenol was not reported, hence recommendations beyond this volume of drink is difficult. Meanwhile, the athlete can consume a diet high in fruit and vegetables thus obtaining many of the polyphenols discussed.

Table 15: Details of studies investigating effects of various polyphenol supplements on exercise performance

Author	Dietary Antioxidant	Supplement Duration	Subjects	Performance Test[a]	Performance Outcome[b]
Lafay, et al., 2009	Grape extract 400 $mg.d^{-1}$	1 month	20 elite male athletes	Flight time in 45 s of vertical rebound jumps; Crossover design	↑24% , $p<0.05$
Oh, et al., 2010	Ecklonia cava (dose unstated)	30-min prior to trial	23 untrained males	Cycle time to fatigue in an incremental test (9 min); Crossover design	↑22%, $P<0.05$
Bailey, et al., 2010	Beetroot juice $0.5L.d^{-1}$	6 days	7 untrained males	Cycle time to fatigue in an incremental test (10 min); Crossover design	↑20% , $p<0.01$
Bailey, et al., 2009	Beetroot juice $0.5L.d^{-1}$	6 days	8 untrained males	Cycle time to fatigue in an incremental test (11 min); Crossover design	↑14% , $p<0.05$
Lafay, et al., 2009	Grape extract 400 $mg.d^{-1}$	1 month	20 elite male athletes	Power in 10 rebound jumps; Crossover design	↑6.4%, $p>0.05$
Sadowska-Krepa, et al., 2008	Red grape skin Extract	6 weeks	14 active males	50m swim time, following 5 x 50m swim repetitions	↑5.0%, $p<0.05$

	1170 mg.d^{-1}			(1-min); Controlled trial	
MacRae & Mefford, 2006	Quercetin 600 mg.d^{-1}	6 weeks	11 elite male cyclists	Time taken to complete a 30-km cycle test (50 min); Crossover design	↑3.1%, p≤0.01
Cureton, et al., 2009	Quercetin 1000 mg.d^{-1}	7-16 days	30 untrained males	Work performed during a 10-min cycle test, following a 1h cycle; Controlled trial	↑2.5%, p>0.05
Nieman, et al., 2010	Quercetin 1000 mg.d^{-1}	2 weeks	26 untrained males	Distance covered in 12 minutes of 60 min @ 60% $V0_2$ maximum and a 12-min run; Crossover design	↑2.9%, p=0.04
Vanhatalo, et al., 2010	Beetroot juice 0.5L.d^{-1}	15 days	8 untrained males	Peak power (watts) in an incremental cycle test (9-min); Crossover design	↑ 2.5%, P<0.05
Skarpanska-Stejnborn, et al., 2006	Ground black-currant fruit (dose unstated)	6 weeks	19 elite male rowers	Time taken to complete a 2000m rowing ergometer test; Controlled trial	↑0.2%, p>0.05

[a] Max: maximum; Approximate exercise times have been included if reported
[b] ↑performance improvement versus control; ↓performance decrement versus control

MIXED DIETARY ANTIOXIDANTS

The evidence for a change in performance using mixed antioxidants appears unconvincing, with only one of the studies listed in Table 16 reaching significance. The likely reason for the trivial effect of mixed antioxidants is the use of a lower dose of each supplemented antioxidant than previous studies discussed in this review.

Despite the diversity in the performance data, studies have demonstrated reductions in vascular function with mixed antioxidants. Twenty-five healthy males, taking an acute dose of vitamin C and E demonstrated reduced brachial artery vasodilatation, compared with placebo (Richardson, et al., 2007). Excess reactive species limit vasodilation and blood flow by reducing nitric oxide availability (Eskurza, Monahan, Robinson, & Seals, 2004); however moderate concentrations increase vasodilation directly due to their vasoactive properties (Richardson, et al., 2007; Wray, Uberoi, Lawrenson, Bailey, & Richardson, 2009). Exercise-induced vasodilation is attenuated in those who supplement with vitamin C and E suggesting that chronic supplementation with large doses of antioxidant mixtures is likely to be unwise (Richardson, et al., 2007).

Low-level increases in reactive species with muscle contraction is imperative for optimal muscle function (Copp, et al., 2009; Reid, et al., 1994) and regulation of vascular smooth muscle (Richardson, et al., 2007). Accumulation of reactive species exceeding the capacity of the

endogenous antioxidant system, result in muscle fatigue (Finaud, et al., 2006; Ji, 1996). Consequently antioxidant supplementation has been proposed as a potential treatment to curtail the adverse effects of reactive species. However, we would caution the assumption that a reduction in reactive species is a good thing and will allow an athlete to train harder, as there is no evidence of this.

Table 16: Details of studies investigating effects of mixed dietary antioxidants on exercise performance

Authors	Dietary Antioxidants	Supplement Duration	Subjects	Performance Test[a]	Performance Outcome[b]
Snider, Bazzarre, Murdoch & Goldfarb , 1992	CoenzymeQ10, cytochrome C, inosine, vitE (300 mg+1500 mg,+300 mg +545 mg.d^{-1})	4 weeks	11highly trained male triathletes	Run time to fatigue in a 90 min run at 70% $V0_2$ max; Crossover design	↑7.3%, p=0.57
Yfanti C, et al., 2010	vitC and vitE (500 mg and 363 mg.d^{-1}) d^{-1}	16 weeks	21 untrained males (11 treatment, 10 placebo)	Maximal power during an incremental cycle test; Controlled trial	↑4.0%, p>0.05
Romano-Ely, et al., 2006	VitE & VitC (amounts not stated)	During exercise only	14 male cyclists	Cycle time to fatigue during a 70% $V0_2$ max test (1.5 h); Crossover design	↑2.0%, p>0.05
Oostenbrug, et al., 1997	Fish oil (6 g.d^{-1}) +/- vitamin E (300 mg.d^{-1})	3-weeks	24 trained male cyclists	Cycle time to fatigue @ 70% maximal watts (1 h); Controlled trial	↑0.3%, p=0.09
Teixeira, et al., 2009	VitE136 mg, VitC 200 mg, βcarotene15 mg, lutein1mg, selenium 200 µg, zinc 15 mg & magnesium 300 mg.d^{-1}	1 month	20 trained kayakers (14 males, 6 females)	Time to complete 1000m kayak race; Crossover design	↓1.5%, p>0.05
Larcombe, et al., 2008	VitE, βcarotene, lutein, zeaxanthin, (amounts unstated)	1 month	Captive adult budgerigars (flight birds)	Flight speed (10 s); Crossover design	↓1.8%, p>0.05
Senturk, et al., 2005	VitA (β carotene), VitE & VitC (50 mg, 1 g +800 mg.d^{-1})	2 months	20 trained, 9 untrained males	Cycle time to fatigue in a incremental cycle test (10 min); Controlled trial	↓4.7%, p>0.05
Copp, et al., 2009	VitC & tempol (76 mg + 52 mg. kg^{-1} iv)	Immediately before	6 mice	Muscle force produced during 1Hz contractions for 3 min; Uncontrolled trial	↓25%, p<0.05

[a] Max: maximum; Approximate exercise times have been included if reported

[b] ↑performance improvement versus control; ↓performance decrement versus control

Abbreviations: VitE: Vitamin E; VitC: Vitamin C: VitA: Vitamin A

CONCLUSION

Antioxidant supplements are widely used by athletes to avoid elevated oxidative stress, the consequences of which include muscle damage, immune dysfunction and fatigue. It appears to be an erroneous assumption to provide athletes with mega-doses of antioxidant supplements to

avoid oxidative stress and subsequently performance. Unfortunately, very few studies have investigated the efficacy of antioxidant supplements making recommendations very difficult.

Despite the lack of evidence on antioxidant supplementation, vitamin C is a well researched antioxidant which decreases oxidative stress taken in doses of 0.2-1 $g.d^{-1}$. Vitamin C in large doses appears to reduce training induced adaptations by reducing mitochondrial biogenesis, altering fuel utilization via reductions in IL-6 or by acting as a pro-oxidant, when mega-dosing (>1 $g.d^{-1}$). A small dose of vitamin C (200-400 $mg.d^{-1}$) may be sufficient to reduce oxidative stress but not past a threshold that will impair optimal training adaptations. Further research is required to clarify a dose response to vitamin C.

Vitamin E appears to have little impact on human athletic performance, unless at altitude. NAC decreases oxidative stress and improves cycling performance in trained athletes, but may have toxic effects taken chronically. Polyphenols appear to improve flow-mediated dilatation of blood vessels and alter peripheral circulation dynamics (Ghosh & Scheepens, 2009). A particular class of polyphenols, quercetin, increases mitochondrial biogenesis and appears to improve maximal endurance performance. Beetroot juice contains the polyphenol, anthocyanin, and improved cycling performance in 3 of 3 studies. Beetroot juice contains naturally occurring nitrates and further research is required to elicit effects of these separate from the polyphenols. Mixed antioxidants appear to have little effect on performance in humans, possibly due to the lower doses usually provided in these studies. Further research is required on most of the antioxidants supplements, however promising results are seen with polyphenols and are worthy of further investigation.

CHAPTER 6

EFFECTS OF DIETARY ANTIOXIDANTS ON TRAINING AND PERFORMANCE IN FEMALE RUNNERS

This chapter comprises the following paper submitted to Medicine and Science for Sports and Exercise, October, 2010.

Braakhuis AJ, Hopkins WG, Lowe TE, Effects of dietary antioxidants on training and performance in female runners, October 2010.

(Author contribution percentages: AB: 90%, WH: 5%, TL: 5%)

ABSTRACT

Purpose: Exercise-induced oxidative stress is implicated in muscle damage, immune dysfunction and fatigue and has led athletes to believe that antioxidant supplementation may assist recovery. This study investigated the effects of daily ingestion of vitamin C (1000 mg), blackcurrant juice (15 mg vitamin C, 300 mg anthocyanins) and placebo in isocaloric drink form for 3 weeks on training progression, an incremental running test, and 5-km time-trial performance.

Methods: Twenty-three trained female runners (age, 31± 8 y; mean ± SD) completed 3 blocks of high-intensity training for 3 weeks, separated by a washout period. Each block was preceded by a 5-km time trial and ended with a short taper, an incremental test and 5-km time trial. Changes in training and performance with each treatment were analyzed with a mixed linear model, adjusting for performance at the beginning each training block. Markers of oxidative status were also measured in blood.

Results: There was a harmful clear effect on mean running speed during training on vitamin C (1.3%; 90% confidence limits ±1.3%) and blackcurrant juice (1.0%; ±1.3%) compared with placebo, but by the end of the block effects on training speed were unclear. Effects of the two treatments relative to placebo on mean performance in the incremental test and time trial were unclear, but runners faster by 1 SD of peak speed showed a clear benefit on peak running speed with blackcurrant juice (1.9%; ±2.5%).

Markers of oxidative stress after taking vitamin C were higher: erythrocyte catalase at rest (23%; ±21%), protein carbonyls at rest (27%; ±38%) and erythrocyte superoxide dismutase post-exercise (8.3%; ±9.3%), compared with placebo.

Conclusion: Chronic vitamin C or blackcurrant intake should be avoided, however acutely blackcurrant may improve performance and warrants further investigation.

INTRODUCTION

Exercise-induced oxidative stress, due to an accumulation of reactive species, is implicated in muscle damage, immune dysfunction and fatigue. Reactive species produced in excess, and

beyond the athlete's antioxidant capacity may cause damage to lipids, proteins, DNA, increase the release of cytosolic enzymes and result in poor recovery and performance. Measures of in vivo antioxidant status are useful to understand how exercise and diet influence oxidative stress. Antioxidant status can be assessed by measuring individual plasma concentrations of antioxidants and by measuring the activity of the enzymes responsible for scavenging reactive species. Erythrocytes' antioxidant enzyme activity has been used as an indicator of oxidative stress, with higher activity suggesting higher reactive species present (Cazzola, et al., 2003). Oxidative stress can be determined by measuring the damage to proteins or lipids caused by reactive species. Oxidative modification of protein incorporates a carbonyl group into the protein, thus an effective biomarker of oxidative stress. MDA is the reaction product of lipid peroxidation and can be determined from plasma. In addition to the markers of oxidative stress, in vitro assays can determine the degree to which a biological sample copes with a fixed addition of reactive species, therefore indicating how successfully a fixed sample of erythrocytes resist oxidative damage.

Athletes are of the opinion that dietary antioxidant supplements will assist recovery from exercise. Given the conventional wisdom that oxidant stress is harmful, many athletes supplement their diet with vitamin C and other antioxidants to protect against oxidant stress (Schwenk & Costley, 2002). However, reactive species not only cause damage but may play a role in cell signaling for training adaptation (Gomez-Cabrera, et al., 2008; Ristow, et al., 2009). Recent research has shown that antioxidant supplements block expression of RNA of proteins related to adaptations to exercise, including antioxidant enzymes and mitochondrial biogenesis in sedentary and trained males (Ristow, et al., 2009). Reducing concentration of reactive species via antioxidant supplementation could therefore have negative consequences by attenuating adaptations to training.

Besides antioxidants in supplement form, whole foods have been studied due to their favorable antioxidant content. The health benefits of blackcurrants are traditionally attributed to their vitamin C content, however the effect of vitamins, anthocyanins and other polyphenol compounds in blackcurrants may be additive. Blackcurrants have been reported to have a very high antioxidant activity and to be amongst the top dietary sources of antioxidants (Halvorsen, et al., 2006; Lister, et al., 2002). Blackcurrant juice extract increased the lifespan of mice (Jones & Hughes, 1982) and blackcurrant flavonoids protect against exercise induced oxidative stress in humans (Skarpanska-Stejnborn, et al., 2006). The interaction of food compounds in blackcurrant drinks containing a mixture of polyphenols (phenolic acid, flavonols, flavan-3-ol), quercetin, fiber, vitamins and tannins, may have an impact on physiological parameters not seen in blackcurrant extract alone and is worthy of investigation (Kähkönen, Hopia, & Heinonen, 2001).

To add further complexity, it is possible that gender independently influences oxidative stress. Estrogen activates a pathway that up-regulates antioxidant enzymes and has strong antioxidant

properties (Vina, Sastre, Pallardo, Gambini, & Borras, 2006) which may alter biochemical indices of post-exercise muscle damage and leukocyte invasion (Goldfarb, McKenzie, & Bloomer, 2007; Bloomer, et al., 2009). Also, the rate of oxidant production from mitochondria of females is lower than that of males (Vina, et al., 2006). Overall dietary antioxidant supplementation may impact females differently to males. To date, the impact of training and antioxidant supplementation on training efficacy and performance in competitive females has not been investigated.

This study is a crossover study including 23 female runners, who performed three 3-wk blocks of overload training each followed by a taper and performance tests, with consumption in each block of an isocaloric drink containing either blackcurrant extract, megadoses of vitamin C, or placebo, with 4-wk washouts and performance pre-tests before each block to adjust for individual changes in performance over the extended period of the study.

MATERIALS AND METHODS

Subjects and study design

Twenty three trained female runners volunteered to take part in this study. The characteristics of the runners are presented in Table 17. Using a two treatment and placebo cross-over design, each runner completed three, 3-week blocks of high-intensity training separated by a washout period of normal training lasting 4 weeks. Each block was preceded by a 5-km time trial and after a short taper was followed by an incremental test and 5-km time trial. The runners were non-smokers, and did not take any antioxidant supplements for 1 month prior to the study. There was one withdrawal from the study as a result of injury. Runners completed a training log and drink compliance tick-box daily. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by Auckland University of Technology Ethical Committee. Runners attended a voluntary presentation on the demands of the study before giving their written consent to participate. See Appendix 11, 12 and 13 for the ethical approval, sample consent form and information sheet.

