THE UNIVERSITY OF HULL

The Effects of Fruit Vegetable Concentrate upon oxidative stress and buffering capacity in *vivo*.

being a Thesis submitted for the Degree of Master of Science

in the University of Hull

by

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February 2012

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Acknowledgements

There are a number of people I would like to acknowledge for their support and guidance during the completion of this thesis. Firstly I would like that thank my supervisors Dr Jason Siegler, Dr Grant Abt and Nigel Mitchell who gave me the opportunity to undertake this research and make it possible to collaborate with one of the most successful sporting organisations currently in the UK in British Cycling.

I would like to thank the post-graduate research students and laboratory technicians (James, Chris and Chris) in the department, mainly Daniel Peart, Bryna Chrismas and Richard Kirk who have not just been a friendly face every day, but their support in data collection and analysis would have not made the research that I have taken on possible.

A special mention to Dr Angela Hillman who I owe immeasurable gratitude for her time, patience, expertise, support and, guidance with which I would not have been able complete this thesis or to have gained the skills and knowledge that I have today. She has picked me up when things have been tough and has always believed in my capabilities even when I didn't believe in them myself. A special mention also has to go to Richard Page for being a great friend and colleague on the rollercoaster that is research. I hope that we will continue to work together for a long time to come.

Thanks must also go to Dr Rebecca Vince and Dr Ric Lovell and, Dr Adrian Midgley for their time and patient in helping with data collection and biochemical analysis of my samples, without their help I would not have any data to write about.

Finally I would personally like to thank my family in particular Rachael Bessinger whose wise words lead me to take on a research masters but most importantly my mum who as always supported me on every front to ensure that I have the opportunity to progress my education to the highest level.

Publish Work and Work in Review

Turner, M., Page, R., Mitchell, N., Siegler, J. (2011). The Effects of Energised Greens upon blood base balance during resting conditions. *Journal of the International Society of Sports Nutrition*, 8 (14).

Turner, M.C., Page, R., Hillman, A.R., Chrismas, B., Bray, J.W., Peart, D.J., Mitchell, N., Siegler, J.C. (2012). The effects of acute antioxidant supplementation upon markers of oxidative stress during intermittent cycling exercise. *International Journal of Sports Nutrition and Exercise Metabolism, In Review.*

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Abbreviations

°C	Degrees Centigrade
BE	Base Excess
FR	Free Radicals
FVC	Fruit Vegetable Concentrate
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
GXT	Graded Exercise Test
HR	Heart Rate
HCO_3^-	Bicarbonate
MDA	Malondialdehyde
NAC	N-Acetylcysteine
$NaHCO_3$	Sodium Bicarbonate
PC	Protein Carbonyls
PLC	Placebo
RDA	Recommended daily allowance
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
RPM	Revolutions per Minute
TAC	Total Antioxidant Capacity
TBARS	Thiobarbituric Acid Reactive Substances
TEAC	Trolox Equivalent Antioxidant Capacity
TGSH	Total Glutathione
TR	Trained
UT	Untrained
VAS	Visual Analogue Scale

VO_{2max}	Maximal Oxygen Consumption
W	Watts
W_{max}	Maximal Wattage

Abstract

Western diets are high in protein and insufficient in fruit and vegetable consumption. This can result in low-grade acidosis, which is linked with various metabolic diseases and can also impact exercise performance. Supplementing with fruit vegetable concentrate (FVC) could potentially attenuate the effects of low-grade acidosis and provide protection against the effects of oxidative stress due to the high antioxidant potential within these nutritional supplements.

The first study investigated the dose response of acute FVC supplementation upon blood acid-base levels during resting conditions. Eight physically active males (age: 23 ± 2 yr; height 180.1 ± 6.2 cm; weight 76.9 ± 7.2 Kg) consumed either a manufacturer recommended dose (9 g) of energised greens (FVC), an equal dose placebo (PLC), or a known acid-base regulator ($NaHCO_3$) in a single blind, randomised crossover design. Capillary blood samples were taken every 15 minutes to measure changes in blood pH, bicarbonate (HCO_3^-) and base-excess (BE). A visual analogue scale (VAS) was used to analyse potential changes in gastrointestinal discomfort. Blood pH rose in all trials but was only significantly elevated in the $NaHCO_3$ trial ($P<0.001$) compared to FVC and PLC trials. Similarly, HCO_3^- only rose following $NaHCO_3$ ingestion ($P<0.001$) with no significant increases in the FVC or PLC trials ($P>0.05$). BE significantly increased during the $NaHCO_3$ trial ($P<0.001$) with no changes following FVC or PLC ingestion.

The second study investigated the effects of acute FVC supplementation upon markers of oxidative stress during an acute bout of intermittent exercise. Seven physically active males (age: 25 ± 6 yr; height 179.0 ± 6.7 cm; weight 73.2 ± 9.1 kg, 54.7 ± 7.3 VO_{2max}) consumed either a manufacturer recommended dose of FVC (9 g) or a placebo with 750 ml of water after an overnight fast. One-hour post ingestion subjects completed an acute

bout of intermittent exercise consisting of eight 2-minute intervals at 85% W_{max} interspersed with seven 2-minute intervals at 50% W_{max} of the subject's pre-determined maximal wattage (W_{max}). Heart rate and RPE were measured at the end of each interval. Venous blood samples were drawn pre, post ingestion, immediately post, one and two hours post exercise for changes in Thiobarbituric Acid Reactive Substances (TBARS), Total Antioxidant Capacity (TAC), and Total Glutathione (TGSH). Body mass, urine osmolality and urine pH was measured pre and post ingestion and immediately post exercise. There were no significant main for condition (P=0.80) or time (P=0.56) or interaction effects (P=0.17) for changes in TGSH concentrations. There was no significant main effect for condition (P=0.47), or time (P=0.62) respectively for changes TAC concentrations, nor were any interaction effects observed (p=0.39). However there were no main (P=0.53), or interaction effects (P=0.97), TBARS concentrations were lower following FVC supplementation but were not significantly different. Furthermore there was no significant differences as a result of PLC supplementation (P=0.37). There were no effects for time (P=0.99) or conditions (P=0.92) for changes in body mass, noir where significant differences observed for changes in urine pH between conditions (P=0.84) or at any time point (P=0.47). Urine osmolality declined post-ingestion with no differences between conditions (P>0.05) however, were significantly different between pre-ingestion and post-exercise concentrations (P<0.05).

The results from the current thesis show that acute FVC supplementation does not have an effect upon blood acid base levels at rest and therefore it is unlikely to attenuate changes in acid-base balance as a result of exercise. Furthermore, despite containing a high abundance of antioxidant compounds, acute supplementation with FVC does not attenuate the effects of exercise induced oxidative stress in healthy males during intermittent exercise. Further research is required as to the effects of acute and chronic

supplementation upon acid-base regulation and antioxidant capacity as a result of FVC supplementation, as well as the effects of such supplementation upon populations with poor diets that lack fruit and vegetables.

Keywords: Antioxidants, Cycling, Intermittent Exercise, Acidosis.

Chapter 1. Introduction

1.1. Introduction

The three-fold increase in oxygen consumption associated with exercise primarily aids the generation of energy through aerobic metabolism, with 95-99% of oxygen used in the phosphorylation of ATP (Sen, 1999). The remaining percentile, which is incomplete in the reduction of oxygen during aerobic metabolism, results in the formation of reactive oxygen species (ROS) (Powers & Jackson, 2008). Although various tissues (e.g. skeletal muscle, liver and, brain), have been reported to contribute to the production of ROS (Fisher-Wellman & Bloomer, 2009), authors have attributed excess ROS production and subsequent muscle fatigue to the high concentration present within skeletal muscle (Powers & Lennon, 1999; Reid, Stokic, Koch, Khawli, & Leis, 1994).

The knowledge of ROS and the association with exercise has only recently been discovered. Initial research by Dillard and colleagues was the first to show that exercise contributes to ROS production (Dillard, Litov, & Tappel, 1978). Following this, research has identified the increase in ROS in contracting skeletal muscle of animals and humans (Davies, Quintanilha, Brooks, & Packer, 1982; Koren, Sauber, Sentjurc, & Schara, 1983), while most recently researchers have sought to explain the implications of ROS upon muscle force production (Reid, Khawli, & Moody, 1993), as well as their role as signalling molecules (Powers, Duarte, Kavazis, & Talbert, 2010).

Increases in oxidative stress have been found following various exercise modalities (Alessio et al., 2000; Bloomer et al., 2006). As a result, researchers have sought to attenuate the increase in ROS through interventions such as exercise training (Miyazaki et al., 2001; Tessier, Margaritis, Richard, Moynot, & Marconnet, 1995), and nutritional interventions in the form of antioxidant supplementation (Sen, 2001) to maintain the balance between pro- and antioxidants (Sen, 1999). There is a plethora of research that

has investigated supplementing with antioxidants such as ascorbic acid and α-tocopherol, either independently, or in combination (Bryant, Ryder, Martino, Kim, & Craig, 2003; Medved et al., 2004; Sacheck, Milbury, Cannon, Roubenoff, & Blumberg, 2003). However much of the literature is equivocal with regard to the attenuation of ROS formation (Lamprecht, Hofmann, Greilberger, & Schwaberger, 2009), as well as their ergogenic effect (Aguilo et al., 2007; Bryant et al., 2003).

Supplements containing high concentrations of polyphenols, which have a high antioxidant potential, have been found to attenuate the effects of oxidative stress as well as improve exercise performance (Davis, Carlstedt, Chen, Carmichael, & Murphy, 2010; Kalafati et al., 2010; Nieman et al., 2010). The consumption of fruit and vegetables containing high concentrations of metabolising anions is important in reducing the development of potential metabolic disorders and coronary artery disease (Mirmiran, Noori, Zavareh, & Azizi, 2009). Despite campaigns within countries recommending a daily intake of five fruits and vegetables, as well as international guidelines recommending the consumption of 400g per day (World Health Organization, 2003), these targets are rarely met within western society. Research has shown that UK households only consume an average of 290g fruits and vegetables per day (Naska et al., 2000), with the majority of antioxidant consumption attained through nutritional supplements (54% and 64%, vitamin c and vitamin e respectively) (Chun et al., 2010). Inadequate consumption of fruit and vegetables can reduce overall body antioxidant capacity (Bloomer, 2007). This, in conjunction with high animal protein intakes, which is prevalent in the majority of western diets, can lead to elevations in kidney net acid excretion resulting in low grade chronic acidosis (Remer, 2001). This resulting induced metabolic acidosis can reduce kidney function (Koeppen, 2009), insulin sensitivity

(DeFronzo & Beckles, 1979) and can lead to muscle atrophy following regulatory compensation of the kidneys through amino acid catabolism (Koeppen, 2009).

The detrimental effects upon performance as a result of both metabolic acidosis (Raymer, Marsh, Kowalchuk, & Thompson, 2004; Robergs, Hutchinson, Hendee, Madden, & Siegler, 2005; Siegler, McNaughton, Midgley, Keatley, & Hillman, 2010) and oxidative stress (Bloomer et al., 2006; Goldfarb, You, Bloomer, Landes, &, Murphy, 2002; Reid et al., 1993), are well known. However, supplementing with FVC has been found to protect trained subjects from lipid peroxidation and protein oxidation following aerobic and eccentric exercise (Bloomer, Goldfarb, & McKenzie, 2006; Goldfarb, Garten, Cho, Chee, & Chambers, 2010). Although the exact mechanisms are currently unknown, chronic FVC supplementation in both untrained males and females has been shown to significantly increase glutathione (GSH) and vitamin E levels while reducing malondialdehyde (MDA) concentrations following 30 minutes running at 80% VO_{2max} (Goldfarb, McKenzie, & Bloomer, 2007). Supporting findings have been presented in trained individuals supplementing for two weeks (Bloomer et al., 2006). The attenuating effects have also shown to be prevalent during the recovery phase in trained males following eccentric exercise, with significantly lower protein carbonyl concentration (a measure of protein oxidation) up to 72 hours post exercise following four weeks supplementation (Goldfarb et al., 2010).

Although chronic supplementation has been shown to have protective effects in both clinical and exercise settings (Bailey, Williams, Betts, Thompson, & Hurst, 2010; Nantz, Rowe, Nieves, & Percival, 2006), the literature administering FVC is currently limited in nature. Despite this, acute doses have shown to attenuate markers of oxidative stress in both trained and untrained males (Alessio, Goldfarb, & Cao, 1997; Morillas-Ruiz et

al., 2005; Nakhostin-Roohi, Babaei, Rahmani-Nia, & Bohlooli, 2008; Thompson et al., 2003). Supplementing prior to and during exercise has been effective in attenuating protein oxidation in trained cyclists following 90 minutes cycling at 70% VO_{2max} (Morillas-Ruiz et al., 2005; Morillas-Ruiz, Villegas Garcia, Lopez, Vidal-Guevara, & Zafrilla, 2006). Recent research has found attenuating effects on both oxidative stress and metabolic acidosis following high intensity cycling as a result of pro-alkalising supplementation (Peart et al., 2011). Due to the pro-alkalising properties and antioxidant concentrations of FVC (Berardi, Logan, & Rao, 2008; Goldfarb et al., 2010), similar effects could be observed following acute supplementation, as administration two hours prior to exercise with high polyphenol supplements has presented protective effects from oxidative stress following high intensity running (Davison, Callister, Williamson, Cooper, & Gleeson, 2011). Therefore, the purpose of this research was to: a) investigate the protective effects of FVC supplementation upon acid-base regulation at rest and: b) determine the effects of acute supplementation upon markers of oxidative stress following high intensity intermittent exercise.

Chapter 2 Literature Review

2.1 Oxidative Stress

The development of oxidative stress can be caused by various stressors such as environmental pollution (Halliwell, 1991), nutrition (Sies, Stahl, & Sevanian, 2005) and physical exercise (Vollaard, Shearman, & Cooper, 2005). The increase in the rate of oxygen consumption resulting from exercise up-regulates the formation of reactive oxygen species (ROS), disturbing the pro-antioxidant balance (Halliwell & Cross, 1994). Although ROS have been shown to play important roles in cellular function (Powers et al., 2010), chronic exposure to ROS can have detrimental effects upon cellular structure and function (Droge, 2002). Early research by Dillard and colleagues (1978) was the first to identify a causal link between ROS production and exercise. Consequently, since these findings research has sought to investigate the effects of differing exercise modalities on oxidative stress (Chen, Kim, Henning, Carpenter, & Li, 2010).

The maintenance of cellular homeostasis is dependent upon the balance between the production of ROS and the antioxidant system, which has been extensively reviewed (Finaud, Lac, & Filaire, 2006; Fisher-Wellman & Bloomer, 2009; Powers & Jackson, 2008). Reactive oxygen species, such as superoxide, hydrogen peroxide and hydroxyl radicals, all have strong oxidising potential due to their ability to permeate membranes, and their relatively long half-life (Powers & Jackson, 2008). While numerous sites have been identified to produce high levels of ROS, the predominant site for ROS formation is in the mitochondria, during oxygen metabolism at complexes I and III of the electron transport chain where singlet oxygen is formed (Lenaz, 1998; Nohl & Jordan, 1986). Contracting skeletal muscle results in major production of ROS (McArdle, Vasilaki, & Jackson, 2002), with the formation of NADPH oxidase in the sarcolemma leading to an increase in superoxide production (Powers & Jackson, 2008). A discrepancy between type I and II muscle fibres has recently been noted, with the latter presenting higher

ROS promoting properties. This in turn would account for the higher ROS production observed during aerobic exercise (Anderson & Neufer, 2006).

