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Effect of Almond-Raisin versus Commercial Sports Beverage on Antioxidant Status of Runners

Anh Thu V. Tran

LOMA LINDA UNIVERSITY
Graduate School

Effect of Almond-Raisin versus Commercial Sports Beverage on Antioxidant Status of Runners

by

Anh Thu V. Tran

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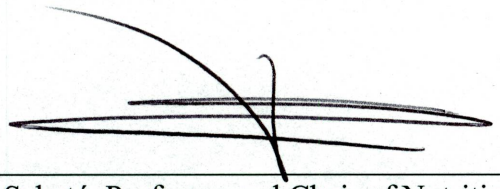
Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science in Nutritional Sciences.

R. Sujatha , Chairperson

Sujatha Rajaram, Assistant Professor of Nutrition

E. Haddad

Ella Haddad, Associate Professor of Nutrition



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ABSTRACT OF THE THESIS

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Master of Science, Graduate Program in Nutritional Science
Loma Linda University, September 2005
Dr. Sujatha Rajaram, Chairperson

Background: Whole foods are readily available antioxidant source and provide beneficial macromolecules and phytochemicals, which may contribute to optimal health. However, the approach to examining whole foods rich in antioxidants to play either an equivalent or better role in defending exercise-induced oxidant stress have not yet been taken.

Objective: The objective of this study was to compare the effects of 2 isocaloric pre-exercise beverages: an almond-raisin beverage and a commercial sports beverage in male endurance runners on selected antioxidant status markers.

Design: In a randomized crossover study, 10 male runners (ages 28$\pm$1.2 years) ran to exhaustion on a treadmill at 70% VO_2max, twice, separated by 2 weeks. Blood samples were drawn 60 minutes pre-exercise, at the start, and every 20 minutes thereafter. Trolox Equivalent Antioxidant Capacity (TEAC) represented antioxidant activity. Gallic Acid Equivalents (GAE) represented total serum polyphenols. Using high-pressure-liquid-chromatography, thiobarbituric acid reactive substance (TBARS) reflected serum malondialdehyde levels. Statistical analyses were conducted using mixed linear models that included time and treatment as fixed factors, and subject as a random factor.

Results: In the longitudinal analysis, there was a significant main treatment effect found when all time points were included for TEAC and GAE, with the almond-raisin beverage having a significantly greater level than the commercial sports beverage ($p = 0.0002$, $p = 0.0239$, respectively); the TBARS showed no significant treatment effect ($p = 0.08278$). Conclusion: The almond-raisin beverage appears to provide a measure of antioxidant protection beyond that of the sports beverage and may be considered as an alternative to the commonly used beverages for endurance runners.

CHAPTER ONE

INTRODUCTION

Statement of the Problem

Driven virtually from gun to tape, conquering a stretch of distance, today's endurance runners strive to perform to near genetic and training limitations. Regular physical exercise alone has been well-documented for its beneficial effects in circulating lipids and lipoproteins, maintaining ideal body weight, reducing blood pressure, improving insulin sensitivity, and decreasing the overall risk of death due to degenerate diseases (Whyte et al 2005). Despite these advantages, it is now clear that rigorous muscular exercise results in an overwhelming production of radicals and other reactive oxygen species (ROS) known as oxidative stress (Ginsburg et al 1996, Leaf et al 1997, Child et al 1998, Whyte et al 2005). During intense exercise, an athlete's ability to approach maximum oxygen consumption (VO_{2max}) can cause the whole body to increase 10 to 20-folds above resting levels of oxygen consumption and potentially raise 200-folds in active muscle fibers (Keul et al 1972, Sacheck et al 2001). This increase has been suggested to generate the "mitochondrial leak," which is associated with approximately 2 to 5 percent of total electron flux through the cytochrome chain resulting in reduction of the remaining oxygen to form superoxide radicals, igniting a cascade of events (Figure 1) (Leaf et al 1997, Child et al 1998; Evans WJ et al 2000, Sen et al 2001, Takanami 2000, Chevion et al 2003).

Previous studies have associated accumulation of ROS as the underlying mechanism to the disturbance of muscle homeostasis (i.e. oxidation-reduction status), suggesting that the imbalance causes muscle fatigue and injury (Schneider et al., 2004).