Exercise Performance Tests

Each 3-wk training block was preceded by a 5-km time trial and after a short taper was followed by an incremental test and 5-km time trial. Familiarization testing occurred in all runners for both the 5-km time trial and incremental treadmill test. All laboratory tests were performed on a calibrated treadmill (Powerjog, Mid Glamorgan, UK) in a temperature-controlled laboratory.

The 5-km time trial was performed on a loop road circuit close to the testing laboratory. The profile of the course was undulating including an ascent of 56 m covering 2.5-km of the total race and a descent of -57 m covering 1.5-km of the total race. All 5-km trials were held at the same time of the day in similar environmental conditions (except for one hot day which was accounted for in the analysis). Runners warmed up and began the time trial together to

maximize the impact of competition on performance results. Runners were instructed to treat each trial as a competition.

For the incremental treadmill test, runners performed a warm-up, stretching then began the test consisting of five to eight stages at an initial gradient of 1% and speed set relative to each runner's best 10-km race time. The speed of the treadmill was increased by 1 $km.h^{-1}$ for each subsequent stage for the first 4 stages, then in incline by 1% every minute until exhaustion.

Training

The training consisted of high-intensity intervals performed competitively on a hilly course, and the performance tests after each taper were a treadmill incremental test and a 5-km road time trial. Training programs were individualized for each runner according to their fitness level and current training load. Compulsory training sessions were completed 2-3 times a week and consisted of 4 near-maximal 3- to 5-min timed repetitions of a hilly course and 6 additional 2-min intense hill repetitions. Runners were categorized into one of two training groups, long or short session, based on their incremental run test during familiarization. The long session involved repetitions of 1-km, the short 650-m, both including steep inclines. Each compulsory training session was completed under supervision on the road accompanied by one of the researchers, with one session a week being timed. Examination of the training diaries showed all runners complied with the standardized training program.

Heart rate monitors (Polar S625x or $FSC3_c$; Polar Electro, Kempele, Finland) and training logs were provided and runners instructed to record every session, including duration, average and maximum heart rates. Morning resting heart rate was also recorded and averaged for the week (HR_{rest}). Calculations for the estimation of the training impulse (TRIMP) were derived from Banister (Banister, Morton, & Fitz-Clarke, 1992). The TRIMP score is equivalent to minutes spent training at a heart rate midway between maximum and resting. For females, TRIMP = training duration x $[(HR_{mean} - HR_{rest})/(HR_{max} - HR_{rest})] \times 0.86\exp[(HR_{mean} - HR_{rest})/(HR_{max} - HR_{rest}) \times 1.67]$, where training duration is the minutes spent training, HR_{mean} is the mean heart rate, HR_{rest} is the morning heart rate at rest and HR_{max} is the maximum heart rate derived from the laboratory exercise test.

Antioxidant Drinks

The three dietary treatments contained equal volumes of fluid and calories. The vitamin C (VC) treatment was prepared combining a commercially available fruit drink (Raro, Cerebos Gregg's Ltd, Auckland, NZ) with vitamin C powder (Melrose ascorbic acid, Melrose Laboratories Pty Ltd, Mitcham, Australia) to provide 1000 mg of vitamin C daily. The blackcurrant treatment (BC) was prepared by combining a fruit drink concentrate (Barkers Fruit, Berrylife, Geraldine, NZ) with blackcurrant juice powder (Tasman Extracts, Nelson, NZ) and blackcurrant extract (containing the following anthocyanins; delphinidin-3-glucoside 4.8%, delphinidin-3-rutinoside 17.6%,

cyaniding-3-rutinoside 14.7% and cyaniding-3-glucoside 2.1%; from Tasman Extracts, Nelson, NZ). The final nutrient content provided from the blackcurrant treatment each day was 300mg anthocyanin and 15 mg vitamin C. Placebo treatment (PL) was prepared using a commercially available sports drink, orange flavor (Replace, Horleys, Auckland, NZ). Drinks were prepared weekly and treatment was blinded to the researchers involved. Two of the runners reported gastrointestinal upset on the blackcurrant treatment, however not serious enough to limit consumption.

The runners completed an antioxidant food frequency questionnaire prior to commencing the study, designed to assess the overall antioxidant intake over the previous three weeks. The antioxidant questionnaire was repeated at the conclusion of each 3-wk training block to determine any changes in dietary habits. On the day of blood testing runners were asked to refrain from foods high in antioxidants including red wine, fruit juice, coffee, tea, chocolate drinks, chocolate and no more than three pieces of fruit. A food diary was completed on the day of blood testing to ensure compliance to the low antioxidant consumption and determine dietary habits for that day. The food diary included all food consumed until the completion of testing, so excludes the evening meal and supper. Examination of the food diaries showed that all runners complied with the pre-trial standardization protocol. The runners also completed a menstrual cycle log at the completion of each 3-week training block.

Blood Testing

Venous blood samples (10mL) were obtained from the antecubital vein of the runner into an EDTA containing vacutainer. Venous samples were obtained between 1-3 hours before, and 20-45 minutes after finishing the 5-km time trial. Blood samples were taken at the same time for each individual runner, during each trial.

A one milliliter aliquot of whole blood was refrigerated for in vitro erythrocyte antioxidant activity. Collected blood was centrifuged at 2700 g at room temperature for 2 min. A 200 µL sample of plasma was immediately transferred to 250 µL of MPA/EDTA for later analysis of vitamin C, and two 200 µL plasma samples were transferred to 40 µL of GSH/EDTA for later analysis of protein carbonyls (PC) and malondialdehyde (MDA). These samples and the remaining plasma were frozen and on dry ice and stored at -80^{o}C. The packed erythrocyte phase was washed three times with PBS and centrifuged as above, then frozen on dry ice and stored at -80^{o}C before analysis of antioxidant enzyme activity. Ascorbic acid and uric acid analysis were completed within a week of sample collection. Biochemical assays were performed in duplicate or triplicate.

Antioxidant enzyme activities and plasma determinations

Superoxide dismutase (SOD) was measured by the method described by Kakkar et al. (Kakkar, et al., 1984). The erythrocytes were first treated with ice cold chloroform and ethanol (ratio 62.5:37.5) and supernatant removed before measuring SOD activity. Glutathione peroxidase (GPx) was measured by the method described by Mannervik (1985). Catalase (CAT) was

measured using the method by Abei (1984). Hemoglobin was measured using Drabkins solution as described by Dacie & Lewis (1984). The average within-run coefficients of variation obtained for all samples, done in triplicate was 5.9% and 2.11% for GPx and SOD activities, respectively, and expressed relative to hemoglobin concentration. Catalase was completed as single measurements and thus coefficients of variation were not determined.

Ascorbic acid and uric acid were measured by HPLC with electrochemical detection as described by Lykkesfeldt (2002).

Oxidative stress markers

Plasma protein carbonyls were measured using a protocol described by Morabito and colleagues (2005). Briefly, plasma (100 µL) was added to an equal volume of 2,4-dinitro-phenylhydrazine (DNPH, Sigma-Aldrich, Auckland, NZ) in 2 M HCl (control =DNPH/HCl in the absence of plasma) and incubated in the dark for 1 h. Protein was precipitated with 50 % trichloroacetate (TCA, Sigma-Aldrich, Auckland, NZ) and washing with equal amounts of ethanol:ethylacetate, resuspended in 1 mL 6 M guanidine (Merck, NZ Ltd., Palmerston North, NZ) and the absorbance of the final suspension measured at 360 nm in a UV visible 1601 spectrophotometer (Shimadza Corporation, Kyoto, Japan). Carbonyl levels were calculated using the absorbance difference between test and control using the molar absorption coefficient (ε): 22,000 M-1cm-1. Plasma protein levels were measured using the Bradford method (Bradford, 1976) using commercial Bradford reagent (BioRad Laboratories Ltd., Auckland, NZ). Results are expressed as nmoles of protein carbonyls/mg total protein.

Malondialdehyde in plasma included the total MDA. Briefly, 100µl plasma was added to 100µl 2M sodium hydroxide and 20µl methanol (0.005% BHT; butylated hydroxy toluene; ICN Eschwege, Germany) and incubated at 60°C for 30 min then 65 µl 4M perchloric acid (PCA) was added. The sample was immediately vortexed and centrifuged at 10000g for 2-min. To 240 µl of the supernatant 24 µl 2M HCL 10 mmol DNPH was added and the mixture and incubated at 60°C for 30-min. The sample was then extracted with Hexane: Dichloromethane (80:20). The samples were blown dry in nitrogen and reconstituted in 15% methanol. The samples were then analyzed by HPLC with UV detection as described by Pilz et al. (2000).

The erythrocyte in vivo antioxidant capacity was determined as follows**.** Whole blood was refrigerated at 4ºC overnight. Samples were centrifuged for 5-min, 1000g at 4ºC and washed 3 times in saline. 33µl of packed cells were added to 300 µl saline and incubated for 5-min at 37ºC then mixed. 25 µl of the sample was added to a 1-ml cuvette then 1.2 ml Drabkins added to cuvette. Absorbance was measured at 540 nm (Dacie & Lewis, 1984). 100ml of 0.4 mmol cumene hydroperoxide was prepared. The cumene hydroperoxide was 88% (5.78 M). Mix 7µL cumene with 100 ml saline. The sample was mixed for 5-min by continuous stirring.

The in vitro incubation was prepared by adding 300 µl of the 0.4 mmol cumene hydroperoxide to the 308 µl of remaining erythrocyte solution, and then mixed. The solution was incubated for 30-min at 37ºC. The cells were lysed and erythrocytes precipitated by adding 300 µl of 0.6M PCA. The solution was immediately centrifuged and 500µL of the clear supernatant added to 100 µL of 8 mmol EDTA. Samples were frozen at -80°C until analysis.

Sample preparation for measurement of free MDA required that 200µL of supernatant had 20µL of 2M HCL 10 mmol DNPH added. The sample was incubated at 60°C for 30-min and analysed by HPLC with UV detection as described by Pilz et. al. (Pilz, et al., 2000).

Refer to Appendix 6 for further detail on the laboratory methods.

Statistical analyses

The data were analyzed using a mixed linear modeling procedure (Proc Mixed) in the Statistical Analysis System (version 9.2, SAS Institute, Cary, NC) to derive estimates of changes in the mean, using 5-km performance before each treatment as a covariate to adjust for individual differences in washout and maintenance of fitness between treatments. The test date, energy and carbohydrate intake were used as covariates for the 5km-time trial post block run. As was the stage of the menstrual cycle, where days 18-24 inclusive of the menstrual phase where coded as mid-luteal and remaining days were coded as other. Average time in weekly running repetitions (running speed in training) was included as a mechanistic covariate in separate analyses to determine the effect of training performance on 5-km race and laboratory speed.
The thresholds for small, moderate, large and very large effects on performance were assumed to be 0.3, 0.9, 1.6 and 2.5 of the race-to-race within-athlete variability in competitive performance of top athletes (Hopkins, et al., 2009). The variability in mean power output for endurance running and cycling in races are ~0.8% (Hopkins, et al., 2005) and ~3.5% (Paton & Hopkins, 2001) respectively, giving smallest effects of 0.25% and ~1.0%. We chose 0.5% as a value to apply to high-intensity endurance sports generally; thresholds for moderate, large and very large effects were therefore 1.5%, 2.7% and 4.2%. We made probabilistic magnitude-based inferences about the true values of outcomes, as described elsewhere (Hopkins, et al., 2009). In brief, an outcome was deemed unclear if its confidence interval overlapped thresholds for smallest worthwhile positive and negative effects; equivalently, effects were unclear if chances of the true value being substantially positive and negative were both >5%. The magnitude of a clear effect was reported as the magnitude of its observed value, sometimes with an assertion about the probability the true value was substantial. The plasma and erythrocyte markers of antioxidant status were standardized following log transformation, with thresholds for small, moderate, large are 0.20, 0.60 and 1.20, respectively (Hopkins, et al., 2009).

RESULTS

Subject characteristics are presented in Table 17.

Table 17: Descriptive characteristics of the 23 runners who completed the study

	Mean ± SD
Age (y)	31 ± 8
Weight (kg)	61 ± 7
Maximal oxygen uptake (ml.min^{-1}.kg^{-1})	49 ± 4
Average training pre-study (min.wk^{-1})	245 ± 130
Average training[a] (min.wk^{-1})	330 ± 100
Repetition run time long course[a] (min:s)	04:37 ± 00:22
Repetition run time short course[a] (min:s)	02:52 ± 00:14
Peak running speed[b] (km.hr^{-1})	16.8 ± 1.7
5-km race time[b] (min:s)	25:06 ± 2:51

[a]Mean over the 3 wk of training on placebo.
[b]After the 3 wk of training on placebo.

Examination of training logs showed all runners complied with the standardized training program, with only trivial differences between dietary interventions on total TRIMP scores for the 3-wk of training (see Table 18). The training completed during the 3-wk was a clear, small to moderate increase in TRIMP compared with the runners' usual training. There was a moderate increase in training from baseline to Week 1 and Week 2 of the study.

Table 18: Intensity and duration of training as quantified by the weekly training impulse (TRIMP) index (min) at baseline and during each week of the 3-wk training blocks

Treatment	Baseline	Week 1	Week 2	Week 3
Blackcurrant	650 ± 430	1130 ± 370	900 ± 260	650 ± 200
Vitamin C	680 ± 450	1020 ± 400	900 ± 330	640 ± 340
Placebo	600 ± 350	1120 ± 400	830 ± 270	610 ± 200

Data are mean ± SD.

All runners reported 100% compliance with the daily drink consumption, determined by the daily drink compliance tick-box. Excluding the antioxidant drinks, all runners consumed a low antioxidant diet on testing day, with trivial differences between dietary interventions. Over the three week intervention period there were trivial differences in antioxidant intake on all treatments, from non-treatment sources (see Table 19). Differences in energy intake on the day of testing were at most small (mean ± SD; BC 4.4 ± 1.2 MJ; VC 4.6 ± 1.9 MJ; PL 4.1 ± 1.5 MJ). Differences in carbohydrate intake on the day of testing were also small at most (BC 147 ± 44 g; PL 134 ± 47 g; VC 169 ± 84 g); carbohydrate intake was nevertheless included as a covariate in the analysis of time-trial time and was shown to improve performance (1.0% per 100g of carbohydrate consumed; 90% confidence limits ±1.1%).

Table 19: Dietary antioxidant intake during the 3-wk training block and on 5-km race day

	During (mmol.wk^{-1})		On race day (mmol)	
Treatment period	from diet[a]	from treatment	from diet[b]	from treatment
Baseline	45 ± 22	-	-	-
Blackcurrant	43 ± 24	36	2.5 ± 1.3	5.1
Vitamin C	45 ± 29	10	2.2 ± 1.0	1.4
Placebo	47 ± 32	0	2.6 ± 1.5	0.0

Data from diet are mean ± SD; data from treatment represent content in the supplements.
[a]Determined via an antioxidant food-frequency questionnaire.
[b]Determined via food diary.

Interval repetition running times were slower during the VC and blackcurrant compared to placebo for the average runner (see Table 20). Faster runners (defined by +1 standard deviation of mean speed on the incremental running test) were also slower on vitamin C and on blackcurrant, compared with placebo (see Table 20). By week 3 the differences in repetition times were trivial between each dietary intervention.