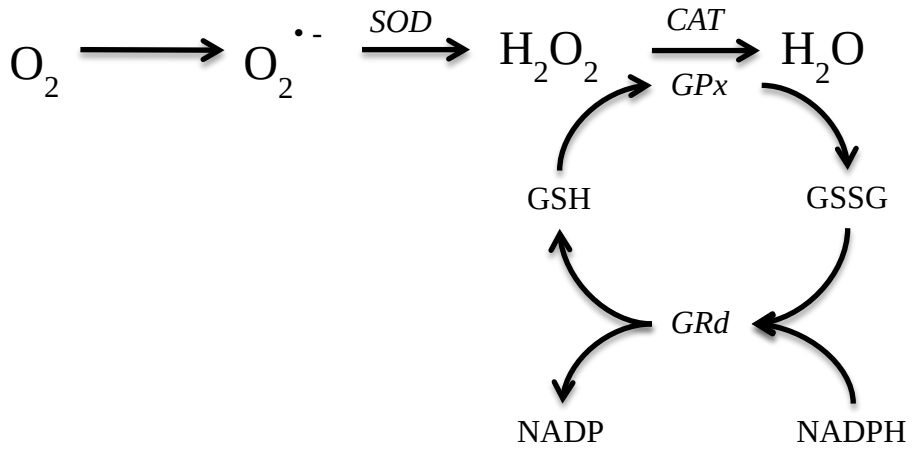


Figure 2.1*: The production and removal of reactive oxygen species (ROS). Glutathione (GSH), Oxidised Glutathione (GSSG), Glutathione Peroxidase (GPx), Glutathione Reductase (GRd), Superoxide Dismutase (SOD), Catalase (CAT), Hydrogen Peroxide (H_2O_2), Singlet Oxygen ($O_2^{\bullet-}$), Oxygen (O_2), Water (H_2O). Figure adapted from Droge (2002).*

The quantification of ROS production following exercise is an important issue due to the variation in half-life of ROS (Halliwell, 1991). Consequently, direct and indirect measurements have been utilised in quantifying changes in lipid and protein oxidation during exercise, all with varying validity and reliability (Powers, Smuder, Kavazis, & Hudson, 2010). The use of direct methods such as electron spin resonance spectroscopy, high performance liquid chromatography, radiolysis, and laser flash photolysis have been previously used *in vivo*, and are considered accurate and reliable measures of ROS production (Finaud et al., 2006). However, due to the variation in half-life as well as the cost-effectiveness of these sampling methods, researchers have employed indirect methods such as assays to quantify changes in by-products of oxidative stress.

Indirect assessment within the literature has commonly utilised the measurement of lipid peroxidation such as malondialdehyde (MDA) and thiobarbituric acid reactive

substances (TBARS). Both MDA and TBARS have been frequently used as a measure of lipid peroxidation, however its use has been subject to caution due to the reported lack of validity within human studies (Alessio et al., 2000; Fisher-Wellman & Bloomer, 2009). This has been due to the effect interaction of products during sampling (Halliwell & Gutteridge, 2007). In addition, the inconsistent changes in theses markers between trained and untrained subjects, despite the significant increases in enzymatic antioxidants following exercise induced oxidative stress, has led to disagreement between researchers as to their suitableness as a marker of lipid peroxidation (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997; Miyazaki et al., 2001; Santos-Silva et al., 2001).

Changes in the antioxidant defence system are primarily investigated through the measurement of endogenous, non-enzymatic antioxidants such as glutathione, which is comprised of total glutathione (TGSH), oxidised glutathione (GSSG) and reduced glutathione (GSH). Other enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The vast range of endogenous antioxidants are beyond the scope of this review, however, the use of glutathione as a supplement has been of particular interest with regard to exercise induced oxidative stress, and has been found to be an important mediator in the regulation of redox homeostasis (Gohil, Viguie, Stanley, Brooks, & Packer, 1988), and will therefore be reviewed in this thesis. Furthermore, exogenous antioxidants such as α-tocopherol (Vitamin E) and ascorbic acid (Vitamin C) have been used as indicators of oxidative stress (Laaksonen et al., 1999), and will also be discussed.

2.2 Oxidative Stress Markers during Exercise

Since early research reported an increase in ROS production in skeletal muscle during exercise (Davies et al., 1982), several researchers have investigated the effects of exercise on ROS accumulation (Gohil et al., 1988; Jammes, Steinberg, Bregeon, & Delliaux, 2004; Reid et al., 1992). Consequently, research has found exercise induced oxidative stress to increase following differing exercise modalities (Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005), although findings are equivocal.

Early research by Viinikka and colleagues found no changes in lipid hydroperoxides (LOOH) following cycling graded exercise test (GXT) within trained males (Viinikka, Vuori, & Ylikorkala, 1984), which has been further supported within the same subject population (Asami, Hirano, Yamaguchi, Itoh, & Kasai, 1998). These findings have also been supported with regard to both aerobic and anaerobic exercise, irrespective of training status (Bloomer et al., 2005; Sumida et al., 1997; Wilber et al., 2004), with more recent research by Orhan and colleagues also reporting no changes following longer duration exercise in trained subjects (Orhan et al., 2004).

2.3. Non-Enzymatic Biomarkers

Lipid peroxidation concentration has been shown to increase in a time dependent fashion in both trained and untrained individuals during ultra-endurance exercise (Kanter, Lesmes, Kaminsky, La Ham-Saeger, & Nequin, 1988; Knez, Jenkins, & Coombes, 2007), with increases persisting up to five days post exercise in trained subjects (Neubauer, Konig, Kern, Nics, & Wagner, 2008). The differences between trained and untrained subjects has been found to be due to the higher concentrations of antioxidant enzymes within skeletal muscle of trained compared to untrained individuals (Ortenblad, Madsen, & Djurhuus, 1997). Additionally, increases have been

linked to the inflammatory responses of exercise (Pinho et al., 2010), which have found to be attenuated by antioxidant supplementation after ultra-endurance exercise (Mastaloudis, Morrow, Hopkins, Devaraj, & Traber, 2004) .

2.3.1 Thiobarbituric Acid Reactive Substances (TBARS) and Malondialdehyde (MDA)

The production of TBARS results from the reaction between MDA and thiobarbituric acid, yet measurement of TBARS may not directly represent MDA production during oxidative stress; therefore the use of such general markers of lipid peroxidation has been subject to caution (Powers et al., 2010). Despite this, a plethora of research has reported increases in TBARS concentrations as a result of prolonged and intermittent exercise (Fisher et al., 2011; Kayatekin, Gonenc, Acikgoz, Uysal, & Dayi, 2002), which can be sustained above baseline for as long as one hour post exercise (Peart et al., 2011; Steinberg, Delliaux, & Jammes, 2006; Steinberg, Ba, Bregeon, Delliaux, & Jammes, 2007) .

The increase in TBARS concentration has been has been shown following a GXT. Nikcolaidis and colleagues reported significant increases in TBARS concentrations following a 90% VO_{2max} treadmill run in healthy males (Nikolaidis et al., 2006). These findings have been further extrapolated to trained males, who have also shown an increase in TBARS concentration immediately post GXT (Szczesniak et al., 1998; Vider et al., 2001) . In addition, increases in TBARS concentrations have been found to increase following more prolonged steady state exercise in both trained and untrained populations (Nikolaidis et al., 2006; Vincent, Morgan, & Vincent, 2004). The findings by Laaksonen and colleagues reported an increase of 50% in TBARS concentrations following 40 minutes cycling at 60% VO_{2max} (Laaksonen et al., 1999).

Furthermore, later work by Nikolaidis and colleagues presented a two fold increase in trained males and females following intermittent exercise (Nikolaidis et al., 2007). More recent research by Pinho and colleagues has presented significant elevations in TBARS concentrations following an Ironman race (Pinho et al., 2010). Such findings could be resultant of the reduced exercise intensity due to the intensity dependent relationship between ROS production and exercise. Alternatively, the differences between findings could be resultant of the lack of specificity of TBARS as a measure of lipid peroxidation due to the minimal changes observed, but also the fact that null findings in TBARS do not concur with the changes in other markers of oxidative stress following exercise (Meijer et al., 2001) .

The production of MDA is a result of fatty acid oxidation; however, as previously mentioned, the measurement of MDA has been indirectly assessed using TBARS. Early research has presented a significantly increased MDA concentration following a GXT (Lovlin, Cottle, Pyke, Kavanagh, & Belcastro, 1987). However, recent research does not concur with the earlier findings following a similar exercise protocol in both untrained and trained subjects (Alessio et al., 2000; Leaf, Kleinman, Hamilton, & Barstow, 1997; Nishiyama, Ikeda, Haramaki, Yoshida, & Imaizumi, 1998). Furthermore both untrained (Chung, Goldfarb, Jamurtas, Hegde, & Lee, 1999; Kanaley & Ji, 1991) and trained subjects (Bloomer et al., 2005; Orhan et al., 2004) have presented little change in MDA concentrations following fixed intensity exercise. Although the large increase in MDA concentrations have be reported following prolonged exercise. Discrepancies within the literature have been attributed to the catabolism, excretion and redistribution of MDA concentrations within the body and therefore the increases in

concentrations that have been reported are reminiscent of the greater exercise intensity or duration of the exercise protocol in question (Leaf et al., 1997).

2.3.4 Glutathione (GSH/GSSG)

Glutathione, the roles of which range from antioxidant defence to immune system regulation, is concentrated primarily within the erythrocytes (Sen, 1999). The multitude of activities performed by the GSH system makes it a primary component in the regulation of pro-oxidant activity. Although concentrations are small, the majority of the GSH pool resides within the cytoplasm of the cell with an additional, less concentrated pool in the mitochondria and nucleus. The decomposition of hydrogen peroxide via the enzymatic antioxidant superoxide dismutase (SOD) is mainly regulated by GSH reactions (Lew, Pyke, & Quintanilha, 1985; Sen, Atalay, & Hanninen, 1994) . Subsequently, the measurement of glutathione as a marker of oxidative stress has been used in both animals (Sen et al., 1994b, Lew et al., 1985), and humans (Gohil et al., 1986, Laaksonen et al., 1999).

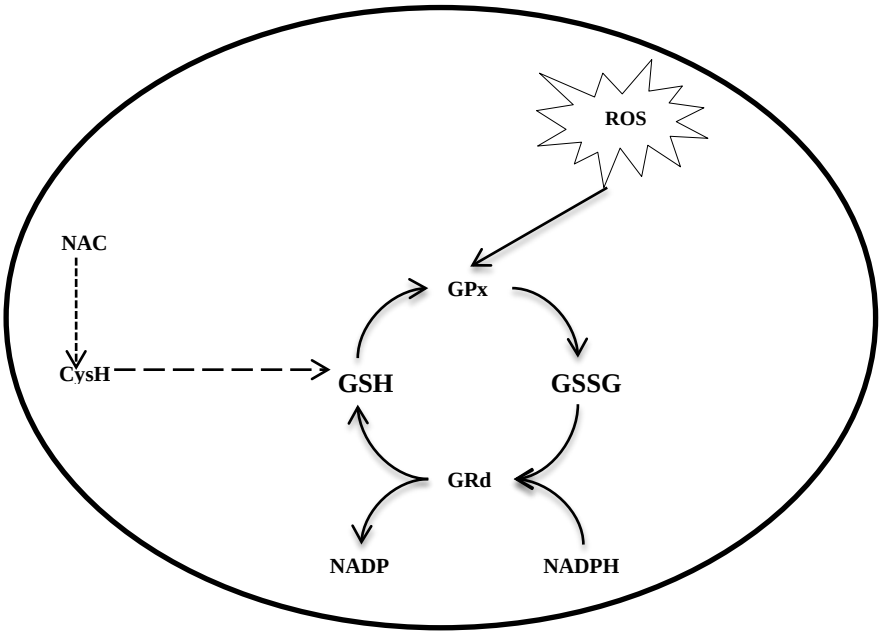


***Figure 2.2**: A simplified schematic of the glutathione cycle and its regulation of ROS, as well as the effects of* N-Acetylcysteine *(NAC) supplementation on the regulation of the glutathione redox balance. Figure adapted from Sen (1999).*

Initial research by Lew and colleagues found that exercise resulted in an increase in oxidised glutathione (GSSG) within liver, plasma and, muscle tissue following running to exhaustion in rats (Lew et al., 1985). More importantly, the authors noted an increase in reduced glutathione (GSH) within the plasma which was attributed to the efflux of GSH from the liver to regulate GSSG production within the muscle, therefore emphasising the mediating role of the glutathione system in ROS regulation. Subsequently, human research has found changes in glutathione status following maximal exercise in untrained (Steinberg et al., 2007) and trained subjects (Vider et al., 2001).

Furthermore, research by Gohil et al. (1988) presented a significant decrease in reduced glutathione in the skeletal muscle of untrained males during prolonged submaximal exercise. Conversely, exercise over three consecutive days at the same exercise intensity as that utilised by Gohil and colleagues depleted the glutathione system, increasing GSSG in trained athletes (Viguie et al., 1993). In addition, maximal incremental exercise to exhaustion has also been shown to deplete the glutathione system in healthy adults (Nikolaidis et al., 2007; Steinberg et al., 2006). The up-regulation of glutathione enzymes, such as glutathione reductase and glutathione peroxidase, plays a crucial role in regulating the glutathione homeostasis, with the removal of peroxides reported to be elevated in trained compared to untrained subjects (Marzatico et al., 1997).

N-Acetylcysteine (NAC) is a potent ROS scavenger which mediates glutathione resynthesis and reduces extracellular cysteine (Kelly, 1998). The detailed effects of NAC are beyond the scope of this review, however due to role of NAC in the regulation of glutathione homeostasis, it is important to understand its role in the regulation of

redox balance. The administration of NAC has been found to delay the onset of muscle fatigue (Reid et al., 1994; Shindoh, DiMarco, Thomas, Manubay, & Supinski, 1990), as well as increase muscle cysteine and GSH concentrations in trained males (Medved et al., 2004; Medved, Brown, Bjorksten, & McKenna, 2004). Subsequent effects have resulted in an increase in time to exhaustion during prolonged cycling in both trained (Medved et al., 2004; Medved, Brown, Bjorksten, & McKenna, 2004) and untrained males (McKenna et al., 2006; Murphy, Medved, Brown, Cameron-Smith, & McKenna, 2008).

2.3.5. Total Antioxidant Capacity

Total antioxidant capacity (TAC) is comprised of an augmentation in a multitude of enzymatic and non-enzymatic antioxidants such as uric acid, glutathione peroxidase, glutathione reductase, and superoxide dismutase (Ortenblad et al., 1997). Both acute exercise bouts and exercise training result in markedly increased antioxidant enzymes within the blood (Marzatico et al., 1997). Trained athletes boast a greater total antioxidant capacity, which is a consequence of the stimulation of redox signalling pathways and the increase of antioxidant enzyme gene expression resultant of exercise training (Powers et al., 2010).