Given that radicals are produced during normal metabolism, it is no surprise that skeletal muscle myocytes contain defense mechanisms to reduce the risk of radical-mediated injuries (Criswell et al, 1993, Venditti et al 1997, Aslan et al 1998, Chevion et al, 2003). As a protective network in the intra- and extracellular environment to detoxify and reduce the deleterious effects of ROS, there are 2 major classes that make up the endogenous protective system: 1) enzymatic (common but not limited to superoxide dismutase, glutathione peroxidase, and catalase) and 2) non-enzymatic (glutathione, vitamin E, vitamin C, lipoic acid, carotenoids, ubiquinone, uric acid, and bilirubin) antioxidants. Although the body has an elaborate antioxidant network to quench free radicals, under certain conditions, such as exhaustive exercise of greater than 60 minutes approaching VO_{2max}, the protective capabilities are compromised (Leaf et al 1997, Vasankari et al 1998, Liu et al 1999, Child et al 1999, Chevion et al 2003).

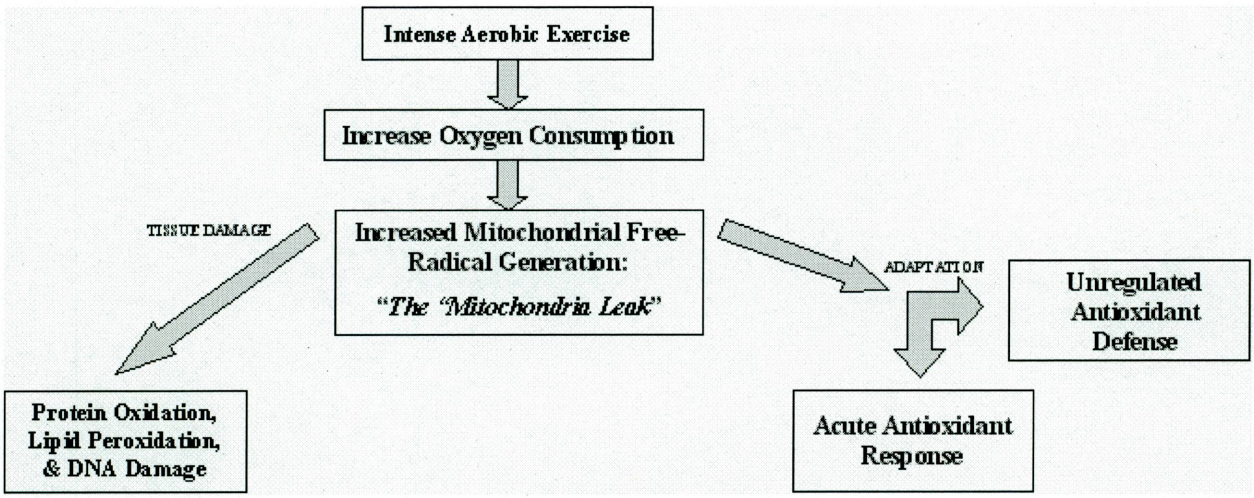


Figure 1. Proposed cascade of events ignited by intense aerobic exercise (Adapted from Adams et al 2002).

Study designs using animal models have shown the significant delay of muscle fatigue and/or injury using antioxidant supplementation, but a few results have been replicated in humans (Witt et al 1992). An observational trail of a single bout ultraendurance exercise on athletes reporting pre-race use of isolated vitamin E supplement demonstrated possible protection against some of the acute effects of exercise-induced oxidative stress (Ginsburg et al 1996). However, no significant effect was found in athletes reporting pre-race usage of isolated vitamin A or C from this study. A more specific design in supplementation reported short-term dosage of pre-race vitamin E resulted in fewer complaints of intestinal injury, occult bleeding, and/or the severity of post-race GI complaints among marathon runners (Buchman et al 1999). The increased vitamin E turnover during endurance exercise compared to sedentary periods is a repeated pattern in numerous studies and suggests its strong contribution in the fight against oxidative stress (Mastaloudis et al 2001).