Table 20: Effects (%) on running speed of blackcurrant (BC) and vitamin C (VC) supplements compared with placebo (PL)

	BC vs PL	VC vs PL
Intervals in training		
All runners	-1.0; ±1.3 (possibly slower)	-1.3; ±1.3 (likely slower)
Faster runners	-1.2; ±1.8 (likely slower)	-2.1; ±1.8 (likely slower)
Peak incremental test		
All runners	0.8; ±1.7 (unclear)	0.7; ±1.8 (unclear)
Faster runners	1.9; ±2.5 (likely faster)	0.9; ±2.5 (unclear)
5-km time trial		
All runners	-0.9; ±1.6 (possibly slower)	0.1; ±1.6 (unclear)
Faster runners	0.6; ±1.8 (unclear)	0.1; ±1.9 (unclear)

Data are mean percent effect with 90% confidence limits; qualitative clinical inference is shown in parentheses. Faster runners are those whose mean performance in the three pre-treatment incremental tests was 1 SD faster than the mean for all runners.

Each 3-wk training block had a moderate to large improvement in 5-km race times (mean ± SD; Block1, 4.0% ± 3.1%; Block 2, 3.0% ± 4.6%; Block 3, 2.4% ± 3.8%). The average runners were possibly slower on blackcurrant, but very fast runners (+2SD) had improved running performance in the 5-km race (3.0%, ± 90% CL 2.9%) and the incremental running test (2.3%, ±3.6%). The effect of the menstrual cycle on 5-km time trial performance was trivial but unclear

(0.3%, ±2.5%). The results of the laboratory incremental running test suggest faster runners benefit from BC, but unclear on vitamin C (see Table 20). The effect of the menstrual cycle on the laboratory running test was trivial, but unclear (-0.1% ± 1.7%). The 5-km times had a moderate to large improvement on all dietary treatments (mean ± SD; PL 3.8% ± 4.3%; BC 2.4% ± 3.9%; VC 3.2% ± 3.6%), with differences between treatments unclear. Training-performance time included in the analysis as a mechanistic covariate revealed a clear relationship between percent change in training-performance time and percent change in 5-km time between treatments (0.8; ±0.4 % /%), accompanied by a slight reduction in the effects of the antioxidant drinks. The effect of inclusion of training performance in the analysis of laboratory run speed was trivial (-0.3; ±0.6 %/%) and there was little accompanying reduction in the effect of the drinks.

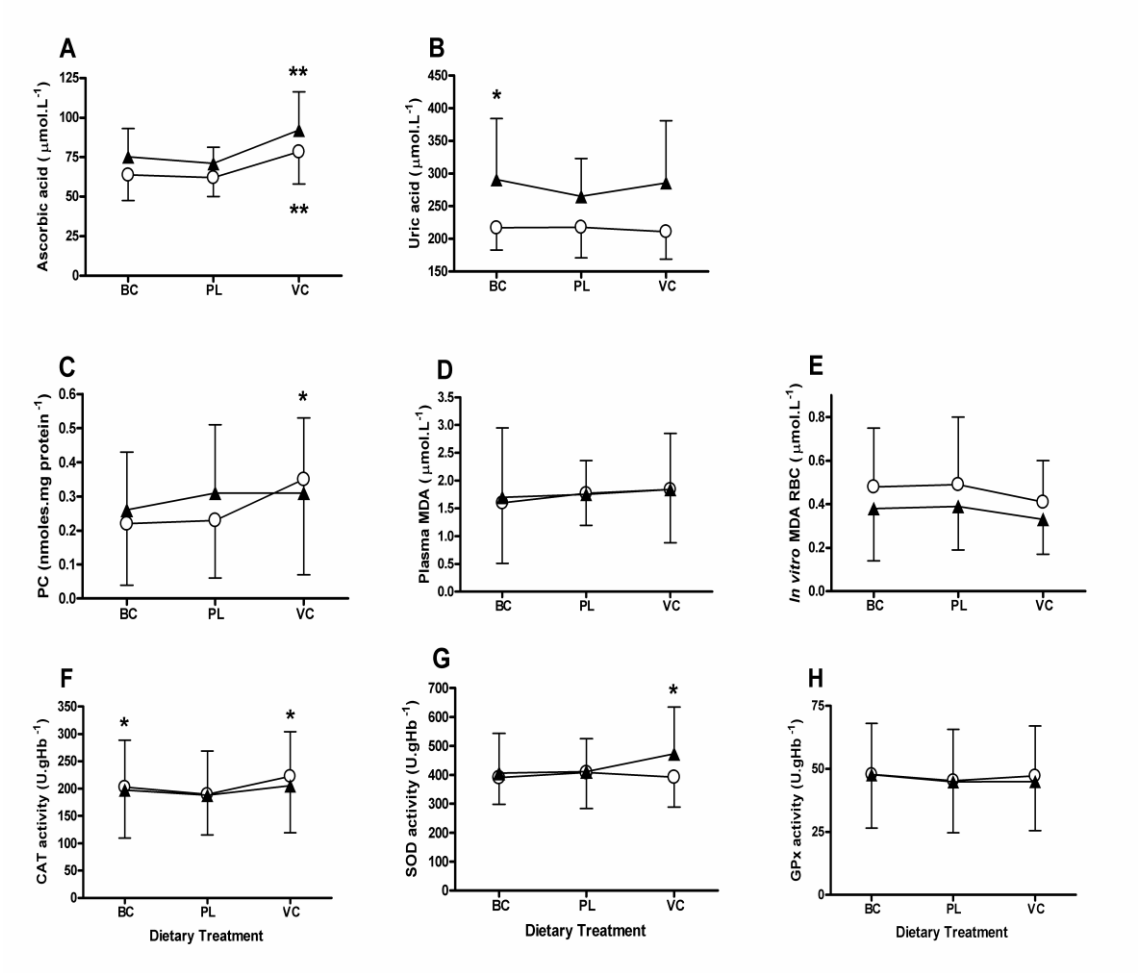

Figure 2: Biomarkers of antioxidant status and oxidative stress, pre (○) and post (▲) exercise, in three dietary antioxidant supplements (BC-blackcurrant, PL-placebo, VC-Vitamin C). Levels of antioxidants in plasma (Plasma ascorbic acid and uric acid concentration in plasma (A and B, respectively). Oxidative stress markers (protein carbonyl and malondialdehyde concentration in plasma (C and D, respectively) and in vitro malondialdehyde concentration in erythrocytes (E)). Erythrocyte antioxidant enzyme activity (F, catalase; G, superoxide dismutase and H, glutathione peroxidise). Data are means; error bars are SD. Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

*Small, clear difference from placebo treatment.

**Moderate, clear difference from placebo treatment.

Plasma concentrations of the antioxidants ascorbic acid and uric acid displayed a clear, small to moderate rise with exercise (Figure 2, A and B). Following the vitamin C runners had a clear, small increase in ascorbic acid levels in the plasma both at rest and following exercise. There were only trivial differences between the plasma levels of uric acid on each of the dietary treatments, at rest and after exercise. There was a small, possible effect of the menstrual cycle on ascorbic acid (8%, ±12%) and uric acid (5.8%, ±10.2%).

The markers of oxidative stress, MDA and PC and an oxidative challenge test using erythrocytes (MDA-erythrocytes) were measured. Blackcurrant had a small, possibly beneficial effect on plasma MDA at rest, compared with placebo (see Figure 2, D). Trivial differences were seen with VC and blackcurrant in plasma MDA, following exercise. Compared with placebo, VC and blackcurrant had trivial differences between erythrocyte MDA, following *in vitro* stimulation, at rest and post exercise (see Figure 2, E). Protein carbonyls at rest showed a small, possibly harmful effect after VC(see Figure 2, C).

The activity of erythrocyte enzymes responsible for the removal of reactive species were determined (glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT)). GPx activity had a trivial difference following any of the dietary interventions (see Figure 2, H). There was a small, possible effect of the menstrual cycle on GPx activity (5.8%, ±7.6%). CAT activity showed a clear decrease with training, from Block 1 to 2 to 3 (mean at rest ± SD; 269 ± 74; 188 ± 77; 162 ± 57 $U.gHb^{-1}$). Vitamin C had a clear, harmful effect on CAT activity at rest (see Figure 2, F). and SOD activity post exercise (see Figure 2, G). There was a small, possible effect of the menstrual cycle on SOD activity (-6.3%, ±9.1%).

DISCUSSION

The aim of this study was to investigate the effects of three weeks of hard training in combination with dietary antioxidant intake on training speed, racing speed and antioxidant status of the blood. Vitamin C and blackcurrant extract impaired training intensity clearly by ~1%, but in spite of the large sample size and sophisticated analysis, mean effects on the performance tests were unclear (i.e., possibility of small beneficial effects but unacceptable risk of small harmful effects). It is possible that there were beneficial acute effects from the blackcurrant on the day of the tests that were offset by harmful chronic effects on training, an issue that requires further research. Blackcurrant had little effect on antioxidant status, but vitamin C appeared to increase markers of oxidative stress, including protein carbonyls (PC), catalase (CAT) and superoxide dismutase (SOD) activity, suggesting it may have acted as a pro-oxidant.

The weekly training load represented by the TRIMP scores (a mean of 938) was similar to that in another study of competitive runners (900) (Iwasaki, Zhang, Zuckerman, & Levine, 2003). This training resulted in large gains in performance in all three treatment conditions. Similar high-intensity training for as little as 1-2 wk produced substantial changes in mitochondrial

enzymes (Green, et al., 2009; Little, Safdar, Wilkin, Tarnopolsky, & Gibala, 2010) and muscle capillarisation (Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003) that could explain the improvements in aerobic performance in the present study. Thus our runners must have experienced substantial physiological adaptations that the supplements could have modified.

Vitamin C is recognized as an excellent antioxidant, but the consumption of excessive amounts may be detrimental to athletic performance via the blockage of exercise-dependent production of reactive species (Gomez-Cabrera, et al., 2008). One of the primary mechanisms by which reactive species are thought to induce training adaptations is by stimulation of mitochondrial growth and antioxidant enzyme up regulation, in skeletal muscle (Little, et al., 2010, Ristow, 2009 #236). Ristow et. al. (2009) demonstrated that 4 weeks of exercise training induced expression of mitochondrial growth markers, which treatment with vitamin C and E prevented.

Previous research has demonstrated the ability of dietary antioxidants to negate training improvements. Vitamin C has been shown to reduce athletic performance in racing greyhound dogs given 1 g of vitamin C daily for 4-wk, with supplemented dogs running an average 0.2 s slower (Marshall, et al., 2002). The exact mechanisms for the decrements in performance are possibly a result of sub-optimal mitochondrial biogenesis, as discussed, and/or the large dose antioxidants acting as a pro-oxidant and hampering muscle force generation or exercise recovery. Vitamin C, and to a lesser extent blackcurrant, were reducing training performance in the runners by possibly acting as a pro-oxidant.

Vitamin C in high concentrations is capable of acting as a pro-oxidant in the presence of available iron (Podmore, et al., 1998; Stadtman, 1991). It is debated whether these findings are relevant to the plasma of athletes, where most of the transition metals are not free but attached to binding proteins, and thus prevented from participating in free radical reactions (Duarte & Lunec, 2005; Levine, 1986). Certainly in trained athletes, serum iron has been shown to increase after exercise, without a concomitant increase in iron binding capacity (Smith & Roberts, 1994). Bryant (2003) investigated the impact of vitamin C and E supplementation on elite cyclists and suggested that 400 IU/day of vitamin E provided adequate oxidative protection but supplementing the diet with 1 g per day of vitamin C may promote cellular damage. Vitamin C as a pro-oxidant may increase tissue damage or reduce force production within the muscle. Like vitamin C, some flavonoids can also have pro-oxidant effects taken in large doses, thus while berry beverages and extracts appear to increase antioxidant protection and reduce exercise induced oxidative stress in moderate intakes (Matsumoto, Nakamoto, Hirayama, Yoshiki, & Okubo, 2002) they are capable of pro-oxidant activity (Laughton, Halliwell, Evans, Robin, & Hoult, 1989).

The vitamin C increased the activity of CAT at rest and SOD post exercise. We also found CAT activity was higher at rest on blackcurrant. A marker of oxidative stress (PC) was higher at rest on vitamin C, but lower on blackcurrant. A secondary marker of oxidative stress, MDA, was

lower on blackcurrant at rest. The results of the blood assays suggest vitamin C increases oxidative stress, and blackcurrant may lower it. The possibility that vitamin C is acting as a pro-oxidant is reflected in the increase in oxidative stress markers.

In contrast to vitamin C, blackcurrant possibly assisted performance in faster runners completing the laboratory incremental running test. Mechanisms by which polyphenols may confer performance benefit involve changes in vascular function such as improvement of the endothelial function and proliferation in blood vessels (Ghosh & Scheepens, 2009; Stoclet, et al., 2004). Studies have indicated that in addition to, and independently of their antioxidant effects, plant polyphenols can enhance the synthesis of vasodilators and inhibit the synthesis of vasoconstrictors by endothelial cells (Schini-Kerth, et al., 2010).The major polyphenols shown to influence vascular function in humans are primarily from cocoa, wine, grape seed, berries, tea, tomatoes, soy and pomegranate (Ghosh & Scheepens, 2009). Grape seed extract has been shown to alter vascular function, endothelial function, improve flow-mediated dilatation of blood vessels and reduce oxidative damage in a randomized, control cross-over study (Clifton, 2004). Blackcurrant has been shown to alter peripheral circulation dynamics, thus improving blood flow, at rest or after typing work in nine healthy males. In athletes provided ground blackcurrant fruit for six weeks, performance in a 2000-m ergometer time trial did not change, but parameters of blood flow did, suggesting an important role of polyphenols even for trained participants (Skarpanska-Stejnborn, et al., 2006).Therefore, polyphenols may enhance performance acutely and warrant further investigation.

The menstrual cycle was found to influence blood measures of ascorbic acid, uric acid, GPx and SOD activity. Southam and Gonzaga (Southam & Gonzaga, 1965) reported decreased ascorbic acid excretion during the luteal phase of the menstrual cycle, although not widely reported in more recent literature. Previous research has found menstrual cycle phase dependent changes in erythrocyte GPx activity, but not SOD or CAT (Massatra, et al., 2000). The menstrual cycle phase did not affect the oxidative stress markers (PC and MDA), as reflected in another study (Chung, Goldfarb, Jamurtas, Hegde, & Lee, 1999). The running parameters had a trivial change with the menstrual cycle, similar to results reported in other studies investigating the impact of the menstrual cycle of endurance performance (de Jonge, 2003).

In conclusion, chronic consumption of vitamin C, and to a lesser extent blackcurrant, decreased training speed in female runners. Vitamin C appears to increase markers of oxidative stress, including PC, CAT and SOD activity, suggesting pro-oxidant action. However, there is a possibility that the faster runners benefitted from blackcurrant in the 5-km time trial and the laboratory test. Further research is needed on acute ingestion of blackcurrant, in case any harmful effect on training offsets possible benefit of ingestion on race day.

CHAPTER 7

CONCLUSION OF THE THESIS

The research presented in this thesis demonstrates the crucial role dietary intake has on the antioxidant status and athletic performance of athletes. There is clear evidence that an increase in reactive species production occurs as a result of exercise. However, prior to the research conducted in this thesis, it was less clear whether the exercise-induced oxidative stress had a harmful impact on performance. Therefore, the aims of this thesis were threefold: to determine dietary antioxidant intake in athletes; investigate the relationships between antioxidant intake and antioxidant status in athletes of an endurance sport, and to study the effects of antioxidant intake on performance.