The changes in TAC following shorter exercise bouts are equivocal. Recent work by Berzosa et al. (2011) reported significant increases in TAC following maximal exercise. Furthermore, increases in TAC have been found immediately post exercise following a treadmill run to exhaustion in trained males (Vider et al., 2001). In addition, an investigation utilising trained runners found a significant reduction in TAC immediately upon exhaustion (Watson et al., 2005). While the differences between the respective studies could be attributed to exercise modalities, the difference methods of analysis

could also contribute to differences to the varying concentrations observed. Despite observed increases in antioxidant enzymes resultant of exhaustive exercise in trained athletes (Tauler et al., 2006), this increase has failed to negate exercise induced oxidative stress production in trained athletes during similar exercise (Tauler et al., 2006). Although research measuring changes in TAC during prolonged exercise is sparse, increases in trolox equivalent antioxidant capacity (TEAC), a measure of TAC, has been found following a simulated self-paced half marathon in trained subjects (Child, Wilkinson, Fallowfield, & Donnelly, 1998).

2.4 Antioxidant supplementation

Antioxidants play an important role in maintaining the redox balance and provide protection from cellular and DNA oxidative damage (Tauler, Ferrer, Romaguera et al., 2008; Tauler, Ferrer, Sureda et al., 2008). Antioxidants such as vitamin C, vitamin E and β-carotene, which are highly concentrated within fruits and vegetables, have been found to improve antioxidant defences in both young and old healthy populations (Agte, Jahagirdar, & Chiplonkar, 2006; Kiefer et al., 2004). Although the recommended intakes of fruits and vegetables are rarely met within western societies (Billson, Pryer, & Nichols, 1999; Naska et al., 2000), and the majority derived from vitamin supplements (Chun et al., 2010), the consumption of fruits and vegetables provide more than an antioxidant potential. There is a plethora of research that has presented the positive benefits of antioxidant supplementation for improving outcomes in both clinical and exercise settings (Aguilo et al., 2007; Alessio et al., 1997; Sastre et al., 1992).

2.4.1. Asorbic Acid

Asorbic acid has been extensively used as an antioxidant supplement (Alessio et al., 1997; Bryer & Goldfarb, 2006; Goldfarb, Patrick, Bryer, & You, 2005; Thompson et al., 2003). Its role as an antioxidant lies in its ability to scavenge ROS such as superoxide, lipid hydroperoxide and hydroxyl radicals, as well as its role in resynthesizing α-tocopherol, aiding the transition of radicals from the lipid to aqueous phase, and finally spare GSH during periods of oxidative stress (Carr & Frei, 1999; Meister, 1994).

Reductions in markers of oxidative stress have been noted following both acute (Ashton et al., 1999), and chronic ascorbic acid supplementation (Alessio et al., 1997; Bryant et al., 2003). Ascorbic acid supplementation has been also noted to be beneficial in reducing the effects of muscle soreness and muscle damage in both untrained (Bryer & Goldfarb, 2006) and trained individuals (Thompson et al., 2004), which could be a resultant of the increased stores of ascorbic acid in the peripheral circulation (Thompson et al., 2004).

Furthermore, higher ascorbic acid dosage protocols have been shown to have positive effects in regulating redox balance. Bryer and Goldfarb (2006) administered high doses of ascorbic acid (3 grams per day ($g \cdot D^{-1}$)) in healthy males performing eccentric exercise, reporting a significant attenuation in oxidised glutathione concentrations (Bryer & Goldfarb, 2006). Positive effects have also been reported following short duration aerobic exercise also using high doses of ascorbic acid (1 $g \cdot D^{-1}$), in which MDA concentrations have been shown to be attenuated in untrained healthy males (Nakhostin-Roohi et al., 2008). Furthermore, high doses have been found to attenuate lipid peroxidation (12% vs. 46%) following acute one day supplementation (1000 mg, 2-hours pre-exercise), suggesting that the dosage administration of ascorbic acid has

more implications on attenuating ROS production compared to administration duration (Alessio et al., 1997). While high doses of ascorbic acid appear to be beneficial, lower doses (200 mg·day^{-1} – 500 mg·day^{-1}) do not appear to have the same effect (Goldfarb et al., 2005; Thompson et al., 2003). Furthermore, three weeks ascorbic acid (400 mg·day^{-1}) can attenuate eccentric exercise induced muscle damage in physically active individuals (Jakeman & Maxwell, 1993). Additionally, although small differences in TAC 24 hours post exercise (30 minutes, at 75% of VO_{2max}) were found in the supplemented group, there were no changes in markers of muscle damage or inflammation within the supplemented group (Nakhostin-Roohi et al., 2008).

While ascorbic acid has the ability to donate a hydrogen ion and reduce oxygen in vivo (Frei, Stocker, & Ames, 1988; Frei, England, & Ames, 1989), in some cases, supplementation, particularly in high concentrations (1-3 g·D^{-1}), has been found to augment rather than attenuate ROS production (Thompson et al., 2003). For example, during the process of α-tocopherol regeneration, semiascorbyl radical, a potent ROS, is formed (Carr & Frei, 1999). The formation of this ROS would explain the negative effects of high doses of ascorbic acid supplementation that have been reported within the literature (Bryant et al., 2003; Thompson et al., 2003). In addition, the administration of vitamin C has been found to prevent mitochondrial biogenesis as well as several endogenous antioxidants in skeletal muscle (Gomez-Cabrera et al., 2008), therefore presenting strong evidence for the negative effects of high does supplementation upon the expression of antioxidant enzymes (Ristow et al., 2009), but also stressing the importance of ROS in skeletal muscle adaption (Powers et al., 2010).

2.4.2. α-tocopherol

α-tocopherol is a lipid soluble exogenous antioxidant which is found to regulate gene expression (Azzi et al., 2004). The effects of α-tocopherol were originally attributed to its ability to increase low oxygen pressure, improve myocardial efficacy and peripheral capillary dilation (Shephard, 1983). Conversely, both early (Helgheim, Hetland, Nilsson, Ingjer, & Stromme, 1979) and more recent research (Hartmann, Niess, Grunert-Fuchs, Poch, & Speit, 1995; Meydani et al., 1993; Sacheck et al., 2003) has presented an ergogenic effect in its ability to attenuate markers of oxidative stress as well as reduce the effects of muscle damage following exercise (Singh & Jialal, 2004).

Original research has suggested improved maximal oxygen uptake (VO_{2max}) resultant of α-tocopherol supplementation (Helgheim et al., 1979; Shephard, 1983), however later research has conclusively shown that such supplementation is in-effective in improve maximal oxygen uptake of trained athletes (Lawrence, Bower, Riehl, & Smith, 1975; Nielsen et al., 1999; Patil, Chaudhuri, & Dhanakshirur, 2009; Rokitzki, Logemann, Huber, Keck, & Keul, 1994). However, research is yet to elucidate the reason for increases in VO_{2max} of untrained populations following α-tocopherol supplementation (Nalbant et al., 2009), and although the performance enhancing effects of α-tocopherol supplementation upon maximal oxygen uptake would appear small, supplementation is imperative within athletic populations to maintain antioxidant milieu but could also improve performance in populations with α-tocopherol deficiency (Takanami, Iwane, Kawai, & Shimomitsu, 2000).

The current RDA of α-tocopherol of 15 $mg \cdot day^{-1}$ (18.14 IU) for both males and females is often exceeded within dietary intake (Sen, 2001). Furthermore, the administered doses used in research often exceed these values, with doses ranging between 400-1200

IU·day^{-1}(Bryant et al., 2003; Lawrence et al., 1975). Although sedentary individuals may require higher doses of 200 mg·day^{-1} as compared 100 mg·day^{-1} for trained individuals (Takanami et al., 2000), the ability for these values to reduce the effects of lipid peroxidation following acute exercise are not conclusive (Kanter, Nolte, & Holloszy, 1993). In fact, some research has presented potential pro-oxidant effects in both trained and untrained populations (McAnulty et al., 2005). The resynthesis of α-tocopherol is mediated through ascorbic acid during increased concentrations of ROS resultant of exercise induced oxidative stress (Knez, Coombes, & Jenkins, 2006). This has been supported by research which has combined both α-tocopherol and ascorbic acid which is discussed below.

2.5. Combined Antioxidant Supplementation

As previously discussed, the attenuating effects of single antioxidant supplementation are equivocal. Therefore, a plethora of research has investigated combined antioxidant supplementation, both at rest and within an exercise environment (Kanter et al., 1993; Lamprecht et al., 2009; Tauler, Aguilo, Fuentespina, Tur, & Pons, 2002). Due to the interaction between α-tocopherol and ascorbic acid it has been hypothesised that the combination of these antioxidants can attenuate the effects of oxidative stress (Bryant et al., 2003). However despite increasing total antioxidant capacity in untrained subjects (Goldfarb et al., 2007), the effects of combined supplementation has not been found to attenuate the increase in lipid and protein oxidation in trained individuals (Bailey et al., 2010; Bloomer et al., 2006).

The addition of β-carotene (30-40 mg), a singlet oxygen free radical scavenger as well as antioxidant chain breaker (Kanter et al., 1993), has been used in combination with antioxidants previously mentioned in this review. Administration of β-carotene with

these antioxidants has shown to have attenuating effects upon lipid peroxidation in both trained and untrained populations (Aguilo et al., 2007; Kanter et al., 1993). However, excessive chronic and imbalanced administration can negate the positive effects associated with combined supplementation, potentially resulting in inefficient α-tocopherol and β-carotene resynthesis (Lamprecht et al., 2009). Therefore, although a combination of antioxidants can attenuate the effects of oxidative stress, caution must be taken in the supplementation period as well as the respective doses of the antioxidants being administered.

2.6. Fruit Vegetable Concentrate

Fruit vegetable concentrate (FVC) is a supplement of growing interest due to the high levels of polyphenols (Goldfarb et al., 2010; Morillas-Ruiz et al., 2006). Polyphenols are secondary metabolites that are mainly found in plants, as well as fruits and vegetables (Pandey & Rizvi, 2009). Different kinds of polyphenols have been identified, of which flavonoids are one of these groups. Such flavonoids as quercetin, which is discussed in brief below (*See section 2.8*), are abundant in polyphenols and have been shown to provide protection against exercise induced oxidative stress (Allgrove, Farrell, Gleeson, Williamson, & Cooper, 2011). As supplements such as FVC contain numerous types of fruits and vegetables, they contain high concentrations of polyphenols which have high antioxidant potential and therefore can provide protection from ROS production as a result of exercise (Bloomer et al., 2006).

The use of FVC has been shown to have a number of health benefits such as increasing antioxidant capacity (Kiefer et al., 2004; Samman et al., 2003), and improving the immune response (Nantz et al., 2006). The effects upon both immune function and antioxidant capacity could be related to the reduction in severe symptoms of illness that

have been reported following FVC supplementation (Roll, Nocon, & Willich, 2011) The consumption of FVC has also been shown to significantly reduce both systolic and diastolic blood pressure of hypertensive subjects following 90 days supplementation (Zhang, Oxinos, & Maher, 2009).

The associated benefits of chronic FVC supplementation have also been found following acute aerobic exercise, reducing markers of lipid and protein oxidation in both untrained (Goldfarb et al., 2007), and trained subjects (Goldfarb et al., 2010). However, the effects of acute administration of FVC are less conclusive. Despite significantly attenuating protein and DNA damage during 90 minutes cycling exercise in trained subjects, acute FVC supplementation has not been found to attenuate the increase in lipid peroxidation (Morillas-Ruiz et al., 2005; Morillas-Ruiz et al., 2006).

Although no increase in antioxidant status was observed following acute FVC supplementation, more recent research administering dark chocolate (70% coca), which contains high concentrations of polyphenolic compounds, has been found to increase antioxidant status when supplementing two hours prior to submaximal exercise (Davison et al., 2011). Although several factors such as differences in training status and antioxidant status of subjects (Bloomer & Fisher-Wellman, 2008; Dekany et al., 2006) could account for differences between respective studies, the effects of acute antioxidant supplementation upon markers of oxidative stress is of increasing interest and yet to be elucidated.

2.7 Spirulina platenisis

Spirulina platenisis, a component of the FVC supplement, is a photosynthetic cyanobacterium and is rich in essential amino acids. Spirulina, which it is more

commonly referred to, has recently been used as a nutritional supplement (McCarty, 2007), in part due to its high antioxidant potential (Dartsch, 2008). Spirulina exhibits greater digestible potential due to its lack of cellulose wall in comparison to other algae supplements (Lu, Hsieh, Hsu, Yang, & Chou, 2006). Initial research in animal models found spirulina supplementation to aid GSH levels as well as reduce lipid peroxidation (Kuhad, Tirkey, Pilkhwal, & Chopra, 2006a; Kuhad, Tirkey, Pilkhwal, & Chopra, 2006b). Although the research investigating this amino acid is sparse, spirulina supplementation in humans has been shown to have numerous health benefits such as reducing cholesterol, preventing cancer and boosting immune function (Belay, Ota, Miyakawa, & Shimamatsu, 1993). Furthermore, recent research by Lu and colleagues has reported significant increases in antioxidant enzyme capacities as well as reductions in markers of lipid peroxidation following three weeks of spirulina supplementation (Lu et al., 2006). In addition to these findings, supplementation with spirulina also presented an ergogenic effect, significantly prolonging time to exhaustion of untrained subjects (Lu et al., 2006). More recently, this ergogenic effect has been shown in moderately trained males, who also exhibited attenuation in lipid peroxidation following supplementation (Kalafati et al., 2010).

2.8. Quercetin Supplementation

Unlike Spirulina which is found in the FVC in question, Quercetin is not a specific component of FVC. However, it is important to briefly discussion the effects of the polyphenolic supplement quercetin, which is a dietary flavinoid mainly found in the skins of fruits and vegetables that poses high antioxidant potential (Wilms, Hollman, Boots, & Kleinjans, 2005). Although *in vitro* research has shown quercetin to potentially improve antioxidant status (Wilms et al., 2005), such effects within trained and untrained human populations are less conclusive and therefore its effectiveness as

an antioxidant supplement is yet to be confirmed (McAnulty et al., 2008; Quindry et al., 2008; Shanely et al., 2010).