The measurement of dietary antioxidant intake has previously been problematic due to the time-consuming nature for both completion and analysis of food diaries and the lack of food composition data on complete antioxidant content/action of foods. Chapter 3 presents the development and validation of a food-frequency questionnaire for use with athletes. The correlation between the blood antioxidant biomarker and the antioxidant intake reported in the food diary was trivial, prompting a journal reviewer to conclude the basis of the questionnaire validation was flawed. However I was able to argue convincingly that while the biomarker is a useful addition to the research, it should not overtake the food diary as the gold standard comparison. Chow and colleagues (Chow, et al., 2007) highlight the concerns with using an antioxidant biomarker as a gold standard measure of dietary antioxidant intake. To interpret a lack of association between the blood biomarker and the food diary does not mean the food diary result is incorrect, but rather the blood biomarker is only capable of determining antioxidant intake over a few days (and therefore correlates with the questionnaire, completed at the same time as the blood test) but not the food diary. The food-frequency questionnaire is less labour intensive for the athlete and researcher than a 7-d diary and appears to be at least as trustworthy for estimating antioxidant intake.

The study described in Chapter 4 assessed associations between dietary nutrients, antioxidants biomarkers, with training and performance. The diet of the rowers appeared to be balanced and sufficient in dietary antioxidants. The majority of dietary factors had weak to insubstantial associations with antioxidant status. The major dietary concern was the mean low intake of vitamin D, likely caused by the limited number of New Zealand foods that contain vitamin D. The implications of inadequate vitamin D intake may include increased stress fracture risk and other musculoskeletal issues (Larson-Meyer & Willis, 2010). The other dietary feature was the high intake of vitamin C by athletes, some of whom where mega-dosing in the order of 1 g daily. The results presented in Chapter 4, consistent with other research, reflect the fact that elite athletes are more likely to supplement than other populations (Sungot-Borgen, et al., 2003). The primary source of vitamin C in rowers consuming greater than 500 mg daily came from supplements

rather diet. The main result in this chapter demonstrated that antioxidant status is associated with hours of training more so than with diet. The impact diet has on antioxidant status in a less nourished population may be quite different from our results.

Given the conventional dogma that oxidant stress is harmful, many athletes supplement their diet with vitamin C and other antioxidants to protect against oxidant stress (Clarkson & Thompson, 2000). However, reactive species not only cause damage but may play a role in cell signalling for training adaptation (Gomez-Cabrera, et al., 2008: Ristow, 2009 #236). Reducing concentration of reactive species via antioxidant supplementation could therefore have negative consequences by attenuating adaptations to training. In Chapter 5 the literature on antioxidants and their impact on athletic performance was discussed. Mega-doses of vitamin C impaired athletic performance substantially in the majority of studies, possibly by reducing mitochondrial biogenesis, altering fuel utilization via reduction in interleukin-6, or by acting as a pro-oxidant. I speculated that smaller doses of vitamin C may be sufficient to reduce oxidative stress without impairing training adaptations. Vitamin E had little effect on athletic performance, although effects of megadoses on endurance performance in rodents were beneficial. Polyphenol supplementation improved endurance performance in all studies, although not all outcomes were clear. Acute N-acetyl-cysteine intake improved performance in the majority of studies, but it has toxic side effects. Overall, the impact of chronic consumption of antioxidants is likely to be harmful to training. I recommend further research on the effects of moderate doses of vitamin C and polyphenols taken chronically and on the effects of all antioxidants taken acutely before exercise.

To investigate the notion that antioxidants may be detrimental to performance Chapter 6 describes the study on vitamin C and blackcurrant on performance in female runners. I expected the consumption of vitamin C to reduce the benefits of training, through reductions in reactive species mediated training adaptations. There was indeed a harmful effect with consumption of vitamin C and blackcurrant on training speed taking vitamin C and blackcurrant juice, but faster runners had a possible benefit on run performance taking blackcurrant juice. Based on this research I would discourage the consumption of megadoses of vitamin C or polyphenols chronically, but am still undecided about the possibility that polyphenols taken acutely may benefit performance.

If I had my time again and were to repeat the experimental studies described in this thesis, I would include a larger group of athletes in the validation of the food-frequency questionnaire, and ideally use athletes from a range of sports and cultural backgrounds. In the correlational study described in Chapter 4, I would have included a measure of oxidative stress, such as protein carbonyls or malondialdehyde, and I would include an objective training monitor to better quantify the training load. I would also have liked to include a larger group of athletes with a wider range of antioxidant intakes. In the final experimental study described in Chapter 6, I

would include an additional study looking at the acute effects of polyphenols on elite athletes, given the possible benefit we saw on performance in the faster runners.

Towards the final stages of completing the doctorate I moved from the New Zealand Academy of Sport to my current role as Sport Dietitian with the United States Olympic Committee. In this new role I have begun to implement the following recommendations, based on the results and my personal reflections of this research.

1. Fruit and vegetables contain a naturally occurring array of plant-based antioxidants and consumption should be encouraged.
2. I would recommend ceasing chronic supplementation of any of the dietary antioxidants is unwise due to the negative impact it may have on training adaptations.
3. Quercetin supplementation is likely to be beneficial for endurance athletes in days, but we would be cautious to recommend for longer.
4. Polyphenols are a promising group of antioxidants for performance enhancement with effects mediated with several possible physiological actions.
5. Moderate doses of antioxidants consumed at particular times of an athlete's training cycle may enhance immunity, improve muscle damage and general well-being, without crossing the threshold to limiting training adaptations.

I would also like to make the following observations:

1. Elite athletes are likely to supplement with antioxidants, but rarely understand or can explain why they do so.
2. As a result of doing this thesis I feel it is too difficult at present to make any recommendation based on antioxidant biomarkers alone.

The most pressing research that I feel needs to be done now is the acute supplementation using the various polyphenols. I would like to make a recommendation to athletes to supplement with polyphenols one week from a competition, but do feel the evidence to date is lacking. Acute supplementation of antioxidants may have substantial benefits to performance that we are missing in the current literature as the majority of studies are chronic, greater than one week. In ideal circumstances dietitians will develop nutrition plans based on the evidence of dosage and timing of antioxidant supplements to achieve optimal health, recovery and performance of athletes.

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APPENDIX 1: REACTIVE SPECIES

The main representatives of reactive species are discussed below:

Superoxide anion, O_2^- is not usually very reactive, but it can generate more toxic products. The O_2^- vigorously seeks to strip an electron from biological molecules to pair up the lone electron in its outer orbit, thus it is able to attack, for example, sugars, proteins, polyunsaturated fatty acids (PUFA) and DNA (H. M. Alessio, et al., 1997). The O_2^- is produced by the activated phagocytes such as neutrophils, monocytes, macrophages, and eosinophils to kill the pathogen organisms. However, this normal protective mechanism may cause damage to the cells (Jackson, 2000).

The **hydroxyl radical, OH*** is extremely reactive and is most likely the final mediator of free radical induced tissue damage (White, et al., 2000). It attacks all proteins, DNA, polyunsaturated fatty acids (PUFA), and almost any biological molecules it touches. The hydroxyl radical is an initiator of lipid peroxidation: OH* removes a hydrogen atom from the unsaturated fatty acids (LH) of membrane phospholipids resulting in the formation of free lipid radical (L*) (Leeuwenburgh & Heinecke, 2001).

$$LH + OH^* \rightarrow L^* + H_2O$$

The lipid radical, in turn, reacts with molecular oxygen and forms a lipid peroxide radical (LOO*)
$L^* + O_2 \rightarrow LOO^*$ Like OH* the lipid peroxide radical can function as an initiator of oxidation by removing another hydrogen atom from a second unsaturated fatty acid

$$LOO^* + LH \rightarrow LOOH + L^*$$

Thus a chain reaction is initiated.

Lipid monohydroperoxidases (LOOH) formed in this reaction can be used as a marker of lipid peroxidation. The result is destruction of the unsaturated fatty acids of phospholipids, loss of membrane integrity and cell death.

Hydroxyl radicals can also modify cell membrane proteins by formation of disulfide (S-S) bonds resulting in aggregation of membrane proteins, forming ion channels and finally disruption of membrane structure and function. Hydroxyl radicals can interact with DNA and inhibit its replication (Asmus & Bonifacic, 2000).

Hydrogen peroxide (H_2O_2) is not a radical as it is electrically neutral. It resembles water in its molecular structure and is very diffusible within and between cells. Hydrogen peroxide has low reactivity but in excess it can attack certain cellular targets, blocking glycolysis and interfering with cell energy metabolism (production of ATP). The H_2O_2 molecule can also damage DNA. Free iron and copper ions can decompose H_2O_2 to OH*; therefore, the “sequestration” of metal ions (bound to transport or storage proteins) is an important body defence mechanism (Asmus & Bonifacic, 2000).

Nitric oxide radical (NO*) is synthesized by vascular endothelial cells, phagocytes, and by the certain cells in brain. Nitric oxide has physiological importance as a vasodilator agent and a neurotransmitter. However, in excess and in combination with oxygen it can be cytotoxic. **Peroxynitrite** ($ONOO^-$) is the product of NO* and O_2^-. Peroxynitrite can cause direct biological damage by oxidizing the thiol (-SH) groups of proteins, can be a powerful initiator of lipid peroxidation (Asmus & Bonifacic, 2000) (Leeuwenburgh & Heinecke, 2001) , can deplete antioxidants such as glutathione and vitamin C, and can inactivate α_1-untiproteinaze, a major inhibitor of proteolyses enzymes in plasma (White, et al., 2000) . The $ONOO^-$ complex can inhibit the mitochondrial respiratory chain leading to energy failure and ultimately cell death.

Free metal ions such as Fe^{3+} are required for partial reduction of oxygen species to injure cells (Leeuwenburgh & Heinecke, 2001). Cells obtain iron from the plasma as ferric iron (Fe^{3+}) bound to **transferrin**. It is delivered to the cytoplasm where an acidic environment releases free ferric iron and then it is stored as ferritin. However, free ferric iron can be reduced by superoxide anions to ferrous iron (Fe^{2+}). Ferrous iron then reacts with the H_2O_2 to produce OH* (Fenton reaction):

$-Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2$

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

The hydroxyl free radical (OH^*) is the end product of the Fenton reaction and is a very reactive and toxic ROS. This ROS causes lipid peroxidation and protein oxidation.

Dependent on their mode of action antioxidants can be divided into three classes:
Primary antioxidants – prevent formation of new ROS. This includes antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and metal binding proteins such as ceruloplasmin (copper binding protein), transferrin (iron binding protein), and ferritin.
Secondary antioxidants – also called the chain breaking antioxidants, include vitamin E, vitamin C, vitamin A, uric acid, and albumin. These antioxidants remove newly formed free radicals before they can initiate a chain reaction. The first three are essential vitamins i.e. they must be consumed as they are only available from foods, drinks or dietary supplements.
Tertiary antioxidants – include repair enzymes as they repair cell structures damaged by free radicals.

The O_2^- anions are removed by **superoxide dismutase (SOD),** which is one of the most important cellular antioxidants. SOD is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) and is the main scavenger of O_2^- (Finaud, et al., 2006).

$-O2 + 2H^+ \rightarrow H2O2$

The conversion of O_2^- to H_2O_2 is a useful temporary fix, but removal of H_2O_2 is critical for cell survival, since although it is a weak oxidative agent, H_2O_2 can cross cell membranes (unlike O_2^-) and directly damage proteins and particular enzymes (Asmus & Bonifacic, 2000).

Glutathione peroxidase (GPx), a mitochondrial enzyme, is another cellular antioxidant. GP uses reduced glutathione (GSH) as a cofactor to reduce H_2O_2 to produce water and 2 molecules of oxidized glutathione (GSSG) (Finaud, et al., 2006).

$$H_2O_2 + 2GSH \rightarrow 2GSSG + 2H_2O$$

GP requires the presence of selenium at the active site and is the main scavenger of H_2O_2 (Finaud, et al., 2006).

Catalase, a heme containing protein, is another free radical trapping enzyme. It acts like GP by reducing H_2O_2 into water and can also detoxify the O_2^- anion (H. M. Alessio, et al., 1997). Catalase is largely located within cells, in peroxisomes which are organelles involved in the nonmitochondrial oxidation of fatty acids and amino acids; thus it plays an important role in the inhibition of lipid peroxidation and preventing damage to DNA and RNA (Finaud, et al., 2006).

APPENDIX 2: COPYRIGHT PERMISSION FOR FIGURE 1 AND TABLE 4

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Sent: Wednesday, 19 May 2010 1:37 p.m.
To: Andrea Braakhuis
Subject: Re: Journal Copyright
Hi Andrea

Confirmation that you have permission

Graham

Graham Pearson
Treasurer
NSNZ
PO Box 2039
Gonville
Whanganui 4543
06-344-1012
027-222-8378

From: Andrea Braakhuis
Sent: Wednesday, 19 May 2010 12:40 p.m.
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Subject: RE: Journal Copyright
Hello,
Following up on the copyright of the table referred to below. I have tried to contact the author through previous place of employment, but had no luck.
I would like to confirm that I have permission to use the table in a thesis and possible journal review article. The thesis is at doctoral level with Auckland University of Technology.

Thank-you for your help.
Andrea Braakhuis
PhD student

From: Andrea Braakhuis [mailto:Andrea.Braakhuis@wintec.ac.nz]
Sent: Tuesday, 30 March 2010 11:41 a.m.
To: Stonehouse, Welma
Subject: Journal Copyright

Hello,
I am a member of the nutrition society and therefore get access to the Proceedings Journal. I am writing a literature review as part of my doctoral thesis and wondered if you could point me in the right direction as to how I obtain permission to reproduce a table from the article referenced below.

Lister CE. Antioxidants: from anthocyanins to zeaxanthin. *Proceedings of the Nutrition Society of New Zealand.* 2006;31:37-50.

Thanks,
Andrea Braakhuis

APPENDIX 3: ETHICAL APPROVAL FOR CHAPTER 3 AND 4

MEMORANDUM

Auckland University of Technology Ethics Committee (AUTEC)

To:Will Hopkins

From: **Madeline Banda** Executive Secretary, AUTEC

Date: 2 March 2007

Subject: Ethics Application Number 06/230 **Associations between vitamin C, antioxidant status, diet and markers of post exercise inflammation and oxidative stress.**

Dear Will

Thank you for providing written evidence as requested. I am pleased to advise that it satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC) at their meeting on 11 December 2006 and that as the Executive Secretary of AUTEC I have approved your ethics application. This delegated approval is made in accordance with section 5.3.2.3 of AUTEC's *Applying for Ethics Approval: Guidelines and Procedures* and is subject to endorsement at AUTEC's meeting on 12 March 2007.

Your ethics application is approved for a period of three years until 2 March 2010.

I advise that as part of the ethics approval process, you are required to submit to AUTEC the following:

A brief annual progress report indicating compliance with the ethical approval given using form EA2, which is available online through *http://www.aut.ac.nz/research/ethics*, including when necessary a request for extension of the approval one month prior to its expiry on 2 March 2010;

A brief report on the status of the project using form EA3, which is available online through *http://www.aut.ac.nz/research/ethics*. This report is to be submitted either when the approval expires on 2 March 2010 or on completion of the project, whichever comes sooner;

It is also a condition of approval that AUTEC is notified of any adverse events or if the research does not commence and that AUTEC approval is sought for any alteration to the research, including any alteration of or addition to the participant documents involved.

You are reminded that, as applicant, you are responsible for ensuring that any research undertaken under this approval is carried out within the parameters approved for your application. Any change to the research outside the parameters of this approval must be submitted to AUTEC for approval before that change is implemented.

Please note that AUTEC grants ethical approval only. If you require management approval from an institution or organisation for your research, then you will need to make the arrangements necessary to obtain this.

To enable us to provide you with efficient service, we ask that you use the application number and study title in all written and verbal correspondence with us. Should you have any further enquiries regarding this matter, you are welcome to contact Charles Grinter, Ethics Coordinator, by email at charles.grinter@aut.ac.nz or by telephone on 921 9999 at extension 8860.

On behalf of the Committee and myself, I wish you success with your research and look forward to reading about it in your reports.

Yours sincerely

Madeline Banda

Executive Secretary

Auckland University of Technology Ethics Committee

APPENDIX 4: SAMPLE OF A PARTICIPANT CONSENT FORM FOR CHAPTER 3 AND 4

CONSENT FORM	AUT UNIVERSITY TE WĀNANGA ARONUI O TAMAKI MAKAU RAU

Project title: Associations between vitamin C, antioxidant status, diet and markers of post exercise oxidative stress.
Project Supervisors: Will Hopkins & Elaine Rush. Tim Lowe (HortResearch)
Researcher: Andrea Braakhuis

○I have read and understood the information provided about this research project in the Information Sheet dated 21 December 2006.
○I have had an opportunity to ask questions and to have them answered.
○I understand that the project will involve writing down what I eat and training that I do for 7 days.
○I understand that the project will involve a blood test before and after a rowing ergometer test.
○I agree my individual information will be viewed by the researchers and personal coach:
Please tick one: Yes○No○
○I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way.
○If I withdraw, I understand that all relevant information will be destroyed.
○I agree to take part in this research.
○I wish to receive a copy of the report from the research (please tick one): Yes○No○

Participant's signature:.....
Participant's name:.....
Participant's Contact Details (if appropriate):
……..
……..
……..
……..
Date:

Approved by the Auckland University of Technology Ethics Committee on 2nd March, 2007. AUTEC Reference number 06/230

Note: The Participant should retain a copy of this form.

APPENDIX 5 SAMPLE OF A PARTICIPANT INFORMATION FORM FOR CHAPTER 3 AND 4

Participant Information Sheet

Date Information Sheet Produced:

20th December 2006.

Project Title

Associations between vitamin C, antioxidant status, diet and markers of post exercise oxidative stress.

You are invited to participate in a study to find out how diet influences exercise and the way the body responds through metabolic stress. Diet is known to influence sporting performance in many ways however it is still unclear as to whether diet, and specifically antioxidants, can influence post exercise antioxidant ability of the body.

This research will contribute towards a doctorate and will be conducted by Andrea Braakhuis. Participation is voluntary and you may withdraw at any time without penalty.

What is the purpose of this research?

The purpose of this research is to investigate how antioxidant nutrients in the diet can influence how a rower responds to an exercise session. Does diet alter the way a rower will respond to training?

In addition to this, the research results will be presented at sport medicine conferences and written up for publication, but results will be presented as averages from 100 rowers. Individual results will not be presented.

How was I chosen for this invitation?

The study requires well trained rowers and therefore as you are associated with New Zealand rowing this makes you an ideal candidate for this research.

What will happen in this research?

You will be given a 7-day food diary, a tick box form regarding usual intake of antioxidant foods and a questionnaire regarding training status, weight history and medication such as the oral contraceptive pill (for females). The 7 day food diary will be completed in your own time before the exercise test. In consultation with your coach an ergometer test will be set up, usually corresponding to a standard training test time, but preferably the morning. A blood test will be taken before and after the ergometer test. You will be asked to refrain from eating on the morning before the exercise test.

In addition to this, if the results suggest that you have; 1) a poor dietary intake of antioxidants and 2) an exaggerated oxidative stress response to exercise you will have the option to take part in a second study which will provide you with a supplement to potentially improve this situation and therefore improve recovery from exercise. This study will be separate, unrelated to the current study but is aimed at decreasing the oxidative stress in athletes. Further details will be provided if it is determined that you may benefit.

What are the discomforts and risks?

A 7-day food diary takes time to complete and will require you to set aside 15-30 minutes daily to do properly. Blood tests can be a little uncomfortable for many athletes, however minimal amounts of blood will be taken and they will be completed by trained individuals. .The research will exclude rowers who have been sick the one week previous, smokers, those on anti-inflammatory medication and those who have exercised the day of testing.

What are the benefits?

You will have a complete and accurate 7 day food diary analysed and you will obtain information relating to measures of oxidative stress following exercise. Together this information can be used to give you personalised feedback regarding the quality of your diet and how the body is coping with an exercise bout.

How will my privacy be protected?

No material which could identify you personally will be used in any reports on this study. The only identifiers to your information will be the researchers and your personal coach. The coach will only receive information if you have given us permission to do so. You will have the opportunity to say that you do not wish anyone other than the researchers to see your results on the consent form.

What are the costs of participating in this research?

The food diary will take 15-30 minutes a day for 7 days to complete. The testing will take approximately one hour.

How do I agree to participate in this research?

You will be given this form to explain to you the basics of the research. You will be asked to sign a consent form on the day of testing if you agree to take part.

Will I receive feedback on the results of this research?

Yes. The results will be presented back to you as soon as they are available. However, given that 100 rowers are likely to be involved this may be some time after the initial testing. This will occur as written information provided to each personal participant with their results. If requested, feedback can be given to the individual athlete and/or their family or partner.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, *Will Hopkins, will.hopkins@aut.ac.nz,* (09) 921 9793 or

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, *madeline.banda@aut.ac.nz* , (09) 921 9999 ext 8044.

Whom do I contact for further information about this research?

RESEARCHER CONTACT DETAILS:

Andrea Braakhuis, andrea.braakhuis@wintec.ac.nz, (07) 834 8800 Ext: 8044.

PROJECT SUPERVISOR CONTACT DETAILS:

Will Hopkins, will.hopkins@aut.ac.nz, (09) 921 9793, *Elaine Rush, elaine.rush@aut.ac.nz,* (09) 917 9999 x8091 *Tim Lowe, tlowe@hortresearch.co.nz,* (07) 858 4650.

Approved by the Auckland University of Technology Ethics Committee on 2nd March, 2007, AUTEC Reference number *06/230*.

APPENDIX 6: DETAILED LABORATORY METHODS

PLASMA FRAP ASSAY

Reference: Benzie, I.F.F. & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239,* 70-76.

Reagent preparation:

Acetate buffer (pH 3.6, 300 mmol/litre)

TPTZ (2,4,6-tripyridyl-*s*-triazine (Sigma Aldrich, USA) in 10 mmol/litre HCl (BDH))

Fe_3Cl (Iron-3-chloride hexahydate, Sigma Aldrich, USA) 0.270 g/50 mL water, made fresh daily.

FRAP reagent (TPTZ/Fe_3Cl 1:1)

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, (Sigma Aldrich, USA))

A stock standard of Trolox (0.20 mM) was aliquoted into small vials for storage at -80 ^{0}C until use. In the standard assay 40 µl Trolox calibration solutions (0.200, 0.150, 1.100 and 0.050 mM) in acetate buffer were pipette into appropriate wells. A new set of stock Trolox vials were removed from the freezer for daily use.

Method:

Add 25 µl plasma to 100 µl acetate buffer (plasma: acetate buffer 1:4).

Add 25 µl diluted plasma sample or standard to 140 µl acetate buffer + 30 µl FRAP reagent. FRAP reagent should be warmed to 37^{0}C prior to adding.

Read absorbance at 593nm immediately on addition of the FRAP reagent, the plate is shaken to ensure thorough mixing prior to reading, and gain at 4-minutes.

The 4-minute time period must be accurately measured as the absorbance does change with time, however the major antioxidants have all reacted with the FRAP reagent by 4 minutes.

The change in absorbance (ΔA_{593nm}) between the final reading at 4-minutes and the initial reading was calculated for each sample and compared to the ΔA_{593nm} of the standard curve.

PLASMA ASCORBIC ACID & URIC ACID CONCENTRATION

Reference: Lykkesfeldt, J. (2002). Measurement of ascorbic acid and dehydroascorbic acid in biological samples. Assessment of the activity of antioxidant enzymes. *Current Protocols in Toxicology. S12.* 7.6.1-7.6.15.

The ascorbate in newly acquired blood, plasma and saliva samples rapidly oxidize to dehydroascorbate which rapidly oxidizes to L-2,3-diketogulonic acid. The conversion of ascorbate to L-2,3-diketogulonic acid is irreversible and therefore samples not treated immediately or exposed to light will suffer this instability resulting in an underestimation of ascorbate.

Plasma samples are best treated with m-phosphoric acid, mixed and placed at -80^{0}C as quickly as possible. Reverse phase HPLC using an electrochemical detection, despite difficulties with electrode drift, is considered the most sensitive method. According to ascorbate samples should be measured within 1-2 weeks, even if stored at -80^{0}C.

Reagent preparation:

McIlvaine buffer

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), (Sigma Aldrich, USA)

Meta-phosphoric acid (MPA), (Sigma Aldrich, USA)

1mM Na_2EDTA prepared from 10mM Na_2EDTA (375mg/100ml)

For requirements regarding the HPLC column refer to Lykkesfeldt (2002).

Methods:

Collect whole blood sample. Centrifuge to separate plasma from the erythrocytes.

Plasma is added to 10% MPA/EDTA in a 1:1 dilution, vortexed immediately and placed onto dry ice as quickly as possible. Samples are then transferred into -80^{0}C for later analysis.

100µl of thawed plasma extract is added to 160µl of ethanol chloroform mix (62.5:37.5) and vortexed immediately, tube by tube.

Spin for 2 minutes at 16000 RCF. Carefully remove 100µl of clear supernatant.

Add 90µl TCEP (made fresh daily), vortex and sit at room temperature for 5-20 minutes.

Add 310µl of cold McIlvaines buffer and vortex. Place into HPLC vials and place into the autosampler of the HPLC machine at 5^{0}C and analyse.

Preparation of Standards

Standard ascorbic acid solutions of 10, 50 and 100µM and 50, 250 and 500µM of uric acid are prepared as per Lykkesfeldt (2002). A standard is run every 12 samples.

Quantify the ascorbic acid and uric acid concentrations by comparing to the prepared standard curve. Dilution factor for the chloroform ethanol dilution of 0.8 should be included.

PLASMA PROTEIN CARBONYL DETERMINATION

Reference: Levine, R.L., Wehr, N., Williams, J.A., Stadman, E.R., Shacter, E. (2000). Determination of carbonyl groups in oxidised proteins, *Methods in Molecular Biology,* 99, 15-24.

Reagent Preparation:

Concentrated Hydrochloric acid (HCl) diluted 1.72ml into 8.28ml water to give 2M HCl

2,4-dinitrophenylhdrazine (DNPH); 19.8mg into 10ml 2M HCl, stir before use

Trichloroacetic acid (J.T Baker, USA), 50% solution in water
Potassium Phosphate Buffer 20mM (Mono-basic crystal powder) 272mg into 100ml water
Ethanol:ethylacetate (1:1 solution)
Guanidine (Sigma Aldrich, USA); 70.9g into 100ml water to make a 6M solution, then adjusted to pH 2.3 with TCA. Store solution in the dark.

Methods:

100µl plasma + 100µl 20mM DNPH in 2M HCl or 100µl 2M HCl (control) are pipette into separate epindorf tubes and incubated in the dark for 1 hour.
Protein is precipitated by adding 200µl 50% TCA, then mixed. Samples are spun down at 16000RCF for 10 minutes.
Pellet is washed three times with 1:1 ethanol:ethylacetate, then gently mix the pellet to ensure thorough washing
Pellet is resuspended in 1ml 6M guanidine at 37^0C for 15 minutes.
150µl of the supernatant is removed and pipette in duplicate or triplicate onto a Greiner 96 well flat bottom UV plate.
Absorbance is measured at 360-366nm. Protein carbonyl is measured at the difference from sample to control. Absorbance at 360nm=22000$M^{-1}cm^{-1}$.
Measure protein concentration using BioRad Reagent (refer to method below)

Results are expressed as nmols of carbonyl/mg protein (duplicate).

TOTAL PROTEIN CONCENTRATION IN PLASMA

Reagent Preparation:
Make up 1ml of 10mg/ml bovine serum albumin (BSA, Sigma Aldrich, USA) in phosphate buffered solution (PBS, Sigma Aldrich, USA). Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, USA).

Method:
Make a serial dilution of the BSA solution to give 10, 5, 2.5, 1.25, 0.62, 0.31, 0.06, 0 mg/ml.

Pipette 10 µl of standard or sample into a well of a flat bottom 96-well plate (BMG Labtech, 96 F-bottom plate). Measure all samples in triplicate.

Dilute protein assay dye reagent 1:5 in PBS.

Add 90 µl of diluted protein assay dye reagent to each well using an automated pipette. Wait 5 minutes for colour change and read on plate reader at 595nm.

DRABKIN'S METHOD FOR THE DETERMINATION OF HEMOGLOBIN

Reference: Dacie, J.V. & Lewis, S.M. (1984). Practical Haematology, 6th Edition, Churchill Livingstone, Edinburgh.

This method was adapted to calculate the haemoglobin concentration from a concentrated erythrocyte preparation.

The erythrocyte lysate (50µL into 1450µL of water) is further diluted by adding 50 into 1250 L of Drabkins solution, in duplicate, and placed into plastic disposable curvettes (LP Italiana Spa, Italy). Turn on the spectrophotometer machine, click on the "UV probe," "windows", "photometric", "M", "attachments", 540nm, 6-cell, initialise. Or simply blank against straight Drabkin's solution and read absorbance at 540nm directly and record.
Wait at least 10 minutes, remix sample before reading and measure at 540nm. Multiply the average absorbance at 540nm by 36.624 to account for the extinction co-efficient for human haemoglobin, the path length through the curvette and conversion to grams. This will provide a value of haemoglobin concentration in the sample (g Hb/L).

MALONDIALDEHYDE IN PLASMA

Reference: Pilz, et. al. (2000). Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *Journal of Chromatography, 742,* 315-325.

Equipment and Reagents:

Prodigy Column 100mm x 4.6 mm 3µ C18(ODS2) and 75mm x 4.6 mm 3µ C18(ODS2). This gradient works best on the old HPLC and the 75mm column. A slower gradient may work better on the newer HPLC.
Measure at 310 nm.

Mobile phase
A = water 0.2% (v/v) Acetic acid
B = 100% MeOH + 0.2% Acetic acid

Run gradient for the older Shimadzu HPLC (LC6) and shorter column

Time (min)	Sol	%
0	B	20
17	B	45
19	B	80
30	B	80
31	B	20
40	B	20

Run gradient for the newer Shimadzu HPLC (LC10) and longer column

Time (min)	Sol	%
0	B	17
17	B	42
19	B	80
30	B	80
31	B	17
40	B	17

Inject 100µl

Preparation of standard

1 mmol MDA

Add 0.85 ml of Conc HCL to a 100 ml vol flask. Put in at least 95 ml water then add 16.5 ul of 1,1,3,3-tetramethoxypropane (TMOP) (MW 164.2, density 0.997, - immiscible in water but dissolves with hydrolysis) fill volumetric flask to 100ml.

Incubate at 60ºC for approx 45 min.

Dilute to 2 µM 40ul into 20ml (0.1% H_2SO_4), further dilutions are 1 and 0.5 µM

PLASMA TOTAL MDA

Collect blood into an EDTA tube. Spin and dilute plasma 5:1 with 8 mmol EDTA. Freeze at -80°C.