2.9. Diet and Exercise Induced Metabolic Acidosis

The high consumption of animal products and sodium chloride along with the lack of fruit and vegetable intake can have an effect upon acid-base regulation (Remer, 2001). Early research first reported the differences in acid loads between plants and animals and subsequently showed that herbivores produced a greater alkaline urine pH compared to their carnivorous peers (Manz, 2001). Fruit and vegetables, which are abundant in metabolising anions, anionic amino acids and glutamate consuming hydrogen ions, maintain the neutral load (Adeva & Souto, 2011). In comparison animal proteins, when oxidised, generate non-metabolising sulphate. As a result, the chronic imbalance between consumption of metabolising and non-metabolising anions can result in levels of low-grade metabolic acidosis (Frassetto, C., & Sebastian, 2007). Chronic low grade metabolic acidosis has been found to have detrimental effects, including a decline in kidney function (Koeppen, 2009), and reduced insulin sensitivity leading to the potential development of insulin resistance (Cameron, Maalouf, Adams-Huet, Moe, & Sakhaee, 2006). Additionally, chronic low grade metabolic acidosis can affect muscle atrophy as a result of the kidneys role in acid-base regulation via amino acid degradation (Garibotto et al., 2009). The regulation of hydrogen ion concentration within the blood is dependent upon multiple factors such as the partial pressure of CO_2 (Madias, Adrogue, Horowitz, Cohen, & Schwartz, 1979), as well as the diet net acid load (Remer, 2001). It is well documented that western diets which are predominantly net-acid producing leading to the development of low-grade metabolic acidosis (Kurtz, Maher, Hulter, Schambelan, & Sebastian, 1983). The increase in acidosis is a result of the retraction of bicarbonate (HCO_3) from urine, resulting in hydrogen ion/bicarbonate

(H^+/HCO_3) transportation, which can have a resulting impact upon renal net acid excretion (Koeppen, 2009). In addition, high animal protein intakes can have an impact upon calcium equilibrium, ultimately resulting in a negative balance within the kidneys, potentially leading to kidney stone formation (Taylor, Stampfer, & Curhan, 2004).

The consumption of protein in parallel with exercise training is often used to aid recovery following exercise, as well as promote skeletal muscle hypertrophy (Hulmi et al., 2009). However, high dietary intakes of protein can have adverse effects upon acid-base regulation, potentially leading to the development of low-grade metabolic acidosis (Frassetto, Todd, C., & Sebastian, 1998). Consequently, the imbalance in acid-base homeostasis can adversely affect the onset of fatigue during exercise (Robergs et al., 2005). Therefore, ingestion and/or supplementation of pro metabolising anions and potassium contained within fruits and vegetables, which have shown to have positive effects upon both urinary and blood pH, could potentially counteract the effects of acidosis in humans (Berardi et al., 2008; Konig, Muser, Dickhuth, Berg, & Deibert, 2009).

The effects of exercise induced metabolic acidosis through H^+ ion concentration accumulation following intense exercise and the subsequent effects upon fatigue as a result are well documented within the literature (Raymer et al., 2004; Robergs et al., 2005). The accumulation of extracellular H^+ is mainly sourced from the hydrolysis of ATP (Robergs, Ghiasvand, & Parker, 2004). Multiple pathways such as moncarboxylic transporters, and the bicarbonate system, aid proton removal to maintain the strong ion differences between respective cellular spaces (Kowalchuk, Heigenhauser, Lindinger, Sutton, & Jones, 1988). However, during exercise induced acidosis, the rate of H^+ accumulation often exceeds the rate of removal (Robergs et al., 2004). Consequently,

multiple researchers have tried to delay the onset of metabolic acidosis through supplementation with pro-alkalising agents prior to exercise (Stephens, McKenna, Canny, Snow, & McConell, 2002). Despite research which has confirmed increases in blood pH and buffering capacities following supplementation in untrained (McNaughton, 1992; Siegler, Midgley, Polman, & Lever, 2010) as well as trained populations (Cameron, McLay-Cooke, Brown, Gray, & Fairbairn, 2010; Lindh, Peyrebrune, Ingham, Bailey, & Folland, 2008), there effects of such supplements upon performance are not as conclusive (Cameron et al., 2010; Lindh et al., 2008; McNaughton, Backx, Palmer, & Strange, 1999). Research investing the effects of pre-exercise supplementation using pro-alkalising supplements ($NaHCO_3$) upon high intensity exercise has found little or no performance enhancement in healthy male subjects (Siegler et al., 2010; Siegler, Marshall, Bray, & Towlson, 2012).

2.10. Summary

In summary, oxidative stress resultant of exercise can alter cellular homeostasis, potentially leading to impaired muscle function, as well as exercise performance. Although a variety of antioxidants have been used to attenuate the effects of exercise induced oxidative stress, it is clear that not one antioxidant is effective in attenuating the effect of exercise induced oxidative stress. Therefore, the use of antioxidants in combinations with one another, or even a supplement which contains a variety of antioxidants and flavonoids, could potentially be a more effective nutritional supplement in attenuating exercise induced oxidative stress.

It is understood that chronic low grade metabolic acidosis can lead to health complications and that metabolic acidosis is a mediating factor of impaired exercise performance. Although various nutritional supplements have been used to regulate acid-

base homeostasis, side effects such as gastrointestinal symptoms discourage individuals from using such supplements.

Nutritional supplements such as fruit vegetable concentrates could provide an effective nutritional supplement to complement both of these factors. There multitude of high antioxidant ingredients and pro-alkalising compounds contained within these supplements mean that it could potentially have a multiple effects such as attenuating exercise induced oxidative stress as well as regulate acid-base levels both at rest and following exercise.

Chapter 3. General Methodologies

3. General method

The general methodology below outlines the protocols that were used within the respective research studies. Any detailed information specific to that particular study is outlined within the experimental chapter.

3.1. Subjects

The participants for both experiment chapters were healthy, physically active but not trained males, who volunteered to take part in both studies, (Ingestion protocol *n*=8, Exercise protocol *n*=7). None of the subjects reported any pre-existing medical conditions, illnesses, or musculoskeletal injuries. Additionally, participants were not consuming any medication or nutritional supplements (vitamin supplements, ergogenic aids or anti-inflammatory medication) prior to, or during the experimental trials. Subjects were asked to report to the lab following an overnight fast and attempt to maintain the same nutritional and habitual routines during all the experimental trials. All subjects prior to participation completed pre-exercise medical questionnaires and gave informed consent, fully understanding the nature of the respective research and its implications. Research was granted ethical approval by the University of Hull's Department of Sport, Health and Exercise Science ethical committee. Subjects provided written informed consent for both studies and where made aware that they could withdraw from participation at any point in accordance with the Declaration of Helsinki (2008). Prior to both experiments, subjects were asked to report to the laboratory in a fasted state, having refrained from the consumption of caffeine or alcohol, as well as participation in any rigorous activity that was not part of their habitual routine 24 hours prior to any of the trials.

3.2. Pre Experimental Protocol

3.2.1. Anthropometric Data

Subject's height and weight were measured using a stadiometer (Holtan Stadiometer, Holtain Ltd, Crymych, Dyfed) and counter-balance scales (SECA balance scales, Vogel & Halke, Hamburg Germany), respectively.

3.2.2. VO_{2max} Testing

To determine a steady state workload, subjects performed a VO_{2max} protocol based on the work of previous research (Jeukendrup, Saris, Brouns, & Kester, 1996). Testing was conducted on an electromagnetically braked cycle ergometer (Ergo Bike Premium, Daum electronic Gmbh, Furth, Germany). The warm-up consisted of 5 minutes at 95 watts (W), after which the workload increased by 35 W every three minutes maintaining an RPM (revolutions per minute) between 70-80. Breath by breath gas analysis was measured using a metabolic analyser (Cortex Metalyzer 3B, Cortex Biophysic, Leipzig, Germany) throughout exercise as well as heart rate (HR) (Polar HRM, Polar Electro, OY, Finland) and rating of perceived exertion (RPE) (Borg, 1973). Testing was terminated when subjects attained volitional exhaustion or were unable to maintain a pedal cadence of 60 rpm. VO_{2max} was considered reached if two of the following criteria, as currently outlined by the American College of Sports Medicine (ACSM) (Thompson, Gordon, & Pescatello, 2010), were attained during the trial: VO_2 plateau, RER >1.10, HR within ± 10 bpm^{-1} of age-predicted heart rate max (220-age) or RPE >17. Breath by breath analysis was averaged every 30 seconds with VO_{2max} determined on the average of the final three values. The metabolic analyser was calibrated for gas volume, ambient conditions and gas values. Volume was calibrated using a three-litre calibration syringe (Hans Rudolph model 5530, Hans Rudolph, Kansas, USA). Barometric pressure was noted and recorded (Fortin Mercury Barometer, Russell Scientific Instruments, Norfolk,

UK). The system was calibrated using pre-mixed known gas concentrations (Microcan calibration gas, Cranlea and Co. Birmingham, UK).

3.2.3. Hydration Assessment

During the exercise study subjects provided a urine sample, which was analysed prior to each trial to ensure they were sufficiently hydrated. Urine was measured for both pH (pH book (pH 2-12) Scientific Laboratory Supplies, UK) and osmolality (Advanced instruments Model 3320, Advanced Instruments Inc, Massachusetts, USA). Subjects were considered euhydrated with osmolality values ranging between 500-700 $mOsmol/kg^{-1}$ as recommended within the current literature (Sawka et al., 2007).

3.3. Nutritional consumption

Fruit Vegetable Concentrate (FVC) was measured on digital scales (Kern EW 120-4NM Electronic Bench-Top Scales, Kern & Sohn GmbH, Balingen, Germany) to the amounts recommended by the manufacturer (Energised Greens®, Nottingham, UK). FVC was packed into opaque capsules (Gelatin Capsules, Capsuline, FL, USA) using a capsule maker (Capsule Machine, MyProtein, Manchester, UK). Capsules were consumed within 15 minutes of administration with 750 ml of water as recommended by the manufacture. Table 1 presents the ingredient contents per FVC dose.

Table 3.1. *Ingredients of Fruit Vegetable Concentrate (Energised Greens®) per manufacturer recommended dose.*

Ingredients	**Per Dose (9g)**
13:1 extract organic whole leaf 13:1 barley grass	4220mg
Fruits and Greens (*concentrated 100:1 extract from 27 different fruit and vegetables*)	1150mg
Chlorella (*containing 30mg·g Chlorophyll*)	300mg
Spirulina	700mg
Enzyme Complex (*Fermented Rice*)	40mg
Fibre Complex (*apple fibre, apple pectin, microironized wheat germ, wheat bran and acacia fibre*)	2000mg
Lactospore culture (*probiotic stomach acid resistant culture*)	90mg/1.49 billion
Policosanol Complex	40mg
Acerola extract (*with 50% vitamin C*)	150mg
Green tea extract (*40% catechins*)	70mg
Natural fruit-based aromas	240mg

3.4. Blood Collections

3.4.1. Capillary blood

Capillary blood samples were taken for the measurement of acid-base values as previously used within the literature (Siegler, Midgley et al., 2010). Samples (95 µl) were taken from the aseptic index finger into heparinised capillary tubes (blood gas capillary tubes (ABL77) Radiometer, West Sussex, UK). The subjects' finger was sterilised using an alcohol wipe (Uhs, Enfield, UK) and allowed to dry prior to being pierced with a retractable disposable lancet (Accu-chek, Roche Diagnostics, Mannheim, Germany). All blood samples were collected in duplicate and stored on ice until analysis using an automated blood gas analyser (ABL800 Basic analyser, Radiometer, West Sussex, UK).

3.4.2. Venous Blood

Venous blood samples were collected aseptically from the subject's antecubital region using a 21 gauge needle (Vacuette needle 21G, Greiner Bio-one Gmbh, Kremsmunster, Austria). Blood samples were collected into vacuette tubes (Vacuette Tube, Greiner Bio-one Gmbh, Kremsmunster, Austria) treated with K_3EDTA (TBARS), sodium citrate (Glutathione) or lithium heparin (TAC). Throughout the procedure subjects lay supine in a semi-recumbent position in order to restrict changes in plasma volume (Rowell, 1993). Time points of the blood draws are presented in the respective chapter.

Samples were processed in a temperature controlled centrifuge (4ºC) (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) for 10-15 minutes at 18,000-22,000 x g. Supernatant was then collected from samples and frozen (Revco Ultima II Freezer, Kendro Laboratory products, Bishops Stortford, UK) until further analysis.

3.5. Blood Markers of Oxidative Stress

3.5.1. Total Glutathione (TGSH)

Immediately after collection of sodium citrate treated blood a 1 ml aliquot was mixed with 4 ml of freshly prepared 5% meta-phosphoric acid (Sigma-Aldrich Company Ltd., Dorset, England) to facilitate removal of proteins that can interfere with the reaction and to preserve the amount of GSH present when the samples were collected. This mixture was transferred to 1.5 ml eppendorf tubes, stored on ice for 15 min, then centrifuged at 13,000 x g and 4˚C for 15 min (Heraeus Biofuge Pico, Kendro Laboratory products, Bishops Stortford, UK). The supernatant was collected and stored at -80^{o}C for later analysis of TGSH using a commercially available kit (Total Glutathione Detection Kit, Assay Designs, Item No: ADI 900-160, Enzo Life Sciences, Exeter, UK). The assay kit

has a sensitivity of 6.25 picomoles (pmoles) per well, with a range of 12.5-100 pmoles·well.

For determination of TGSH previously prepared blood (50 µl, described above) was diluted to 1:40 in assay buffer solution and transferred to a 96 well plate. Assay buffer was mixed in a 1:25 solution with distilled water (dH_2O). A standard curve was produced from serially diluting 50 µl assay buffer and 50 µl GSSG. All wells were treated with a mixture containing 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) and 10 µl of Glutathione reductase (GR) to form a chramagen (5-thio-2-nitrobenzoate (TNB), the concentration of which was measured every minute for 10 min at 405 nm in a microplate reader (Biotek Synergy HT-R, Biotek Instruments, Vermont, USA). All standards and samples were analysed in triplicate with the mean reported.

3.5.2. TBARS

TBARS were measured using a commercially available kit (ZeptoMetrix, Helvetica Health Care, Item No: 0801192, Geneva, Switzerland). An aliquot (100 µl) of previously prepared plasma was added to 100 µl of sodium dodecyl sulfate solution and 500 µl of thiobarbituric acid solution. Samples were incubated for 60 minutes at 95˚C after which time they were cooled to room temperature and centrifuged at 3000 x g for 15 min. Supernatant was collected and added to a 96 well plate and read at 532 nm in a microplate reader as used previously (*See Section 3.5.1*). A malondialdehyde (MDA) standard curve was constructed and TBARS concentration was calculated by interpolation from this standard curve. From our laboratory, the established coefficient of variation for the TBARS assay used was 2% and 1.9%. Results are expressed in MDA equivalents. All standards and samples were analysed in duplicate.

3.5.3. Total Antioxidant Capacity

Total antioxidant capacity (TAC) was measured using a commercially available kit (Sigma-Aldrich Company Ltd., Item No: CS0790, Dorset, England). The variance between samples as measured by the manufacture in blood plasma was 0.003 mM. Assay buffer was diluted ten-fold and mixed with distilled water to make a 1:10 concentration. Myoglobin solutions were reconstituted by adding the myoglobin stock solution to 285 ml of distilled water to form a myoglobin working solution. Before use, the required amount of myoglobin stock solution was diluted 100-fold with 1x assay buffer and mixed. For each well of trolox standard or test sample, 20 ml of the myoglobin working solution was prepared. Trolox working solution was reconstituted by adding 2.67 ml of 1x assay buffer and vortexed until dissolved. This reconstituted 1.5 mM trolox working solution was used to prepare the trolox standard curve. ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) substrate solution was mixed by adding one ABTS tablet and one phosphate-citrate buffer tablet to 100 ml of distilled water until mixed and totally dissolved. Prepared ABTS substrate-working solution was formed by adding 25 µl of 3% hydrogen peroxide solution to 10 ml of ABTS substrate solution as mentioned above. Standard wells were developed through addition of 10 µl of a trolox standard (from tubes 1-6) and 20 µl of myoglobin working solution. An aliquot of test sample (10 µl) was mixed with 20 µl of myoglobin working solution into a 96 well plate. An aliquot of ABTS substrate working solution (150 µl) was added to each well and then incubated for 5 minutes at room temperature. Stop solution was incubated to room temperature and allowed to homogenise following which 100 µl of solution was added to each well following incubation period. All samples were analysed in duplicate at an absorbance of 405 nm using the same micro plate reader (*See Section 3.5.1*). A standard curve was plotted using average absorbance of standards as a function of final trolox concentration (mM). Antioxidant concentration

of the test sample was calculated using the equation obtained from the linear regression of the standard curve.

Chapter 4. Experiment 1: The effects of acute FVC supplementation upon blood pH and acid-base regulation at rest.

This experimental chapter has formed the basis of the publication detailed below:

Turner, M., Page, R., Mitchell, N., Siegler, J. (2011). The effects of Energised Greens™ upon blood base balance during resting conditions. *Journal of the International Society of Sports Nutrition*, 8 (14).

4.1 Introduction

The inadequate consumption of fruit and vegetables in both males and females can augment the development of serious health risks such as coronary artery disease (Law & Morris, 1998; Mirmiran et al., 2009) and certain cancers (Steevens, Schouten, Goldbohm, & van den Brandt, 2011). The excessive consumption of protein, which is associated with western diets, can lead to renal net endogenous acid concentrations. This is resultant of an increase in the concentration of non-metabolising anions, which ultimately lead to the development of low grade metabolic acidosis (Ballmer & Imoberdorf, 1995). The imbalance in acid-base regulation redox has been shown to effect bone mineral density (Maurer, Riesen, Muser, Hulter, & Krapf, 2003), kidney function (Breslau, Brinkley, Hill, & Pak, 1988), insulin sensitivity (DeFronzo & Beckles, 1979), as well as skeletal muscle atrophy as a result of increased ammoniagenesis and amino-acid catabolism (Ballmer & Imoberdorf, 1995).

The maintenance of pH homeostasis has been shown to be imperative for athletes during exercise due to the effects of metabolic acidosis upon fatigue (Robergs et al., 2005). Therefore, both scientists and athletes alike have supplemented with various substances to induce alkalosis with the aim to attenuate the effects of acidosis and fatigue during high intensity exercise (Robergs et al., 2005). Varying dosage protocols using pro-alkalising supplements ($NaHCO_3$) have been used to induce alkalosis with varying effect (Siegler, Midgley et al., 2010). However, supplementing with relative doses to body weight (commonly 0.3 $g \cdot kg \cdot bm^{-1}$) has led to the development of gastrointestinal (GI) symptoms (Cameron et al., 2010; Stephens et al., 2002), which can be reduced with encapsulated supplementation (Carr, Slater, Gore, Dawson, & Burke, 2011).

Many intervention strategies have been utilised to aid fruit and vegetable intake. Of these strategies, supplementation with FVC has been shown to be both popular and effective in improving the health of clinical populations (Holt et al., 2009; Kiefer et al., 2004). The high potassium concentrations found in fruit and vegetables are inversely correlated with kidney net acid excretion through $KHCO_3^-$ formation, aiding acid-base homeostasis (Demigne, Sabboh, Remesy, & Meneton, 2004). Remer et al., (2001) reported attenuated KNAE within vegetarian diets compared to high consumers of dietary protein. Consequently the pro-alkaline forming nutrients contained within FVC have been shown to increases urine pH as a result of chronic supplementation in sedentary individuals (Berardi et al., 2008). Although the effects of FVC upon blood pH and acid-base response have yet to be investigated, both the chronic and acute ingestion of pro-alkaline supplement has been shown to increase blood and urine pH in males and females (Heil, 2010; Konig et al., 2009). Due to the natural alkalising through metabolising anions, the associated consumption of FVC may therefore induce metabolic acidosis whilst attenuating the GI symptoms shown following ingestion of alkaline promoting supplements. Therefore the purpose of this experiment was to investigate the effects of acute FVC supplementation upon acid-base regulation in resting males in comparison to a current pro-alkalising supplement.

4.2. Methods

Subjects

Eight male subjects (age: 23 ± 2 yr; height 180.1 ± 6.2 cm; weight 76.9 ± 7.2 kg) volunteered to take part in the study. All subjects were healthy, non-smokers and were recreationally active. Subjects were instructed to refrain from the consumption of performance supplements during participation of all the trials. Prior to reporting to the laboratory subjects were asked to maintain the same habitual routine for all the trials as

well as refrain from performing intense physical activity or consuming either caffeine or alcohol at least 24 hours before hand.

Experimental Protocol

Subjects completed three trials as part of a randomised cross-over design with a minimum of three, maximum of 7 days wash-out period between trials. Subjects reported to the laboratory at the same time of day for all the trials (9:00 am), following an overnight fast. Subjects consumed 750 ml of water with either a relative 9 g dose of FVC (Energised Greens, Nottingham, UK) as recommend by the manufacture, 9 g of $NaHCO_3$ (Bicarbonate of Soda, Tesco, UK) or a placebo (Plain Flour, Sainsbury's, UK) in opaque encapsulated pills (Gelatin Capsules, Capsuline, FL, USA) within 15 minutes of administration.

Blood capillary finger prick samples (95 µl) were drawn as previously described (*See methods section 3.6.1*) pre-ingestion and every 15 minutes following ingesting the supplement for 120 minutes. Samples were analysed in duplicate for changes in in blood pH (pH), bicarbonate (HCO_3^-) and base-excess (BE).

Subjects were provided with a visual analogue scale (VAS) questionnaire, as previously used (Cameron et al., 2010), containing nine symptoms of gastrointestinal (GI) discomfort compromising of a measured 100 mm line in which subjects were asked to rate each symptom with a mark. The questionnaire was administered to subjects every 30 minutes throughout the protocol.

Statistical Analysis

All statistical analyses were conducted using SPSS software (SPSS PASW 18, Chicago, Illinois, USA). Descriptive statistics and VAS data are presented as means ± SD. A two-way repeated measures ANOVA with Sidek post-hoc analysis was utilised to measure main effects for time and treatment for changes in blood pH, HCO_3^- and BE in addition to interaction effects between treatments (condition*time) to an alpha level of $p < 0.05$. Where sphericity was violated, the greenhouse geisser correction was used to produce a valid *F* ratio.

4.3 Results

Blood pH

Blood pH rose in all trials from baseline, peaking at 45 minutes following $NaHCO_3$ and placebo (7.445 vs. 7.407 respectively) and peaking 60 minutes following FVC ingestion (7.406). Changes in blood pH showed that there were significant differences between time points ($F_{(16.9, 2.4)} = 12.59$, $p<0.0001$, partial $\eta^2= 0.643$). In addition there was a significant difference between the respective supplements ($F_{(8.5, 1.2)} = 24.58$, $p<0.001$, partial $\eta^2= 0.778$), with NaHCO supplementation significantly different between PLC ($p<0.001$) and FVC ($p<0.001$), respectively. There was a significant interaction effect for time and condition ($F_{(33, 4.7)} = 4.40$, $p=0.004$, partial $\eta^2= 0.386$), with NaHCO supplementation significantly greater at 30 minutes until 120 minutes ($p<0.01$). Furthermore, NaHCO supplementation was significantly greater then FVC ($p<0.03$) and PLC ($p<0.001$) (*See Figure 4.1*).

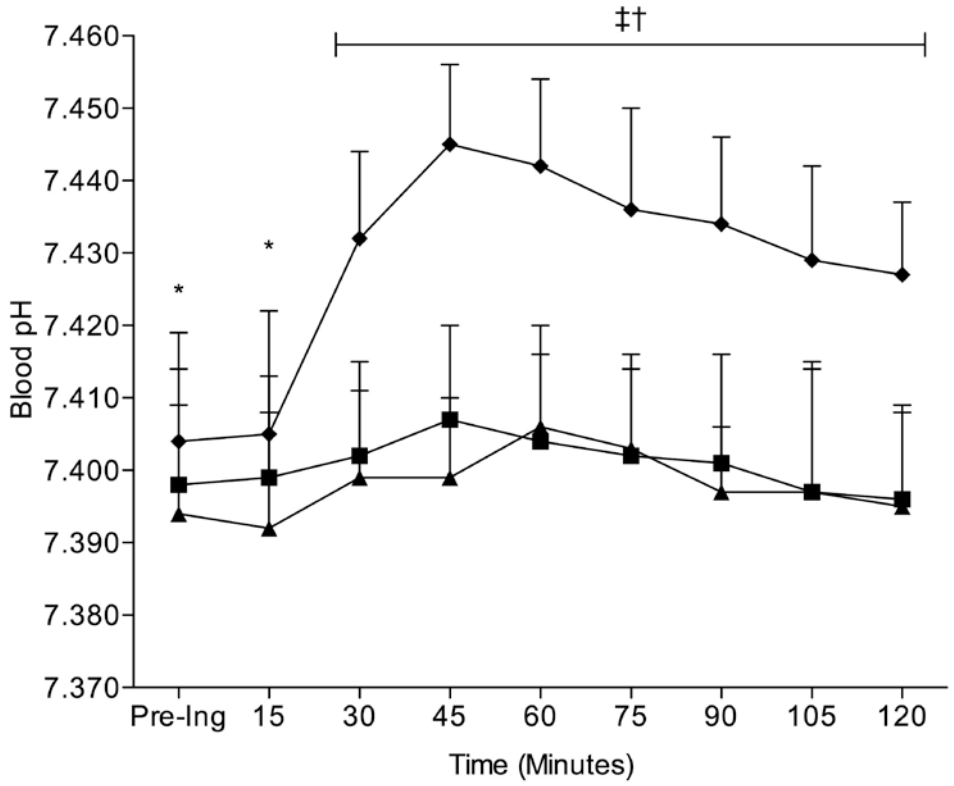


Figure 4.1. *Time dependent response of blood pH (mean ± SD) following ingestion of sodium bicarbonate ($NaHCO_3$, ♦), fruit vegetable concentrate (FVC, ▲) or Placebo (PLC, ■). (P<0.05). * $NaHCO_3$ significantly lower than all other time points. † $NaHCO_3$ significantly greater than FVC and PLC. ‡ Significant interaction effect between $NaHCO_3$ vs. FVC and $NaHCO_3$ vs. PLC trials at the respective time points.*

Bicarbonate

HCO_3^- only rose following $NaHCO_3$ ingestion, peaking 60 minutes post-ingestion; there were no changes following FVC or placebo ingestion. As a result, there was a significant main effect for condition ($F_{(9.7, 1.4)} = 40.0$, $p<0.0001$, partial $\eta^2 = 0.854$), with $NaHCO_3$ significantly greater than FVC ($p<0.05$) and PLC ($p<0.05$) trials respectively. In addition there was a significant effect for time ($F_{(16.4, 2.3)} = 21.3.$, $p<0.0001$, partial $\eta^2 = 0.753$). An interaction effect between time and condition was also found ($F_{(28.8, 4.1)} = 16.9$, $p<0.0001$, partial $\eta^2 = 0.707$), with $NaHCO_3$ supplementation significantly greater than both FVC and PLC supplementation from 30 minutes post ingestion through 120 minutes (*See Figure 4.2*).

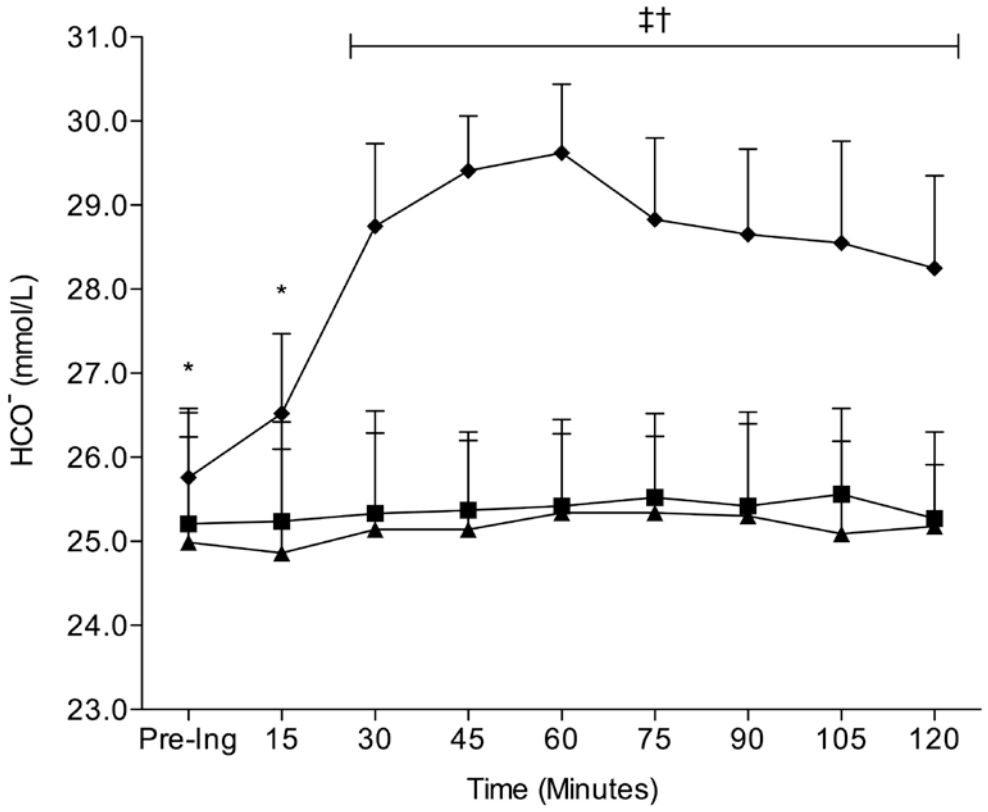


Figure 4.2. *Time dependent response of blood HCO_3^- (mean ± SD) following ingestion of sodium bicarbonate ($NaHCO_3$, ♦), fruit vegetable concentrate (FVC, ▲) or Placebo (PLC, ■). * $NaHCO_3$ significantly lower than all other time points. † $NaHCO_3$ significantly greater than FVC and PLC. ‡ Significant interaction effect between $NaHCO_3$ vs. FVC and $NaHCO_3$ vs. PLC trials at the respective time points.*

Base-Excess

BE only rose following $NaHCO_3$ ingestion, peaking 45 and 60 minutes post-ingestion (5.7 mmol/L). There was a significant main effect for condition ($F_{(10.4, 1.4)} = 33.2$, $p<0.0001$, partial $\eta^2 = 0.826$) There were no changes following FVC or placebo ingestion. Significant main effects for condition were noted between $NaHCO_3$ and other trials respectively from 30 minutes onwards. There was a significant main effect for time ($F_{(14.1, 2.0)} = 22.2$, $p<0.0001$, partial $\eta^2 = 0.761$). An interaction effect between time and condition was also found ($F_{(28.1, 4.0)} = 17.4$, $p<0.0001$, partial $\eta^2 = 0.713$) (*See Figure 4.3*).