Total MDA (NB this must be extracted to clean up sample)
100µl plasma + 100µl 2M NaOH+ 20µl MeOH(0.005% BHT)
Incubate 60°C for 30 min
65 µl 4M PCA. (total vol 285), vortex

Spin 2 minutes

240 of supernatant + 24 2M HCL 10 mmol DNPH
Incubate 60°C 30 min.

Extract with Hexane: Dichloromethane (80:20)
Add 750 hexane, take off 600, then another 750 and take off 700. dry in nitrogen
reconstitute in 160 ul 15%MeOH, inject 100.

RED BLOOD CELL IN VIVO ANTIOXIDANT CAPACITY

Refrigerate whole blood
Centrifuge 5 min 1000g at 4ºC. remove plasma and white cells. Wash 3 times in saline.

33µl packed cells into 300 µl saline, incubate 5 min at 37ºC. Mix well and mix well before the next step as the rbc's will settle very quickly.
Pipette 25 µl into 1 ml cuvette add 1.2 ml Drabkins to cuvette. Measure absorbance at 540 nm. Do this at the end of the in vitro test.

Prepare 100ml of 0.4 mmol Cumene hydroperoxide
The cumene hydroperoxide is 88% and 5.78 M. Mix 7µL Cumene with 100ml saline. Mix 5 min by continuous stirring.

The *in vitro* incubation
(1) Add 300 µl of the 0.4 mmol Cumene hydroperoxide to the 308 µl of remaining rbc solution. Mix
(2) Incubate 30 min at 37ºC.
(3) Lyse and precipitate the rbc's by adding 300 µl of 0.6M PCA.
Immediately centrifuge and mix 500µL of the clear supernatant with 100µL of 8 mmol EDTA. Freeze at -80C until analysis.

Sample preparation for free MDA
200 of supernatant + 20 2M HCL 10 mmol DNPH
Incubate 60°C 30 min.

Inject onto column within 12 hours.

ERYTHROCYTE CATALASE ACTIVITY

Reference: Adapted from Aebi, H.E. (1984) Catalase. In: Bergmeyer, H.U. (ed) Methods in enzymatic analysis. Verlag Chemie, Basel, pp 273-286.

Reagent Preparation:
Potassium Phosphate Buffer (pH 7.0, 0.05M): Weigh 3.4022g KH_2PO_4 (J.T. Baker, USA, molecular weight: 136.09) into 500mL distilled water. Weigh 4.3545g KH_2PO_4 (J.T. Baker, USA; molecular weight: 174.18) into 500mL distilled water.
Add solutions together and pH to 7.0.

Hydrogen peroxide (AnalaR BDH, England)

Method:
Collect whole blood sample. Centrifuge to separate plasma from the erythrocytes.
Erythrocytes are washed in PBS three times.
Concentrated erythrocytes can then be stored in -80^0C and later thawed for analysis.

Turn on the spectrophotometer and set to 25^0C. Set the spectrophotometer to PC control, under the "mode" setting. Set the computer onto UV Probe, kinetics method, cycle time: 3, number of readings: 15, wavelength: 240nm.
The thawed concentrated erythrocytes are diluted by adding 50µL into 1450µL of water (called the lysate).
The lysate is further diluted by adding 50µL into 9.9mL of potassium phosphate buffer.

When all diluted samples are ready, add 380µL hydrogen peroxide, to 100mL of potassium phosphate buffer and 190mL of distilled water. Move to the spectrophotometer machine.
Blank the machine using water in quartz curvettes.

Testing one sample at a time, add 2.9mL of H_20_2/KPhosBuffer/water solution into the curvette and wait at least 5 minutes to reach temperature, add 108µL of lysate, mix with pipette and start the spectrophotometer machine as quickly as possible.
Results are presented as catalase activity/g Hb/L.

ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY

Reference: Adapted from Mannervik, B. (1985). Glutathione peroxidase. *Methods Enzymol, 113*, 490-495.
Set temperature for plate reader 30ºC
0.1 mmol TRIS pH 7.6 (use TRIS-HCL) as buffer. 1 mmol EDTA
Working solution (150 ul/well)
2 mmol reduced glutathione (mw 307)

For one plate
16 mls TRIS
10 mg GSH
2.66 mg NADPH reduced form (N1630 – 100mg or N6505 – 100mg)
16 U GSSG-R, 40 ul (G3664 sigma, 500 or 100 units)

Cumene hydroperoxide
25µl into 10ml H_2O

Incubate 10ul sample with 150ul working buffer
Start by adding 20 ul cumene hydroperoxide solution
Use a blank and subtract the rate.
Results are presented as glutathione activity/g Hb/L.

ERYTHROCYTE SUPEROXIDE DISMUTASE ACTIVITY

Reference: Kakkar, P., Das, B., & Viswanathan, P. N. (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys, 21*(2), 130-132.

Extraction of the SOD from washed RBCs

***Note for this clean up to work all reagents must be ice cold. Place all tubes, rack and EtOH:Chloroform mix in an ice/water slurry*.**

The washed RBCs are usually lysed by freezing and thawing. Mix the concentrated lysate by inverting and vortexing.

Add 50ul of the concentrated rbc lysate into 1450µl of water and vortex. This is the Lysate.

Pipette 500µl of the rbc lysate in duplicate eppendorf tubes. Place in an ice slurry for 5 mins to cool. Next add 200 µl of ice cold EtOH:Chloroform (62.5:37.5) and vortex **immediately**. That is tube by tube. In this step it is critical that congealed lumps of Hb are avoided as this critically deduces the extraction efficiency.

Continue to vortex and return to slurry until there are no lumps of congealed Hb in the extraction mixture. Allow to completely cool again

Spin 16000 RCF for 1 min (ideally in refrigerated centrifuge set at 2-4°C). There should be a clear, Hb free aqueous layer in the top of the tube.

Carefully take 100ul of the aqueous layer (there may be a layer on top) and dilute 1:3 with water. This diluted extract can be used directly in the SOD assay.

SOD ASSAY

Chemicals

SOD S-2515 30000 Units 2690 units/mg protein

NBT- Nitroblue tetrazolium (Tablets) Sigma N5514 10 mg/tablet

PMS-Phenazine Methosulphate

SPP- Sodium Pyrophosphate Sigma S-6422

Tween 20

NAD

Solutions

NBT- dissolve 10mg/10 ml water. Stock in fridge

PMS- Stock dissolve 10 mg/10ml water. Stock to be stored in fridge for up to 3 months. Make fresh working solution immediately before use of PMS (approx 10 µl of stock into 10ml water, the amount needed will increase as the stock ages)

SPP- 1.15 g/50 ml pH 8.3, 5% Tween 40. (The Tween is a non-ionic detergent and is critical for keeping the nitroblue chromogen that is produced in solution).

NAD 1 mg/ml (make fresh daily in amber bottle).

SOD standard

200U/ml standard in -80°C freezer.

Makes enough for one plate if samples are in triplicate

SOD Conc	Vol of 200 U/l stock	Vol of UHQ water	Vol of 10% MeOH UHQ water
0	0	200	200
2.5	5	195	200
5	10	190	200
7.5	15	185	200
10	20	180	200
12.5	25	175	200
15.0	30	170	200

Procedure

Using a standard clear microtiter plate run standards and samples in triplicate using the layout below

Pipette 100ul of either std or sample in triplicate into the microtiter plate a typical layout is shown on the next page

For one complete plate (96 wells). Mix 1.320 ml NBT with 7.480 ml SPP. (NBT SPP mix 12µl and 68ul/well)

Add 80µl of this NBT SPP mix to each well. Mix for at least one minute on a plate vortex.

Using an 8 channel multipipettor, add 25ul of PMS working solution to each well. Mix for approximately 40 seconds.

Using an 8 channel multipipettor, add 25ul of NAD solution to each well. Mix for approximate 40 seconds then immediately read the absorbance at 540nm a subtraction of the absorbance at 650nm can be used as a correction factor.

Read the plate a second time after approximately 10min or when the absorbance in the zero standard reaches approximately 0.900. The rate of change in absorbance should be about 0.05-0.1 units per min.

This assay relied on the detection of the ability of the enzyme to inhibit the phenazine methosulphate mediated reduction of nitroblue tetrazolium dye by colorimetric method.

Erythrocyte SOD activity was calculated by using the following equation:

SOD (U/gm Hb) = % inhibition x 3.75 x 1/gm Hb used per litre

Where Percent inhibition= MA control – MA sample/MA control x 100 and MA control= the change in absorbance at 560 nm over 5 minutes following the addition of PMS to the reaction mixture

Plate LayoutDateFile

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 std	0 std	0 Std									
B	2.5 std	2.5 std	2.5 std									
C	5.0 std	5.0 std	5.0 std									
D	7.5 std	7.5 std	7.5 std									
E	10.0 std	10.0 std	10.0 std									
F	12.5 std	12.5 std	12.5 std									
G	15.0 std	15.0 std	15.0 std									
H												

Results are presented as superoxide dismutase activity/g Hb/L.

APPENDIX 7: DIETARY ANTIOXIDANT INTAKE QUESTIONNAIRE

(CHAPTER 4)

Dietary Antioxidant Questionnaire

GENERAL INSTRUCTIONS

- **Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.**
- **Use only a black or blue ball-point pen. Do not use a pencil or felt-tip pen. Do not fold, staple, or tear the pages.**
- **Put an X in the box next to your answer.**
- **If you make any changes, cross out the incorrect answer and put an X in the box next to the correct answer. Also draw a circle around the correct answer.**
- **If you mark NEVER, NO, or DON'T KNOW for a question, please follow any arrows or instructions that direct you to the next question.**

BEFORE TURNING THE PAGE, PLEASE COMPLETE THE FOLLOWING QUESTIONS.

Today's date:
In what month were you born?
In what year were you born?
Are you male or female?
Are you taking oral contraceptive pills?
Oral contraceptive may act as an antioxidant hence we need to ask this question.
What is your current weight?
How long have you been at your current weight?
What is your lightest adult weight?
How many years have you been involved in the sport?
How many hours do you spend doing on water training a week?
How many hours do you spend doing resistance training (weights) a week?
How many hours do you spend doing additional aerobic training a week?
Have you required ongoing consumption of anti-inflammatory medication?

1. Over the past 1 month, how often did you drink **tomato juice** or **vegetable juice**?

☐ NEVER (GO TO QUESTION 2)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

1a. Each time you drank **tomato juice** or **vegetable juice**, how much did you usually drink?

a☐ Less than ¾ cup (200mL)
b☐ ¾ to 1¼ cups (200 to 300mL)
c☐ More than 1¼ cups (300mL)

2. Over the past 1 month, how often did you drink **orange juice, apple**, **pineapple, cranberry** or **grape juice ?**

a☐ NEVER (GO TO QUESTION 3)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

2a. Each time you drank **orange juice**, **pineapple, apple, cranberry** or **grape juice**, how much did you usually drink?

a☐ Less than ¾ cup (200mL)
b☐ ¾ to 1¼ cups (200 to 300mL)
c☐ More than 1¼ cups (300mL)

3. Over the past 1 month, how often did you drink **fruit drinks** containing **blackberry, strawberry, cranberry, raspberry, blackcurrant** or **blueberry**?

a☐ NEVER (GO TO QUESTION 4)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

3a. Each time you drank **fruit drinks** containing **blackberry, strawberry, cranberry, raspberry, blackcurrant** or **blueberry**, how much did you usually drink?

a☐ Less than ¾ cup (200mL)
b☐ ¾ to 1½ cups (200-300mL)
c☐ More than 1½ cups (300mL)

Over the past 1 month….

4. How often did you drink other **fruit drinks** (such as thriftee, Vitafresh, Kool-Aid, sports drink, diet or regular)?

a☐ NEVER (GO TO QUESTION 5)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

4a. Each time you drank other **fruit drinks**, how much did you usually drink?

a☐ Less than 1 cup (250mL)
b☐ 1 to 2 cups (250-500mL)
c☐ More than 2 cups (500mL)

4b. How often were your fruit drinks **enriched (added) with vitamin C**?

a☐ Almost never or never
b☐ About ¼ of the time
c☐ About ½ of the time
d☐ About ¾ of the time
e☐ Almost always or always
f ☐ Don't know

5. How often did you drink hot drinks such as **coffee, black, green** or **oolong tea**?

a☐ NEVER (GO TO QUESTION 6)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

5a. Each time you drank **coffee, black, green** or **oolong tea**, how much did you usually drink?

a☐ Less than 1 cup (150mL)
b☐ 1 to 2 cups (150-300mL)
c☐ 3 to 4 cups (450-600-mL)
d☐ More than 4 cups (600mL)

6. How many glasses of **ICED tea,** caffeinated or decaffeinated, did you drink?

a☐ NEVER (GO TO QUESTION 7)

b☐ Less than 1 cup per month
c☐ 1–3 cups per month
d☐ 1 cup per week
e☐ 2–4 cups per week
f☐ 5–6 cups per week
g☐ 1 cup per day
h☐ 2–3 cups per day
i☐ 4–5 cups per day
j☐ 6 or more cups per day

7. **Over the <u>past 1 month</u>,** did you drink red wine?

a☐ NO (GO TO QUESTION 8)
b☐ YES

↓

7a. How often did you drink **red wine**?

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

7b. Each time you drank **red wine**, how much did you usually drink?

a☐ 1-2 glasses (110-220mL)
b☐ 3-4 glasses (330-440mL)
c☐ 5-6 glasses (550-660mL)
d☐ More than 7 glasses (770mL)

Over the <u>past 1 month</u>…..

Question 8 continues on the next page

8. How often did you drink **beer**?

a☐ NEVER (GO TO QUESTION 9)

b☐ 1 time per month or less
c☐ 2–3 times per month
d☐ 1–2 times per week
e☐ 3–4 times per week
f☐ 5–6 times per week
g☐ 1 time per day
h☐ 2–3 times per day
i☐ 4–5 times per day
j☐ 6 or more times per day

8a. Each time you drank **beer**, how much did you usually drink?

a☐ 1 glass to 1 can (110-375mL)
b☐ More than 1 to 2 cans (400ml-750mL)
c☐ More than 2 to 3 cans (800-1125mL)
d☐ More than 3 cans (1125mL)

9. How often did you eat **blackberries** or **blackcurrants** (fresh, canned, or frozen)?

a☐ NEVER (GO TO QUESTION 10)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

9a. Each time you ate **blackberries** or **blackcurrants**, how many did you usually eat?

a☐ Less than 10 berries
b☐ Between 10 and 20 berries
c☐ More than 20 berries

10. How often did you eat **dried fruit**, such as prunes, raisins or dates?

a☐ NEVER (GO TO QUESTION 11)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

10a. Each time you ate **dried fruit**, how much did you usually eat?

a☐ Less than 2 tablespoons
b☐ 2 to 5 tablespoons
c☐ More than 5 tablespoons

11. Over the <u>past 1 month</u>, did you eat **strawberries, boysenberries, blueberries,** or **raspberries**?

a☐ NO (GO TO QUESTION 12)

b☐ YES

↓

11a. How often did you eat **strawberries**, **blueberries, boysenberries**, or **raspberries**?

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week

e☐ 2–3 times per month
f☐ 1 time per week
j☐ 1 time per day
k☐ 2 or more times per day

11b. Each time you ate **strawberries, blueberries, boysenberries** or **raspberries,** how much did you usually eat?

a☐ Less than ½ cup
b☐ ½ to ¾ cup
c☐ More than ¾ cup

12. How often did you eat **cranberries,** or **cherries**?

a☐ NEVER (GO TO QUESTION 13)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

12a. Each time you ate **cranberries** or **cherries**, how much did you usually eat?

a☐ Less than ½ cup or less than 10 fruit
b☐ ½ to 1 cup or 10 to 30 fruit
c☐ More than 1 cup or more than 30 fruit

13. Over the <u>past 1 month</u>, did you eat **plums** or **pineapple**?

a☐ NO (GO TO QUESTION 14)

b☐ YES

13a. How often did you eat **plums** or **pineapple** (canned or fresh)?