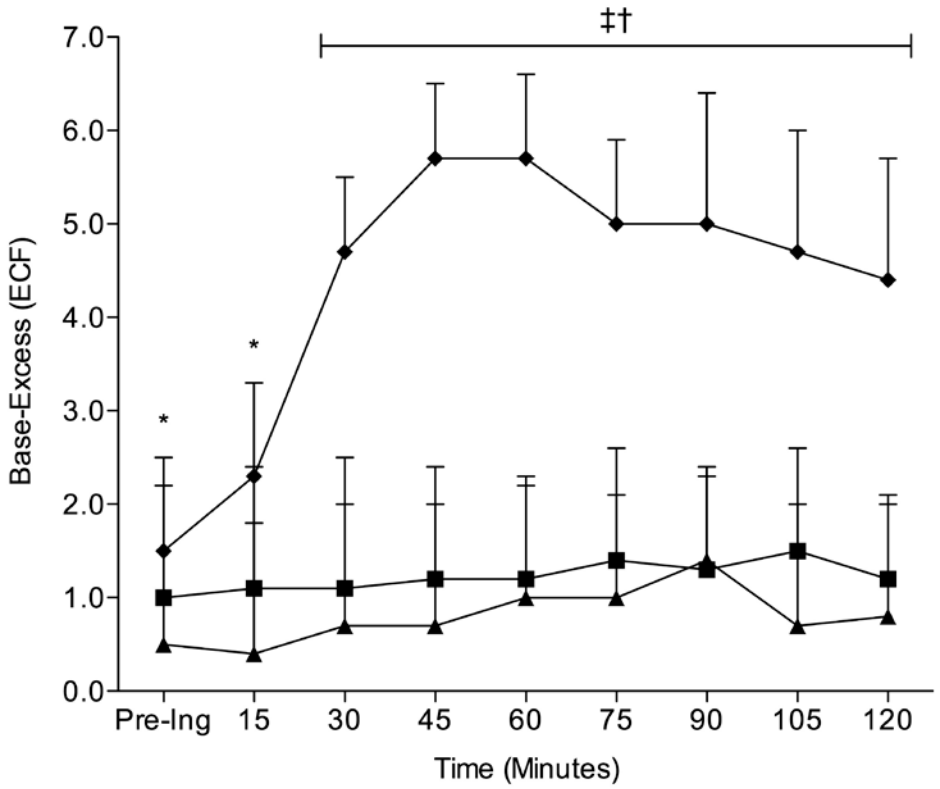


Figure 4.3. *Time dependent response of blood base excess (BE) levels (mean ± SD) following ingestion of sodium bicarbonate ($NaHCO_3$, ♦), fruit vegetable concentrate (FVC, ▲) or Placebo (PLC, ■). * $NaHCO_3$ significantly lower than all other time points. † $NaHCO_3$ significantly greater than FVC and PLC. ‡ Significant interaction effect between $NaHCO_3$ vs. FVC and $NaHCO_3$ vs. PLC trials at the respective time points.*

Gastrointestinal Symptoms

The prevalence of GI symptoms is presented following ingestion of respective supplements (*Figure 4.4*). There was little difference in means between supplements, with all three showing the greatest number of GI symptoms 30 minutes following ingestion ($NaHCO_3^-$: 3.51 ± 2.69; FVC: 3.11 ± 3.64; PLC: 2.33 ± 3.32). The increase was reflective of the observation of increased belching (*Figure 4.5*).

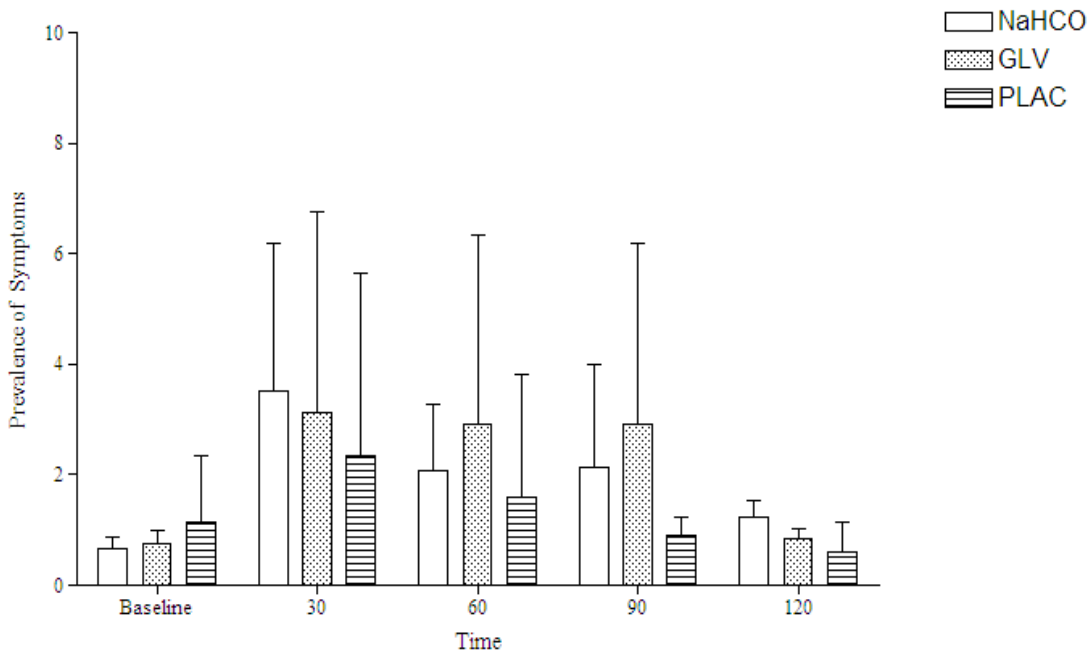


Figure 4.4. *Visual Analogue Scale (VAS (Means ± SD) time dependent response of gastrointestinal symptom prevalence following ingestion of $NaHCO_3$, GLV or Placebo.*

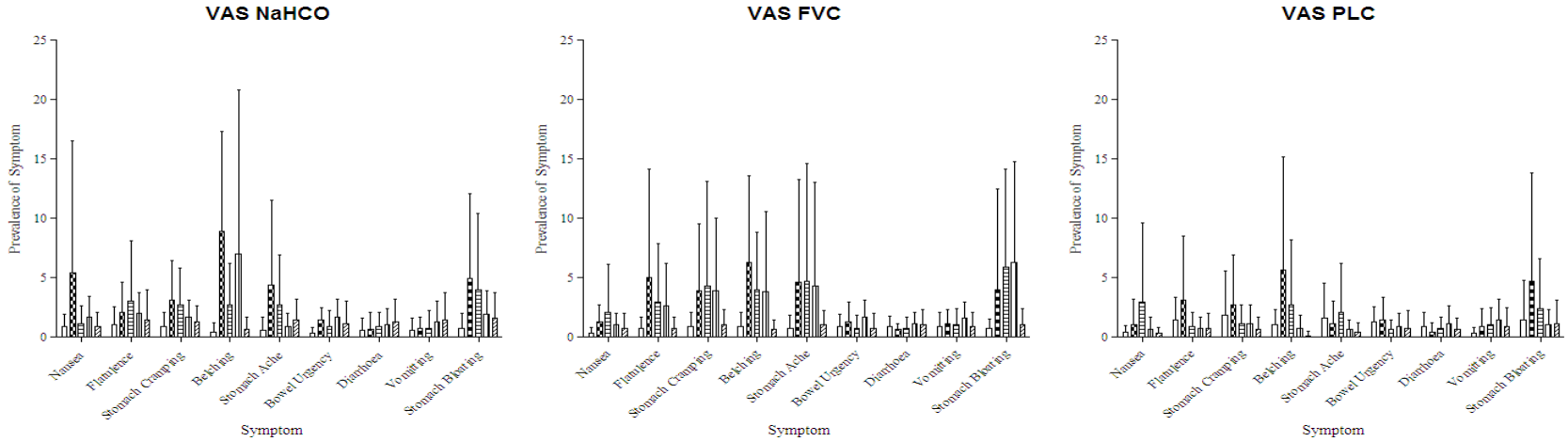


Figure 4.5 A-C. *Individual symptom responses (means ± SD) following ingestion of supplements at baseline (blank), 30 (check), 60 (horizontal lines), 90 (vertical lines) and, 120 minutes (diagonal lines).*

4.4 Discussion

The current findings suggest that an acute dose of FVC supplementation does not have any effect upon blood pH or acid-base regulation compared to a placebo or an equivalent dose of a known buffering ergogenic aid ($NaHCO_3$). The current findings are the first to investigate the effects of FVC supplementation upon blood pH. These findings progress previous research presenting the effects of FVC supplementation on urinary pH changes (Berardi et al., 2008). However, there are limitations with the research presented by Berardi et al. (2008) as findings showed a third of subjects to decrease or exhibit no change pH following prolonged FVC supplementation. Furthermore, those presenting the greater changes in pH were already in a state of low-grade metabolic acidosis. Although not reported, the findings could be influenced by individual dietary intake and the differing levels in kidney net acid excretion (Remer, 2001) or the dietary animal protein intake of individuals (Adeva & Souto, 2011).

The current findings are in contrast to those following ingestion of acute alkaline mineral supplements which found significant increases in blood acid-base as well as urinary pH up to three hours following supplementation (Konig et al., 2009). Pilot work prior to the current investigation showed no changes in blood pH or acid-base following supplementation after 3 hours by which time values had returned to baseline. However, it is clear that further investigation is required to determine the effects of chronic FVC supplementation upon blood buffering responses. The potential discrepancies between the current findings and those of previous research administering pro-alkalising supplements could be due to the differences in bio-accessibility of the compounds within the respective supplements. The reduced bio-accessibility found in fruits and vegetables could result in a delayed acid-base response which is beyond the window of what is considered an acute supplementation period.

In contrast, previous reports indicate supplementing with a known acid-base regulator is not dose-dependent (McNaughton, 1992; Siegler, Midgley et al., 2010) whereby lower doses have been shown to induce significant metabolic alkalosis (Horswill et al., 1988; Siegler, Midgley et al., 2010). Our current findings show that lower doses could be used to induce metabolic alkalosis within resting individuals without exhibiting the gastrointestinal symptoms associated with high doses of $NaHCO_3$ (Cameron et al., 2010; Stephens et al., 2002). Although further research is required, the improved recovery kinetics following $NaHCO_3$ ingestion (Siegler, Keatley, Midgley, Nevill, & McNaughton, 2008) could be effective with the supplementation of lower $NaHCO_3$ doses without prevalence of gastrointestinal discomfort in sensitive individuals.

Furthermore, recent research by Carr et al. (2011) reported the ingestion of capsulated $NaHCO_3$ with carbohydrate presented lower GI discomfort compared to $NaHCO_3$ solution. The use of capsulated supplementation within the current study concurs with these findings (Carr et al., 2011). The current findings showed little differences in the prevalence of GI symptoms in any of the trials, with the most common symptoms being belching, stomach aches and cramps. These findings are similar to the recent work by Carr et al. (2011) who reported similar symptoms although later in the protocol and on a greater scale. Due to the small observed differences in GI symptoms across all supplements it could be suggested that the physiological responses are a consequence of the large quantity of capsules ingested (Carr et al., 2011).

4.5 Conclusions

In conclusion, the ingestion of an acute dose of FVC does not significantly induce any changes in blood pH or buffering capacity compared to placebo supplementation or an equivalent dose of a known ergogenic buffer. Therefore, further research is required to

determine the time dependent responses of chronic FVC supplementation within resting individuals upon metabolic alkalosis.

Chapter 5. Experiment 2: The effects of acute FVC supplementation upon markers of lipid peroxidation, antioxidant status and urine pH during intense intermittent exercise.

This experimental chapter has formed the basis of the publications detailed below:

Turner, M.C., Hillman, A.R., Peart, D.J., Chrismas, B., Mitchell, N., Siegler, J.C. (2012). The effects of acute antioxidant supplementation upon markers of oxidative stress during intermittent cycling exercise. *International Journal of Sports Nutrition and Exercise Metabolism, In Review*.

5.1 Introduction

Since early research identified an increase in the production of reactive oxygen species (ROS) as a result of exercise (Davies et al., 1982; Dillard et al., 1978), a plethora of research has investigated the effects of exercise-induced oxidative stress (Alessio et al., 2000; Bloomer, 2008; Gohil et al., 1988). Increases in ROS have been linked with reductions in muscle force production (Reid et al., 1993), immune function and increased inflammation (Pinho et al., 2010).

Both aerobic and anaerobic exercise has been shown to induce oxidative stress (Bloomer et al., 2005). Although both short and prolonged moderate intensity exercise increases free radical production, intermittent high intensity protocols have been shown to displace the pro-oxidant balance to a greater extent (Bailey et al., 2010; Thompson et al., 2003). Thompson and colleagues reported significant increases in MDA and markers of inflammation (IL-6) in healthy males up to 24 hours post 90-minute intermittent shuttle running (Thompson et al., 2003). These results have been more recently supported in both trained and untrained males during high intensity interval cycling (Leggate, Nowell, Jones, & Nimmo, 2010; Schlader et al., 2008). In addition, high intensity cycling exercise has also been found to increase lipid peroxidation (measured via thiobarbituric acid reactive species (TBARS) concentration) in untrained subjects (Fisher et al., 2011).

Supplementing with antioxidants has been of increasing interest in the literature due to the premise that their use can attenuate exercise induced oxidative stress (Nakhostin-Roohi et al., 2008). The high antioxidant capacity contained within polyphenolic supplements, such as FVC, have been found to have numerous health benefits, including improving antioxidant status and immune function (Lamprecht, Oettl,

Schwaberger, Hofmann, & Greilberger, 2007; Nantz et al., 2006) as well as neutralising chronic low-grade metabolic acidosis (Berardi et al., 2008). The effects of both oxidative stress and metabolic acidosis during exercise are of particular interest because of their known effects upon fatigue and muscle contractility (Reid et al., 1993; R. Robergs et al., 2005). Research by Sastre et al. (1992) reported a positive linear relationship between lactate/pyruvate and lipid peroxidation in animals and humans during exercise which was attenuated through supplementation of N-acetylcysteine, a precursor for glutathione (GSH) formation. Additionally, supplementing with FVC both chronically and acutely has been shown to have protective effects against the increase in protein oxidation within trained athletes (Bloomer et al., 2006; Morillas-Ruiz et al., 2005; Morillas-Ruiz et al., 2006).

Although the exact mechanisms are currently unknown, supplementing with high polyphenol extracts has been shown to augment total antioxidant capacity (TAC) resulting in attenuation of exercise induced oxidative stress in healthy males (Davison et al., 2011). Furthermore, due to the high levels of pro-alkalising minerals contained within FVC, acute supplementation can benefit individuals with low-grade metabolic acidosis (Berardi et al., 2008), which has been attributed to the high levels of pro metabolising anions and potassium bicarbonate (Remer, 2001). Recent research by Peart and colleagues showed supplementing with alternative pro-alkalising agents (sodium bicarbonate) can attenuate metabolic acidosis and elevations in TBARS concentration during high intensity cycling (Peart et al., 2011). However, the effects of acute supplementation of FVC upon both metabolic acidosis and oxidative stress during high intensity intermittent cycling have not been investigated. Therefore, the purpose of this research was to investigate the effects of intermittent cycling exercise upon

oxidative stress and to investigate if this stress could be attenuated by the acute ingestion of FVC.