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

13b. Each time you ate either **plums** or **pineapple**, how much did you usually eat?

a☐ Less than 1 plum or less than ½ cup
b☐ Between 2 – 4 plums or 1-2 cups
c☐ More than 4 plums or more than 2 cups

14. How often did you eat **pears**?

a☐ NEVER (GO TO QUESTION 15)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

14a. Each time you ate **pears**, how much did you usually eat?

a☐ Less than 1
b☐ 1 to 2 pears

Question 14a continues on the next page

c☐ More than 2 pears

Over the <u>past 1 month</u>…..

15. How often did you eat **oranges** or **kiwifruit**?

a☐NEVER (GO TO QUESTION 16)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

15a. Each time you ate **oranges** or **kiwifruit**, how much did you usually eat?

a☐ Less than 1 orange or 2 kiwifruit
b☐ 1-3 oranges or 2-4 kiwifruit
c☐ More than 3 oranges or 4 kiwifruit

Over the <u>past 1 month</u>…

16. How often did you eat **bran flakes** or **whole grain (Weet-bix, VitaBrits)** breakfast cereal?

a☐ NEVER (GO TO QUESTION 17)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

16a. Each time you ate **bran flakes** or **whole grain** breakfast cereal**,** how many did you usually eat?

a☐ Less than 1 cup
b☐ 1-2 cups
c☐ More than 3 cups

17. Over the <u>past 1 month</u>, did you eat **All Bran, Sultana Bran** or **Cocoa Krispies (or other chocolate cereal)** breakfast cereal?

a☐ NO (GO TO QUESTION 18)

b☐ YES

17a. How often did you eat **All Bran, Sultana Bran** or **Cocoa Krispies**?

a☐NEVER

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

17b. Each time you ate **All Bran, Sultana Bran** or **Cocoa Krispies**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1-2 cups

Question 17b continues on the next page

c☐ More than 2 cups

18. How often did you eat **Cornflakes, Rice crispies** or **Grinners** breakfast cereal?

a☐ NEVER (GO TO QUESTION 19)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

18a. Each time you ate **Cornflakes, Rice crispies** or **Grinners**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

Over the <u>past 1 month</u>…

19. How often did you eat **artichokes** or **artichoke hearts?**

a☐ NEVER (GO TO QUESTION 20)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

19a. Each time you ate **artichokes**, how much did you usually eat?

a☐ Less than ½ cup
b☐ ½ to 1 cup
c☐ More than 1 cup

20. How often did you eat **cabbage** (red or white)?

a☐ NEVER (GO TO QUESTION 21)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

20a. Each time you ate **cabbage** (red or white), how much did you usually eat?

a☐ Less than ½ cup
b☐ ½ to 1 cup
c☐ More than 1 cup

Over the <u>past 1 month</u>…

21. How often did you eat **potatoes** (such as red (Maori) or orange kumera). regardless of cooking method?

a☐ NEVER (GO TO QUESTION 22)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day

f☐ 1 time per week
k☐ 2 or more times per day

21a. Each time you ate **potatoes**, how much did you usually eat?

a☐ Less than 1 medium potato
b☐ 1-2 medium potatoes
c☐ More than 2 potatoes

22. How often did you eat **spinach**?

a☐ NEVER (GO TO QUESTION 23)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

22a. Each time you ate **spinach**, how much did you usually eat?

a☐ Less than ¼ cup
b☐ ¼ to 1 cup
c☐ More than 1 cup

23. How often did you eat **capsicums** (red, green or yellow)?

a☐ NEVER (GO TO QUESTION 24)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

23a. Each time you ate **capsicum**, how much did you usually eat?

a☐ Less than ½ cup
b☐ ½ cup to 1 cup
c☐ More than 1 cup

24. How often did you eat **broccoli** (fresh or frozen)?

a☐ NEVER (GO TO QUESTION 25)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

24a. Each time you ate **broccoli**, how much did you usually eat?

a☐ Less than ½ cup
b☐ Less than ½ cup to 1 cup
c☐ More than 1 cup

Over the <u>past 1 month</u>….

25. How often did you eat **pecans** or **walnuts** ?

a☐ NEVER (GO TO QUESTION 26)

b☐ 1–6 times per year
c☐ 7–11 times per year
g☐ 2 times per week
h☐ 3–4 times per week

Question 25 continues on the next page

d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

25a. Each time you ate **pecans** or **walnuts**, how much did you usually eat?

a☐ Less than ¼ cup
b☐ ¼ to ¾ cup
c☐ More than ¾ cup

Over the <u>past 1 month</u>…

26. Did you eat **canned spaghetti, baked beans** (navy beans) or **kidney beans**?

a☐ NO (GO TO QUESTION 27)

b☐ YES

↓

26a. How often did you eat **canned spaghetti, baked beans** or **kidney beans**?

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

26b. Each time you ate **canned spaghetti, baked beans** or **kidney beans**, how much did you usually eat?

a☐ Less than ½ cup
b☐ ½ to 1 cup
c☐ More than 1 cup

27. Over the <u>past 1 month</u>, how often did you eat **unsweetened cooking chocolate** or **sugar free dark chocolate**?

a☐ NEVER (GO TO QUESTION 28)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

27a. Each time you ate **unsweetened cooking chocolate** or **sugar free dark chocolate,** how much did you usually eat?

a☐ Less than ¼ cup (fun size bar)
b☐ ¼ to 1 cup (standard size bar)
c☐ More than 1 cup (1/2 block of chocolate)

28. Over the <u>past 1 month</u>, how often did you eat **milk chocolate** or **dark chocolate** (not already mentioned above)?

a☐ NEVER (GO TO QUESTION 29)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

28a. Each time you ate **milk** or **dark chocolate,** how much did you usually eat?

a☐ Less than ¼ cup (fun size bar)
b☐ ¼ to 1 cup (standard size bar)
c☐ More than 1 cup (1/2 block of chocolate)

29. How often did you eat **chocolate cake** or **chocolate chip cookies**?

a☐ NEVER (GO TO QUESTION 30)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

29a. Each time you ate **chocolate cake** or **chocolate chip cookies**, how much did you usually eat?

a☐ Less than 1 slice or 3 cookies
b☐ 1 slice or 3 cookies
c☐ More than 1 slice or 3 cookies

30. How often did you eat **lasagna with meat** (fresh or frozen and cooked)?

a☐ NEVER (GO TO QUESTION 32)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

30a. Each time you ate **lasagna with meat**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

Over the <u>past 1 month</u>…

31. How often did you eat **condensed (tinned) tomato soup**?

a☐ NEVER (GO TO QUESTION 33)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

31a. Each time you ate **condensed (tinned) tomato soup**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

32. How often did you eat **chocolate ice-cream** (full, reduced or low fat)?

a☐ NEVER (GO TO QUESTION 33)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day

Question 32 continues on the next page

f☐ 1 time per week
k☐ 2 or more times per day

32a. Each time you ate **chocolate ice-cream**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

33. How often did you consume **milk** (full, reduced or low fat)?

a☐ NEVER (GO TO QUESTION 34)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

33a. Each time you consumed **milk**, how much did you usually eat?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

34. How often did you drink **flavoured milk** or eat **yoghurt** (full or reduced fat)?

a☐ NEVER (GO TO QUESTION 35)

b☐ 1–6 times per year
c☐ 7–11 times per year
d☐ 1 time per month
e☐ 2–3 times per month
f☐ 1 time per week
g☐ 2 times per week
h☐ 3–4 times per week
i☐ 5–6 times per week
j☐ 1 time per day
k☐ 2 or more times per day

34a. Each time you drank **flavoured milk** or ate **yoghurt** how much did you usually consume?

a☐ Less than 1 cup
b☐ 1 to 2 cups
c☐ More than 2 cups

35. Please mark any of the following **single herbs** and **spices** you consumed more than once per week either fresh or dried:

a ☐ cinnamon
b ☐ cloves
c ☐ oregano leaf
d ☐ ginger
e ☐ mustard seeds
f ☐ tumeric

Over the past 1 month…

36. How many servings of **fruit** (not including juices) did you eat per week or per day?

a☐ Less than 1 per week
b☐ 1–2 per week
c☐ 3–4 per week
d☐ 5–6 per week
e☐ 1 per day
f☐ 2 per day
g☐ 3 per day
h☐ 4 per day
i☐ 5 or more per day

The next questions are about your use of antioxidant or vitamin pills.

37. Over the past 1 month, did you take any **multivitamins**, such as One-a-Day-, Theragran-, or Centrum-type multivitamins (as pills, liquids, or packets)?

a☐ NO (GO TO QUESTION 38c)
b☐ YES

38. How often did you take **One-a-day-, Theragran-, or Centrum-type** multivitamins?

a☐ Less than 1 day per month
b☐ 1–3 days per month
c☐ 1–3 days per week
d☐ 4–6 days per week
e☐ Every day

38a. Does your **multivitamin** usually contain **antioxidants** (such as vitamin C, vitamin E or selenium.)?

a☐ NO
b☐ YES
c☐ Don't know

38b. For how many years have you taken **multivitamins**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

38c. Did you take any **vitamins, minerals**, or other **herbal supplements** other than your multivitamin?

a☐ NO (GO TO QUESTION 44)

b☐ YES (GO TO INTRODUCTION TO QUESTION 39.)

These last questions are about the vitamins, minerals, or herbal supplements you took that are NOT part of a One-a-day-, Theragran-, or Centrum-type of multivitamin.

Please include vitamins taken as part of an antioxidant supplement.

39. How often did you take **Beta-carotene** (**NOT** as part of a multivitamin in Question 38)?

a☐ NEVER (GO TO QUESTION 40)

b☐ Less than 1 day per month
c☐ 1–3 days per month
d☐ 1–3 days per week
e☐ 4–6 days per week
f☐ Every day

39a. When you took **Beta-carotene**, about how much did you take in one day?

a☐ Less than 10,000 IU
b☐ 10,000–14,999 IU
c☐ 15,000–19,999 IU
d☐ 20,000–24,999 IU
e☐ 25,000 IU or more
f☐ Don't know

39b. For how many years have you taken **Beta-carotene**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

40. How often did you take **Vitamin A** (**NOT** as part of a multivitamin in Question 38)?

a☐ NEVER (GO TO QUESTION 41)

Question 40 continues on the next page

b☐ Less than 1 day per month
c☐ 1–3 days per month
d☐ 1–3 days per week
e☐ 4–6 days per week
f☐ Every day

40a. When you took **Vitamin A**, about how much did you take in one day?

a☐ Less than 8,000 IU
b☐ 8,000–9,999 IU
c☐ 10,000–14,999 IU
d☐ 15,000–24,999 IU
e☐ 25,000 IU or more
f☐ Don't know

40b. For how many years have you taken **Vitamin A**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

41. How often did you take **Vitamin C** (**NOT** as part of a multivitamin in Question 38)?

a☐ NEVER (GO TO QUESTION 42)

b☐ Less than 1 day per month
c☐ 1–3 days per month
d☐ 1–3 days per week
e☐ 4–6 days per week
f☐ Every day

41a. When you took **Vitamin C**, about how much did you take in one day?

a☐ Less than 500 mg
b☐ 500–999 mg
c☐ 1,000–1,499 mg
d☐ 1,500–1,999 mg
e☐ 2,000 mg or more
f☐ Don't know

41b. For how many years have you taken **Vitamin C**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

Over the <u>past 1 month</u>…

42. How often did you take **Vitamin E** (**NOT** as part of a multivitamin in Question 38)?

a☐ NEVER (GO TO QUESTION 43)

b☐ Less than 1 day per month
c☐ 1–3 days per month
d☐ 1–3 days per week
e☐ 4–6 days per week
f☐ Every day

42a. When you took **Vitamin E**, about how much did you take in one day?

a☐ Less than 400 IU
b☐ 400–799 IU
c☐ 800–999 IU

Question 42a continues on the next page

d☐ 1,000 IU or more
e☐ Don't know

42b. For how many years have you taken **Vitamin E**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

43. How often did you take **selenium** (**NOT** as part of a multivitamin in Question 38)?

a☐ NEVER (GO TO QUESTION 44)

b☐ Less than 1 day per month
c☐ 1–3 days per month
d☐ 1–3 days per week
e☐ 4–6 days per week
f☐ Every day

43a. When you took **selenium**, about how much did you take in one day?

a☐ Less than 20 μg
b☐ 21–100 μg
c☐ 101–200 μg
d☐ 201 μg or more
e☐ Don't know

43b. For how many years have you taken **selenium**?

a☐ Less than 1 year
b☐ 1–4 years
c☐ 5–9 years
d☐ 10 or more years

APPENDIX 8: ADDITIONAL CORRELATION DATA TABLES, NOT PRESENTED IN CHAPTER 4.

Table 21: Correlations between resting antioxidant blood biomarkers and dietary intake

	TAC	AA	UA	CAT	GPx	SOD
Antiox-total	0.29	-0.02	0.08	0.05	-0.12	-0.03
Antiox-cereal	0.22	-0.15	0.29	0.05	-0.18	0.07
Antiox-fruit	0.07	0.17	0.03	-0.04	-0.11	-0.19
Antiox-drinks	0.23	0.13	-0.05	-0.04	-0.16	-0.13
Antiox-vegetables	0.00	-0.18	-0.16	-0.04	0.10	-0.12
Antiox-coffee/tea	-0.17	0.03	-0.04	-0.12	-0.16	0.12
Antiox-beer/wine	0.10	-0.01	0.15	0.17	-0.22	0.22
Antiox-chocolate	-0.22	0.01	0.00	-0.09	-0.09	0.05
Fructose	-0.05	-0.20	-0.17	-0.24	-0.24	-0.18
Vitamin E	-0.15	0.02	-0.03	0.13	0.11	-0.06
Vitamin C	-0.14	0.09	0.04	0.04	-0.06	0.20
VitCSupp	-0.08	0.17	-0.18	-0.12	0.13	-0.02
Vitamin D	0.12	-0.11	0.16	-0.03	0.09	-0.02
Vitamin A	0.13	0.25	0.00	0.05	0.04	-0.18
Beta-carotene	0.03	0.22	0.06	0.17	0.16	-0.04
Selenium	0.14	-0.06	0.15	-0.11	0.09	-0.18
Fruit serves	-0.09	-0.02	-0.09	-0.05	-0.18	-0.04

Abbreviations: TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid, CAT: erythrocyte catalase activity, GPx: glutathione peroxidase, SOD: superoxide dismutase, VitCSupp: Vitamin C supplements. Confidence limits for all correlations ~±0.20. Correlations of magnitude greater than 0.10 are clear and substantial. Interpretation of magnitude: <0.10 trivial; 0.10-0.29 small; 0.30-0.49 moderate.