5.2 Methods

Subjects

Seven moderately trained males (*Table 5.1*) volunteered to participate in the study. All subjects were healthy non-smokers and reported no pre-existing medical conditions. None of the subjects were consuming any nutritional supplements or medication at the time or throughout the duration of the trials. Prior to the experimental trials subjects were asked to refrain from any high-intensity physical activity as well as the consumption of caffeine or alcohol for the 24 hours before trials and reported to the laboratory in a fasted (~10 hours) state.

Table 5.1. *Descriptive statistics (mean ± SD (n=7)) of moderately trained males.*

Age (yr)	Height (cm)	Weight (kg)	VO_{2max} ($ml \cdot kg \cdot min^{-1}$)	W_{max} (Watts)	85%W_{max} (Watts)	50%W_{max} (Watts)
25 ±6	179.0 ± 6.7	73.2 ± 9.1	54.7 ± 7.3	285 ± 32	242 ± 27	142 ± 16

Pre-Test Experimental Protocol

Pre experimental testing for determination of VO2max and maximal wattage (W_{max}) were determined as outlined in the general methodologies (*Section 3.2.2*). The calculation of W_{max} was calculated using the equation below as previously developed (Citation).

$$Wmax = Wout + [(\frac{t}{180}) \times 35]$$

Where W_{out} is the workload of the final completed stage, *t* is the time in seconds in the final exhausted stage, 180 is the stage duration in seconds and 35 is the wattage

increment of each stage. From the above equation steady state exercise was determined at 85% and 50% of W_{max}, respectively. Following initial VO_{2max} testing, subjects then rested for 15 minutes followed by familiarisation with the procedures of the experimental trials.

Supplementation Protocol

The supplementation protocol was identical to that used in Experiment 1, as outlined in the general methodologies (*See section 3.3*). The same dosage was used due to the volume of opaque capsules required to meet the manufacture dose. This resulting in the number of capsules taken (20 capsules), to be double the daily amount taken during prolonged supplementation protocols using FVC supplements (Bloomer et al., 2006; Goldfarb et al., 2010).

Experimental Trials

Subjects reported to the laboratory at the same time (8:30 am) for both trials in this single blind cross-over design experiment. Subjects were asked to consume 500 ml of water two hours prior to reporting to the laboratory in order to ensure they were sufficiently hydrated prior to exercise (Sawka et al., 2007). Upon arrival to the laboratory, subjects provided pre-ingestion body mass and urine sample. Urine was analysed for osmolality (Advanced instruments Model 3320, Advanced Instruments Inc, Massachusetts, USA) and pH (pH book (pH 2-12) Scientific Laboratory Supplies, UK.). Following a pre-ingestion blood sample subjects were provided with 750 ml of water and opaque capsules containing either FVC or a placebo, which were consumed within 15 minutes of administration. Subjects returned to the laboratory one hour following ingestion where body mass, urine and blood samples were obtained.

Subjects then cycled on the same electromagnetically braked cycle ergometer as pre-experimental protocol (Ergo Bike Premium, Daum electronic Gmbh, Furth, Germany) in a thermally controlled environment (Temperature 21.2 ± 1.1ºC; Humidity 45.5 ± 4.4%; Pressure 1009 ± 10 mmHg). Following a standardised five minute warm-up (100 W), subjects completed eight 2-minute bouts at 85% W_{max} interspersed by two minutes of recovery at 50% W_{max}. Exercise intensity was based on pilot work and modified from previous research which presented significant increases in markers of inflammation in trained cyclists (Schlader et al., 2008). Subjects were asked to maintain their own self-selected cadence between 60-70 rpm with verbal encouragement provided if the cadence fell below the threshold. Breath-by-breath analysis was measured throughout the protocol with HR and RPE measured at the termination of each two-minute interval (Borg, 1973). Following completion, body mass, urine and blood samples were taken. Further blood samples were drawn one and two hours post exercise with subjects remaining in a fasted state, but permitted to consume water *ad libitum*. Washout period between trials was between five and seven days.

Blood Measurements

Blood was drawn from an antecubital vein into vacuette tubes (Greiner Bio-One, Gloucestershire, UK) containing sodium citrate (TGSH), clot activated serum (TBARS) and lithium heparin (TAC). Plasma and serum supernatants were collected, stored at -80˚C and assayed retrospectively for TBARS and TAC according to manufacturer's instructions and as outlined in the general methodologies (*See section 3.6.2*).

Statistical Analysis

Data are presented as mean ± SD. Analyses were conducted using PASW Statistics 18 (Chicago, Illinois, USA). Linear mixed models (LMM) were used to determine effects

for condition, time and interaction effects (condition*time) for changes in HR, RPE, lactate and markers of oxidative stress. Different covariance structures were assumed and the one that minimised the Hurvich and Tsai's criterion was chosen for the final model. Where a significant F ratio was observed, post hoc comparisons with Sidak-adjusted p values were used to identify which pairs of means were significantly different. Two-tailed statistical significance was accepted at an alpha level of $P < 0.05$.

5.3 Results

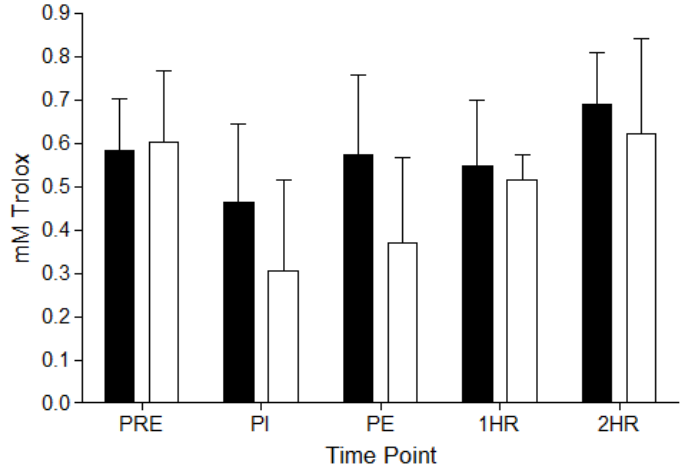
Markers of Oxidative Stress

TGSH concentrations were greater post-ingestion and post exercise during the FVC condition compared with PLC. However, there were no significant differences between conditions ($F_{(1, 11)} = 0.065$, p=0.80). Furthermore, there were no significant differences between time points ($F_{(4, 41)} = 0.759$, $p = 0.56$), although, main effect did approach significance an effect for concentration did approach significance within FVC ($F_{(4, 40)} = 2.152$, p=0.09), in comparison to PLC trial ($F_{(4, 40)} = 0.213$, p=0.93). Furthermore, concentrations did not present any interaction effects ($F_{(4, 41)} = 1.689$, p=0.17; *Figure 5.3*).

There was no significant main effect of supplementation upon TAC concentrations ($F_{(1, 12)} =0.547$, p=0.47), nor was there a main effect for time time ($F_{(4, 14)} =0.697$, p=0.62). Finally time, condition interaction effects presented no significant differences for changes in TAC concentrations ($F_{(1, 14)} =1.090$, p=0.39), (*Figure 5.4).*

TBARS concentrations were lower following FVC supplementation but were not significantly different ($F_{(4, 39)} = 0.803$, p=0.531). However, although differences between in concentrations between conditions were not different values did approach

significance ($F_{(1, 42)} = 3.069$, $p=0.09$). Furthermore there was no significant differences as a result of PLC supplementation ($F_{(4, 39)} = 1.102$, $p=0.370$). In addition, there were no significant differences between time points ($F_{(4, 40)} = 1.723$, $p=0.164$), nor were any interaction effects noted at any point ($F_{(4, 39)} = 0.126$, $p=0.97$; *Figure 5.5*).



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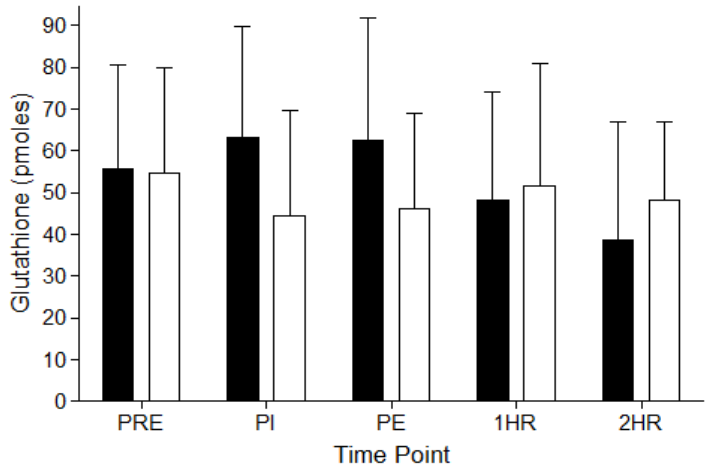

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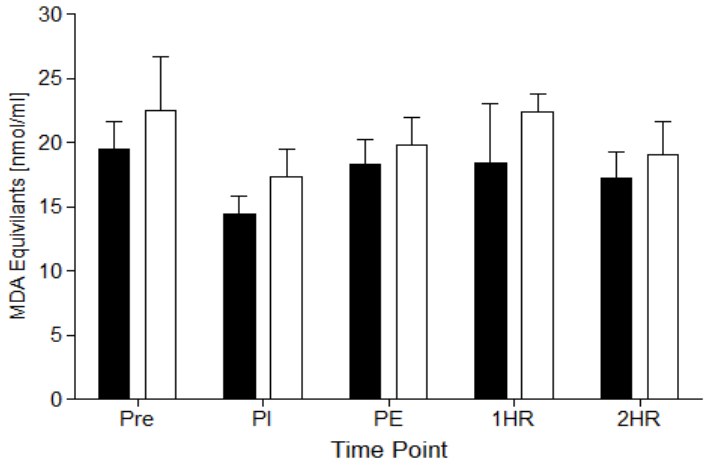

C

Figure 5.1. *The means ± SD (n=7) changes in markers of A) trolox equivilent antioxidant capacity (TEAC); B) total glutathione (TGSH); C) thiobarbituic reactive substances (TBARS) following FVC (dark bars) or PLC (light Bars) antioxidant supplementation. pre-Ingestion (PRE), post-Ingestion (PI), post-Exercise (PE), one-hour post exercise (1HR), two-hour post exercise (2HR).*

Heart Rate

Heart rate continually increased throughout exercise during both high intensity ($F_{(7, 91)}$ = 84.82, P<0.01) and recovery intervals ($F_{(6, 54)}$ = 14.50, P<0.01; *Figure 5.1*) peaking during the final respective interval (FVC: 175±8; PLC: 174±8 vs. FVC: 155±15; PLC: 154±15). HR was greater at all times points compared to initial intervals in both the first high intensity and recovery bouts (P<0.01). There were no significant differences between conditions for changes in HR during high intensity ($F_{(1, 6)}$ = 0.029, P>0.05) or recovery phases ($F_{(1, 13)}$ = 0.253, P>0.05), respectively.

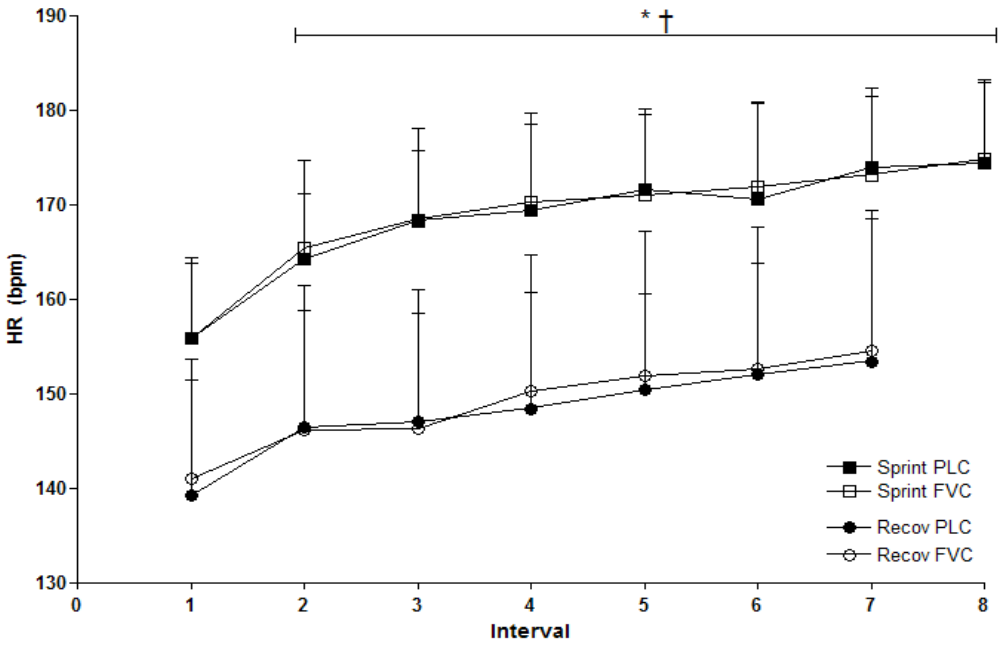

Figure 5.2. *Means ± SD (n = 7) data of heart rate responses to 30 minutes intermittent exercise at 85% W_{max} (sprint) and 50% W_{max} (recov) following fruit vegetable concentrate (FVC) or placebo (PLC) supplementation.*Significantly greater than interval one of sprints.† Significantly greater than first interval during recovery interval (P<0.05).*

Rating of Perceived Exertion (RPE)

Similar to HR, RPE measured at the end of each interval progressively increased over time during high intensity exercise ($F_{(7, 84)}$ = 30.43, p<0.01) although not during recovery bouts despite approaching significance ($F_{(6, 76)}$ = 2.18, p=0.054). Additionally, there were no main effects between conditions during high intensity exercise ($F_{(1, 6)}$ =

0.82, p>0.05) or recovery bouts ($F_{(1, 76)} = 0.311$, p>0.05), nor were there any interaction effects observed during high intensity exercise ($F_{(7, 84)} = 0.382$, p> 0.05); however an interaction effect was observed during recovery intervals ($F_{(6, 76)} = 3.665$, p<0.05) for changes in RPE (*Figure 5.3*).

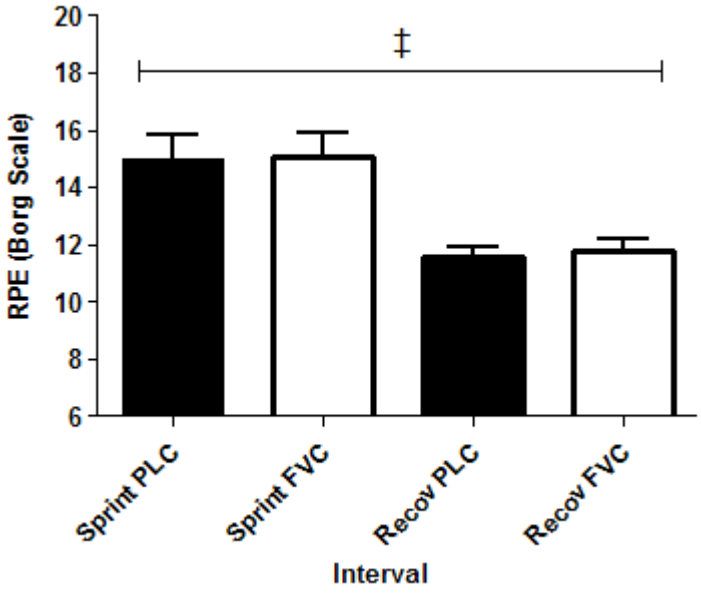

Figure 5.3. *Means ± SD (n=7) of average RPE responses to 30minutes intermittent exercise at 85% W_{max} (sprint) and 50% W_{max} (recovery) respectively. ‡ Sprint RPE significantly greater than recovery values (P<0.05).*

Body Mass, Urine Osmolality and Urine pH

Although it was not hypothesised that body mass would changes during experimental trials the large consumption of fluid that was recommended with supplementation as well as the effects of exercise upon changes in body mass due to sweat loss could have potential effects upon body mass changes. There were no change in body mass as a result of supplementation or exercise throughout with no significant effects for condition ($F_{(1, 36)} = 0.011$, p=0.92) or time ($F_{(1, 36)} = 0.009$, p=0.99) observed.