Table 22: Correlations between antioxidant dietary intake, chronic training and acute performance test parameters

	Total training (years)	Av. training (hrs.wk^{-1})	Distance covered (m)
Antiox-total	-0.15	0.15	0.08
Antiox-cereal	-0.19	-0.06	0.16
Antiox-fruit	0.07	0.18	-0.10
Antiox-drinks	0.03	0.31	0.02
Antiox- vegetables	-0.05	0.09	0.18
Antiox-coffee/tea	0.15	-0.02	-0.15
Antiox-beer/wine	-0.03	0.05	0.04
Antiox-chocolate	0.25	0.23	-0.05
Fructose	0.12	-0.11	-0.07
Vitamin E	-0.03	0.05	-0.23
Vitamin C	0.16	0.02	-0.10
VitCSupp	0.09	0.06	0.22
Vitamin D	-0.07	0.28	-0.23
Vitamin A	0.35	0.09	0.15
Beta-carotene	0.07	0.10	-0.10
Selenium	-0.15	0.12	0.01
Fruit serves	0.11	-0.10	-0.19

Abbreviations: TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid, CAT: erythrocyte catalase activity, GPx: glutathione peroxidase, SOD: superoxide dismutase, VitCSupp: vitamin C supplement, Antiox: antioxidant (determined by the FRAP assay). Confidence limits for all correlations ~±0.20. Correlations of magnitude greater than 0.10 are clear and substantial. Interpretation of magnitude: <0.10 trivial; 0.10-0.29 small; 0.30-0.49 moderate.

Table 23: Associations of the exercise-induced change in antioxidant blood biomarkers with dietary intake. Data shown are the effect of the difference of 2SD of the dietary measure on the change in the biomarker, expressed in units standardized with the resting biomarker SD.

	Δ TAC	Δ AA	Δ UA	Δ CAT	Δ GPx	Δ SOD
Antiox-total	-0.23	0.12	0.00	-0.10	-0.23	-0.08
Antiox-cereal	-0.24	0.03	0.01	0.07	-0.26	0.04
Antiox-fruit	0.05	0.11	0.08	-0.23	-0.17	-0.23
Antiox-drinks	-0.02	-0.03	-0.20	0.24	0.33	-0.36
Antiox- vegetables	-0.20	0.05	0.15	-0.32	-0.07	0.11
Antiox-coffee/tea	0.04	0.01	0.06	-0.21	-0.29	-0.12
Antiox-beer/wine	-0.11	0.09	-0.21	0.12	-0.02	0.09
Antiox-chocolate	-0.07	-0.06	-0.02	-0.25	-0.32	0.22
Fructose	0.11	0.09	0.32	-0.13	-0.11	-0.04
Vitamin E	0.13	0.04	0.12	-0.39	-0.16	-0.14
Vitamin C	0.19	0.03	0.28	-0.79	-0.58	-0.03
VitCSupp	0.07	0.05	0.11	0.28	-0.20	-0.03
Vitamin D	0.16	-0.07	-0.14	-0.18	0.08	-0.11
Vitamin A	0.25	0.13	0.16	0.13	-0.10	-0.08
Beta-carotene	0.05	0.01	-0.02	0.03	-0.24	0.04
Selenium	-0.07	0.02	0.16	-0.01	-0.06	-0.13
Fruit serves	0.08	0.08	0.12	-0.35	0.10	-0.22

Abbreviations: Δ: post – pre change in the change the performance test, TAC: total antioxidant capacity, AA: ascorbic acid, UA: uric acid; CAT: erythrocyte catalase activiy, GPx: glutathione peroxidase, SOD: superoxide dismutase, VitCSupp: Vitamin C supplements, Antiox: antioxidant (determined by the FRAP assay). Interpretation of magnitude: <0.20 trivial; 0.20-0.79 small.

APPENDIX 9: THE IMPACT OF TRAINING ON OXIDATIVE PROTECTION

The focus of the doctoral thesis is to discuss the impact dietary antioxidant intake has on sport performance, however it is important to consider the additive effect training and diet may have. Research results completed on trained individuals may be quite different to untrained; due to the influence physical activity has on oxidative defence mechanisms. Physiological training adaptations have been demonstrated in trained athletes and the susceptibility of plasma lipids to peroxidation following exercise is reduced in this group (Ginsburg, et al., 1996). Shern-Brewer et al. (1998) have shown that regular aerobic stress for an overall shorter time span creates more oxidative by products in the body and increases the susceptibility for lipid peroxidation, whereas chronic exercise over several months or years decreases the susceptibility of lipids to undergo oxidation. Acute exercise increases oxidative stress, following strenuous exercise (Alessio, et al., 1997; Alessio, et al., 2000). Therefore trained athletes are likely to experience less oxidative stress at rest than their sedentary counterparts.

The degree of oxidative stress and muscle damage does not depend on the intensity of exercise, but more accurately related to the degree of exhaustion of the person performing the exercise. Regular exercise can initiate adaptations to enhance the enzymatic defense system against free radical activity. Regular, moderate-intensity exercise increases the number and the size of mitochondria (Ginsburg, et al., 1996), and the amount of antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD) (Adams & Best, 2002), and cytochromes (Alessio, et al., 1997). While we typically regard oxidative stress as an unwanted by-product of exercise, the downstream consequences of radical species may act as potent vasodilators and improve vascular responsiveness (Richardson, et al., 2007).

Lower oxidised proteins are also detected in active individuals or animals, suggesting a training induced antioxidant protective mechanism. Lower concentrations of reactive protein carbonyl derivatives were measured in the brain of exercised rats (Radak, et al., 1998). The decreased level of reactive carbonyl derivatives in the brain was associated with enhanced cognitive functions as measured through avoidance tests. Thus, regular exercise appears to decrease the accumulation of oxidately modified proteins in the brain induced by exercise (Carney, et al., 1991); (Forster, et al., 1996). So while an acute exercise session increase both lipid peroxidation and carbonyl derivatives, it is unlikely these species accumulate as exercise appears to increase the rate of protein turnover. The increased protein breakdown, primarily in the rate of protein turnover, might improve the antioxidant defence of cells (Davies, 1986). It could also be one of the beneficial effects of regular exercise that up-regulates resistance against oxidative stress.

Regular physical activity may be crucial to naturally promote the function of the endogenous antioxidant defence and reduce the risk of reactive species mediated diseases. However, increased physical activity is not the only way to modify the effects of the oxidative stress. The

antioxidant defence system of the body depends significantly on the exogenous antioxidants such as vitamins and trace minerals.

APPENDIX 10: STUDIES REPORTING PERFORMANCE DATA, BUT EXCLUDED FROM CHAPTER 5 DUE TO DESIGN

The studies outlined in Table 23 represent those of either design; parallel group, controlled trial, with no pre-exercise trial, or; no placebo control. The primary aim of these studies was to investigate factors other than athletic performance, and thus the uncertainty in the measure is too high to base conclusions upon. A prime example is Utter (2009), investigating the impact of quercetin on 160-km time trial performance. Quercetin was shown to improve performance on average, by 4.2%, however calculating the confidence limits with 90% certainty show the true result is give or take 660%, with a reported p value of 0.992. Clearly this represents excessive uncertainty regarding quercetin supplementation.

Table 24: Parallel groups, controlled trial studies with no pre-exercise trial time or non-antioxidant control group

Authors	Dietary Antioxidants	Supplement Duration	Subjects	Performance Test[a]	Performance Outcome[b]
Davison, 2005 {Davison, 2005 #5}	Mixed antioxidants	7-days	14 males	Incremental run test -Controlled trial -Time to exhaustion	↑14%, p>0.05
Mastaloudis, 2006 {Mastaloudis, 2006 #245}	Vitamin C & E (1g +300mg.d^{-1}) Vs placebo	6 weeks	13 trained males	50-km time trial -Controlled trial -Time trial	↑5.4%, p>0.05
Utter, 2009[c] {Utter, 2009 #328}	Quercetin 250mg.d^{-1} vs placebo	3 weeks	63 runners	160-km running race -Parallel groups, controlled trial -Time trial	↑4.2% p=0.992[c]
Mastaloudis, 2006 {Mastaloudis, 2006 #245}	Vitamin C & E (1g +300mg.d^{-1}) Vs placebo	6 weeks	9 trained females	50-km time trial -Controlled trial -Time trial	↑3.3%, p>0.05
Senturk, 2005 (Umit K. Senturk, 2005b)	Vitamins A, C, E (β-Carotene 50m 1000mg, α Tocopherol, 800 mg.d)	2 months	9 trained and 9 untrained males	Cycling test -Controlled trial -Time to exhaustion	No data reported p>0.05
Watson, 2005 {Watson, 2005 #246}	None Athletes placed on a low antioxidant diet for 2-weeks	N/A	17 trained male runners	30-min at 60%$V0_2$ max+ run to exhaustion -Controlled trial -Time to exhaustion	↓1.6% on high antioxidant diet, p>0.05

[a] Max: maximum

[b] ↑performance improvement versus control; ↓performance decrement versus control

APPENDIX 11: ETHICAL APPROVAL FOR CHAPTER 6

MEMORANDUM

Auckland University of Technology Ethics Committee (AUTEC)

To:Will Hopkins

From:**Madeline Banda** Executive Secretary, AUTEC

Date:22 August 2008

Subject: Ethics Application Number 08/103 **The effect of vitamin C supplementation on training induced adaptations and endurance performance in athletes.**

Dear Will

I am pleased to advise I have approved the requested minor amendments to the design of your research. This delegated approval is made in accordance with section 5.3.2 of AUTEC's *Applying for Ethics Approval: Guidelines and Procedures* and is subject to endorsement at AUTEC's meeting on 8 September 2008.

I remind you that as part of the ethics approval process, you are required to submit the following to AUTEC:

- A brief annual progress report using form EA2, which is available online through *http://www.aut.ac.nz/about/ethics*. When necessary this form may also be used to request an extension of the approval at least one month prior to its expiry on 25 June 2011;
- A brief report on the status of the project using form EA3, which is available online through *http://www.aut.ac.nz/about/ethics*. This report is to be submitted either when the approval expires on 25 June 2011 or on completion of the project, whichever comes sooner;

It is a condition of approval that AUTEC is notified of any adverse events or if the research does not commence. AUTEC approval needs to be sought for any alteration to the research, including any alteration of or addition to any documents that are provided to participants. You are reminded that, as applicant, you are responsible for ensuring that research undertaken under this approval occurs within the parameters outlined in the approved application.

Please note that AUTEC grants ethical approval only. If you require management approval from an institution or organisation for your research, then you will need to make the arrangements necessary to obtain this.

When communicating with us about this application, we ask that you use the application number and study title to enable us to provide you with prompt service. Should you have any further enquiries regarding this matter, you are welcome to contact Charles Grinter, Ethics Coordinator, by email at charles.grinter@aut.ac.nz or by telephone on 921 9999 at extension 8860.

On behalf of the AUTEC and myself, I wish you success with your research and look forward to reading about it in your reports.

Yours sincerely

Madeline Banda

Executive Secretary

Auckland University of Technology Ethics Committee

Cc:Andrea Braakhuis andrea.braakhuis@wintec.ac.nz, Elaine Rush

APPENDIX 12: SAMPLE OF A PARTICIPANT CONSENT FORM FOR CHAPTER 6

CONSENT FORM	

*Project title:*The effect of vitamin C supplementation on training induced adaptations and endurance performance in athletes

Project Supervisor: ***Professor Will Hopkins***

Researcher: ***Andrea Braakhuis***

- ○ I have read and understood the information provided about this research project in the Information Sheet dated 16th April 2008.
- ○ I have had an opportunity to ask questions and to have them answered.
- ○ I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way.
- ○ I am not suffering from any illness or injury that impairs my physical performance, or any infection.
- ○ I agree to refrain from taking nutritional supplements (vitamin C, E, A or selenium) for one month prior to and whilst taking part in the study.
- ○ I agree to provide blood samples (on four occasions) and complete the four maximal incremental running tests (10 minute cycle test).
- ○ I agree to undertake a three week training program, three times, to the best of my ability.
- ○ I agree to take part in this research.
- ○ I wish to receive a copy of the report from the research which I may choose to share with my family (please tick one): Yes○No○

Participant's signature:.....……

Participant's name:.....……

Participant's Contact Details (if appropriate): .....

Date:

Approved by the Auckland University of Technology Ethics Committee on 2nd March, 2008 AUTEC Reference number 06/230

Note: The Participant should retain a copy of this for their records.

APPENDIX 13: SAMPLE OF A PARTICIPANT INFORMATION FORM FOR CHAPTER 6

Participant Information Sheet

Date Information Sheet Produced:

16th April, 2008

Project Title: The effect of antioxidants on training induced adaptations and endurance performance in athletes

An Invitation

You are invited to participate in a study to find out how nutritional supplements influence exercise and the way the body responds through metabolic stress. Diet is known to influence sporting performance in many ways however it is still unclear as to whether supplements, and specifically vitamin C and antioxidant status, can influence an athlete's response to endurance training. This research will contribute towards a doctorate and will be conducted by Andrea Braakhuis. Participation is voluntary and you may withdraw at any time without penalty.

What is the purpose of this research?

Athletes often consume supplements and foods rich in antioxidants as a strategy to reduce muscle damage or enhance recovery. We wish to compare three types of supplements, in capsule or drink form. Therefore, this research will benefit athletes using antioxidants to assist them in better understanding the dynamics of when supplements or food sources of vitamin C may actually be helpful to athletic performance.

How was I chosen for this invitation?

Participation requires that you are actively training and competing in athletics or multisport. You must be able to comply with the set training schedules. The following people will have been excluded; those that are injured, under 16 years old and not deemed medically fit to take part and those taking supplements (they will be asked to refrain one month prior to testing).

What will happen in this research?

This study will require 20 trained runners or multisport athletes to be trained for 3x3 weeks. At each 3 week period you will be given either weak commercial orange juice, black-currant juice or sports drink to be taken daily.

Three week training study

You will undergo an initial maximal incremental (peak power) test and a 5km time trial (at the local East Side Tavern race), prior to undergoing a three week program of set, monitored training. Training program will be given to all subjects based on your heart rate to ensure comparable training efforts by all subjects. The training will includes 8 compulsory sessions, with the remaining sessions up to the participant to design.

A maximal incremental (peak power) test and 5km time trial will be completed at the end of the three week training program. The laboratory you will visit is well set up to deal with the safety aspects of testing. You will complete an antioxidant questionnaire prior to testing, to quantify the usual dietary antioxidant intake. You will be asked to refrain from taking supplements one month prior to and whilst in the study.

Blood samples will be taken from a vein in your arm prior to and after the 5km time trial by a trained phlebotomist.

What are the discomforts and risks?

This research requires in total four incremental maximal running tests in the laboratory, after which you will undergo a three week training program, followed by the last incremental test. The time commitment is high. During the three week training program, you will need to visit the testing laboratory twice a week for training and to check in your training log and heart rate monitor. The research also involves a total of six blood tests taken, which can be uncomfortable for some people.

How will these discomforts and risks be alleviated? What are the benefits?

You will be reimbursed for your travel and training costs for a total of three weeks. An additional benefit is access to a personalised training program designed by a running-specific physiologist.

How will my privacy be protected?

No material which could identify you personally will be used in any reports on this study. The only identifiers to your information will be the researchers.

What are the costs of participating in this research?

This will be a time consuming project. It is expected that you will visit the laboratory once a week for nine weeks, either for an incremental exercise test or to provide a training log and heart rate monitor. The three week training program will require around 10 hours of training a week, however it is envisaged that you will already be undertaking this training time already.

How do I agree to participate in this research?

You will be given this form to explain to you the basics of the research. You will be asked to sign a consent form on the day of testing if you agree to take part.

Will I receive feedback on the results of this research?

Yes. The results will be presented back to you as soon as they are available. However, given that 60 people are likely to be involved this may be some time after the initial testing. This will occur as written information provided to each personal participant with their results. If requested, feedback can be given to the individual athlete and/or their family or partner.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, *Will Hopkins, will.hopkins@aut.ac.nz,* (09) 921 9793.

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, *madeline.banda@aut.ac.nz* , (09) 921 9999 ext 8044.

Whom do I contact for further information about this research?

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