Urine pH increased following supplementation irrespective of condition (pre-ingestion pH: 6.0 ± 1.0 vs. post-ingestion and post-exercise pH: 7.0 ± 0.0), but was not significantly different between conditions ($F_{(1, 6)} = 0.043$, $p=0.84$) or time ($F_{(2, 18)} = 0.799$, $p=0.47$) (*See Table 5.2*).

For urine osmolality there were no difference between conditions ($F_{(1, 25)} = 0.107$, $p>0.05$) nor was there any interaction effect observed ($F_{(2, 22)} = 1.155$, $p<0.05$). However, a significant effect for time was observed within the FVC trial ($F_{(2, 22)} = 10.126$, $p<0.05$) with urine osmolality higher at baseline to post-ingestion ($P<0.01$) and post-exercise values ($P<0.05$).

Table 5.2. *Means ± SD (n = 7) for changes in body mass (kg), urine osmolality (Urine Osm in mOsm·kg^{-1}) and urine pH pre-ingestion, post-ingestion and post-exercise supplementation with placebo (PLC) or fruit vegetable concentrate (FVC).*

	Pre-Ingestion		Post-Ingestion		Post-Exercise	
	PLC	FVC	PLC	FVC	PLC	FVC
Body Mass	72.6±9.1	72.9 ± 8.8	72.6±9.3	72.9 ± 8.8	72.2±9.2	72.5 ± 8.7
Urine Osm	396±253	589±331	227±188	202±150*	280±242	209±91*
Urine pH	6.0±1.0	6.0±1.0	7.0±0.0	7.0±0.0	7.0±0.0	7.0±0.0

**Significantly different from pre-ingestion value (P<0.05).*

5.4 Discussion

The purpose of the current research was to investigate the effects of acute antioxidant supplementation upon markers of oxidative stress during intermittent cycling exercise. Supplements containing high concentrations of polyphenols, such as FVC (Morillas-Ruiz et al., 2006) and dark chocolate (Davison et al., 2011), have become of increasing interest to researchers due to the protective effects from exercise induced oxidative stress. Although investigations exist examining the relationship between intermittent exercise and oxidative stress both supplementing with (Thompson et al., 2001) and

without (Fisher et al., 2011) antioxidants, the current investigation combines these themes to make the research both current and novel. The current findings did not present any changes in markers of oxidative stress or total antioxidant capacity, supporting research that has also investigated similar acute polyphenol supplementation prior to and during exercise (Morillas-Ruiz et al., 2006).

The use of intermittent exercise protocols are of increasing interest due to the similar physiological adaptations induced in relation to conventional continuous exercise (Gibala et al., 2006), as well as the augmented psychological perceptions of enjoyment (Bartlett et al., 2011). Although increases in oxidative stress are duration dependent (Bloomer et al., 2006), protocols as short as 30 minutes steady state exercise have been shown to result in exercise induced oxidative stress (Bloomer et al., 2005). However despite the current study employing a similar duration protocol, changes in markers of oxidative stress in the current study were not observed.

Previous investigations have demonstrated that intermittent exercise induces an increase in TBARS concentrations in untrained subjects (Quindry, Stone, King, & Broeder, 2003). Additionally, high intensity intermittent exercise has been shown to induce a modest increase in lipid peroxidation in healthy males (Fisher et al., 2011). Despite an evident tendency, the findings of the current study did not demonstrate a significant increase in TBARS concentration immediately post exercise. The potential differences between the current study and that of Fisher and colleagues could be a result of the difference in training status of the subjects, as the subjects in the current investigation were of higher aerobic fitness (VO_{2max}: 55 ml/kg/min vs. 44.6 ml/kg/min), and therefore may have had superior antioxidant defences (Alessio et al., 2000).

Supplementing with a combination of antioxidants has reported mixed findings (Bryant et al., 2003; Lamprecht et al., 2009). Of those that have reported an attenuation in exercise induced oxidative stress as a result of FVC supplementation (Bloomer et al., 2006; Goldfarb et al., 2007; Goldfarb et al., 2010), it is difficult to establish specifically which compound within the supplement is the primary mediator in attenuating exercise induced oxidative stress. One of the ingredients in FVC, spirulina platensis, has been shown to independently attenuate TBARS concentrations prior to and post exercise (Kalafati et al., 2010). Whilst the FVC powder used in the current investigation contained spirulina platensis, TBARS were not attenuated. This would suggest that other compounds in the FVC may have decreased its effectiveness, or the concentration of spirulina platensis in the FVC may not have been sufficient to attenuate oxidative stress.

TAC is a common measure of changes in redox status in the literature (Bloomer et al., 2006). Although changes in TAC weren't significantly different between conditions, the concentration did tend to increase with time which supports previous findings (Alessio et al., 2000). It has been speculated that the changes in TAC following exercise could be resultant of the release of antioxidants into the plasma to provide protection as a result of an increase in lipid peroxidation (Prior & Cao, 1999).

Glutathione is a multi-functional molecule that is mainly implicated in antioxidant defence and cellular redox balance (Sen, 1999). Total glutathione has been shown to be reduced in both animals (Sen et al., 1994) and humans following aerobic exercise (Goldfarb et al., 2005), as well as in trained athletes during short periods of aerobic exercise (Medved, Brown, Bjorksten, & McKenna, 2004). Furthermore acute antioxidant supplementation has been reported to significantly induce elevations in

TGSH concentrations prior to exercise (Medved et al., 2004). FVC supplementation did not evoke such a response in the current study, although a small attenuation was observed as a result of FVC supplementation. We cannot identify whether the changes in concentrations observed are due to changes in reduced or oxidised glutathione, and therefore it's accepted as limitation of the current study.

Due to the pro-alkalising effects of polyphenol supplementation we also investigated the effects of acute supplementation upon changes in urine pH. Although changes in blood pH and other markers of acid-base regulation were not found, acute supplementation with a pro-alkalising supplement has been shown to significantly increase urine pH following an acute dose of a pro-alkalising mineral supplement (Konig et al., 2009). In the current investigation there were no significant changes in urine pH. The discrepancies between findings could be the result of nutritional control with the subjects, as previous studies lacked dietary control (Konig et al., 2009). Furthermore, although there were no differences between conditions there was a significant increase in urine osmolality between time points, which would account for the small changes in urine pH observed in the current investigation.

5.5 Conclusion

In summary this experiment sought to investigate the effects of acute FVC supplementation upon markers of oxidative stress. It was found that such a supplementation protocol did not significantly augment antioxidant status or attenuate changes in markers of oxidative stress during short duration intermittent exercise. Therefore, supplementing with FVC appears to require a chronic loading period in order to observe the benefits associated with increased antioxidant status and reduction in markers of exercise induced oxidative stress.

Chapter 6. General Discussion

Chapter 6. General Discussion

In order to draw together conclusions based on the experimental chapters within this thesis the aims of the respective chapters are presented below.

Experiment 1

1. Investigate the effects acute FVC supplementation in the form of Energised Greens® upon changes in blood pH and acid-base regulation.

There were no significant changes in blood pH or measures of acid-base regulation following acute supplementation of FVC compared with a matched dose of a potent alkaline inducing supplement ($NaHCO_3$).

2. Explore the effects of FVC upon indicators of gastrointestinal discomfort through a visual analogue scale (VAS)

There were no differences at any time point in symptoms of GI discomfort, likely due to large individual variances in visual analogue scores.

Experiment 2

1. Investigate the effects of acute FVC supplementation on lipid peroxidation and antioxidant status.

Despite showing a tendency to attenuate markers of lipid peroxidation (TBARS) and antioxidant status (TAC, TGSH), there was no significant effect of FVC supplementation prior to or during intense intermittent exercise.

2. Investigate the effects of acute FVC supplementation on changes in urine pH prior to and post intense intermittent exercise

There were no changes in urine pH following acute supplementation in subjects with neutral pH levels prior to or following exercise. However, participants who had low pH levels showed a tendency for FVC supplementation to have a greater increase over time compared to subjects with higher basal pH values.

We found that that there were no changes in acid-base status as a result of acute FVC supplementation. Although no changes were observed in the group as a whole, at rest it was observed that subjects with lower basal pH concentrations (pH < 7.350) (as compared to subjects with more neutral pH values (pH 7.400) responded greater to FVC supplementation compared to subjects with a higher basal pH concentrations. Although firm conclusions cannot be drawn upon the effects of acute FVC supplementation therefore, future research should investigate such responses upon subject populations with low basal acid-base levels. It is possible that the disparities between the current findings and that of previous research supplementing with pro-alkalising mineral water could be resultant of a large range of polyphenolic compounds which differ greatly in their bioavailability and bio-accessibility (Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011). Furthermore, unlike more conventional research supplements that induce metabolic alkalosis such as sodium bicarbonate (Price & Singh, 2008) there were no reported changes in GI discomfort with FVC.

Although not comprehensively investigated, acute antioxidant supplementation has been shown to attenuate increases in TBARS (Alessio et al., 1997), as well as augmenting antioxidant defences (Medved, Brown, Bjorksten, & McKenna, 2004). Acute supplementation with FVC in this thesis did not attenuate oxidative stress despite values trending toward a reduction in lipid peroxidation (p=0.08) following intermittent exercise. This may be due to the acute nature of the administration protocol in contrast

to investigations that demonstrate attenuation in oxidative stress from chronic FVC supplementation (Bloomer et al., 2006; Goldfarb et al., 2007).

7.2. Limitations

Despite the author's best efforts to control different confounding variables, there are limitations to the current thesis that have to be addressed. The use of antioxidant supplementation in nutritional intervention studies can be difficult due to the impact of antioxidants upon markers of oxidative stress (Bryant et al., 2003) as well as the interaction of different antioxidants upon one another in order to maintain cellular redox balance (Powers, DeRuisseau, Quindry, & Hamilton, 2004). Although the current research aimed to meet the experimental guidelines that have recently been addressed in the literature, there are some limitations to the current thesis that must be addressed (Powers et al., 2010).

The measurement of lipid peroxidation through the use of TBARS has been criticised due to its lack of specificity (Halliwell & Whiteman, 2004). Heating biological samples following exposure to thiobarbituric acid results in the interaction with malondialdehyde (MDA), however the exposure of samples to heat can in itself result in free radical production (Halliwell & Gutteridge, 2007). Therefore, it is questionable as to whether the changes in TBARS concentrations are representative of the experimental intervention or biological handling of the samples (Powers et al., 2010). Despite this, research has continued to measure changes in lipid peroxidation through the measurement of TBARS in both exercise (Steinberg et al., 2007), and nutritional intervention research (Peart et al., 2011). Alternatively, the use of F_2-Isoprostanes is more reliable due to its stability (Milne, Sanchez, Musiek, & Morrow, 2007). Yet, due to its short half-life (Halliwell & Gutteridge, 2007), its use within the current thesis

would not have been suitable in measuring potential changes in lipid peroxidation due to the prolonged recovery period from exercise.

There are currently three methods to measure changes in total antioxidant capacity, of which trolox equivalent antioxidant capacity (TEAC) is one. Despite extensive literature reviews on the measurement of total antioxidant capacity (TAC) (Cao & Prior, 1998), it remains to be established which method is the most valid for quantifying changes in TAC (Cao & Prior, 1998; Powers et al., 2010). It must be noted the TEAC assay has been previously used to measure changes in TAC following supplementation with FVC as this thesis did, therefore allowing for comparisons to be made between the current and previous research (Bloomer et al., 2006).

The use of glutathione has been used as a marker of redox status following exercise (Sen, 1999). In particular, concentrations are attenuated following acute antioxidant supplementation (Sen, Rankinen, Vaisanen, & Rauramaa, 1994). The second experimental chapter only measured changes in TGSH and therefore it is difficult to conclude if the changes in concentrations are resultant of increases in reduced or oxidised glutathione.

The control of dietary intake is important in any nutritional intervention research (Jeacocke & Burke, 2010). The current investigation controlled nutritional intake through an over-night fast, which has been employed in previous research investigating acute antioxidant supplementation (Davison et al., 2011). However, a one-day dietary recall (Bailey et al., 2010; Goldfarb et al., 2007) would be a more accurate and controlled method to quantify any differences in nutritional intake. In light of the effects of dietary intake upon markers of oxidative stress, as well as changes in acid-base

regulation (Adeva & Souto, 2011), it is therefore accepted as a current limitation of this thesis.

Subjects were instructed to consume sufficient fluid prior to each trial in both research trials. Although there were no changes in body weight observed between trials or time points, which would suggest there to be little change in hydration status as a result of the exercise or fluid consumption. Therefore, although the changes in plasma volume should have been minimal during resting trials, it is not known if the changes in oxidative stress that were observed are consequential of exercise or resultant of the variations in plasma volume. Although subjects were permitted to consume fluid *ad labitium* throughout in both experimental chapters, it is accepted that data on plasma volume changes would potentially be a more accurate method of quantifying any changes or effects upon the concentrations of the markers in question.

6.3 Future Research Recommendations

Although the current thesis presented no changes in acid-base regulation following acute supplementation in healthy individuals, future research could focus upon the effects of both acute and chronic FVC supplementation on changes in acid-base within clinical populations who experience low-grade acidosis.

Further research is also required to investigate the dose response relationship of supplementing with FVC. Although research has found chronic loading to significantly increase antioxidant capacity in both males and females (Nantz et al., 2006), there is currently no research that has investigated the optimal dosage response of FVC supplementation to induce changes in antioxidant status or acid-base levels of healthy individuals.

Furthermore the exact mechanistic responses resultant of supplementation are currently not clear therefore *in vitro* work could potentially identify not just the effects of FVC supplementation upon its regulator role in reducing low-grade metabolic acidosis but also by which mechanisms it initiates the increase in antioxidant capacity.

6.4 Conclusions

In conclusion it is evident that acute fruit vegetable concentrate (FVC) supplementation does not induce changes in acid-base regulation at rest, nor does it increase in antioxidant status prior to intermittent exercise. The consequence of these findings is that supplementing with polyphenol supplements should be loaded chronically in order to attain any metabolic or protective effects. Furthermore, the use of such supplements may be more beneficial to clinical or athletic populations with chronic low-grade metabolic acidosis, or those who have poor dietary intakes of fruit and vegetables. Therefore, it is appropriate that future research should direct the use of this supplement in this area.

Chapter 8. References

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