Although vitamin E plays an important role, single-dose antioxidant studies suffer from experimental design weaknesses due to, but not limited to, the possible additive or synergistic effect in coupling antioxidants. Studies that investigated a mixture of antioxidant supplementation, such as an ascorbic acid, carotenoids, and α-tocopherol cocktail, taken prior to intense aerobic exercise have demonstrated more replicable results in the delay and/or reduction of exercise-induced oxidative stress (Kanter et al 1993, Vasankari et al 1997, Balakrishnan et al 1998). However, evidence suggesting too high a dose of antioxidant supplementation may shift intracellular redox balance to reduce state and cause another source of impair next to the skeletal muscle contractile function and performance (Coombes et al 2001, Marshall et al 2002). A recent study reported that at

overload intensity of exercise training low doses of antioxidant mixture significantly reduced creatine kinase, a common biomarker for muscle damage (Palazzetti et al 2004). There is overwhelming evidence that it is the complete antioxidant defense network balance that combats oxidative stress, not the overload of a single presence of one factor.

There is a strong consensus from the literature that endurance exercise induces oxidative stress and that the pre-exercise usage of combination supplementation aids in the delay of this stress; yet what is lacking in the literature is an aggregate approach. Little progress has been made in the past decade to delineate the contribution of nutrients delivered as whole and unrefined food to the modulation of the pathological consequences of free radicals in the human body. Pre- and post-exercise regimes of high carbohydrate and low fat intake have been a well recognized staple for endurance athletes to maintain the rigors of daily training as well as to optimize performance in regard to achieving proper energy. Recent findings of no significant difference in time-to-exhaustion using a high fat and low carbohydrate combination in a pre-exercise beverage versus the traditional staple among endurance runners suggest plausible flexibility in macronutrient composition (Bazilian et al 2003). Studies have also demonstrated that upon intake of vitamin E supplement, subjects with higher fat intake have a higher plasma vitamin E concentration (Sacheck et al 2000). Therefore, it is reasonable to suggest a high-fat and antioxidant-rich food as a pre-exercise snack. If it can be shown that a whole food approach can combat or even delay exercise-induced oxidative stress, it will help to not only offer an alternative preventive measure to oxidative stress, but also pave the way to investigate natural and inexpensive whole food sources that may confer

equal or better benefits to the athletes compared to commercial sports snacks and beverages.

Purpose of the Study

Objectives

The primary objective of this study is to understand the effects of a pre-exercise almond-raisin beverage, rich in natural antioxidants, specifically vitamin E and polyphenols, versus a commercial sports beverage (Gatorade ®) on exercise-induced oxidative stress among endurance male runners undergoing time-to-exhaustion run at 70 percent VO_2max.

Specific Aims/Hypotheses

The specific aims of this study are:

1) To compare the almond-raisin beverage versus commercial sports beverage (Gatorade ®) effect on serum total antioxidant activity.

Hypothesis: The Almond-raisin beverage will increase serum levels of Trolox Equivalent Antioxidant Capacity (TEAC) compared to the commercial sports beverage (Gatorade ®).

2) To compare the almond-raisin beverage versus commercial sports beverage (Gatorade ®) effect on serum total polyphenols.

Hypothesis: The Almond-raisin beverage will increase serum levels of Gallic Acid Equivalents (GAE) compared to the commercial sports beverage (Gatorade ®).

3) To compare the almond-raisin beverage versus commercial sports beverage (Gatorade ®) effect on lipid-peroxidation by-product – Malondialdehyde (MDA).

Hypothesis: The Almond-raisin beverage will reduce serum levels of thiobarbituric acid reactive substance (TBARS) compared to the commercial sports beverage (Gatorade ®).

CHAPTER TWO

REVIEW OF THE LITERATURE

In the prevention of cellular damage -- the common pathway for aging, cancer, cardiovascular disease, muscle fatigue and/or injury, and a variety of other degenerate diseases, the process to maintain cellular oxidation-reduction homeostasis involves an elaborate system, in which endogenous and exogenous antioxidants are intimately interwoven in an intricate network against their antagonist: the oxidants, also known as free radicals. What appears to be a paradox is that intense exercise increases the production of free radicals by virtue of an increase in oxygen utilization (Figure 1, 2). The purpose of this literature review is to discuss the current understanding of the relationship between antioxidants and exercise-induced oxidative stress.

Oxidants

Chemicals of Free Radicals

Molecules generally contain pairs of electrons that orbit their nucleus, however an electron is occasionally "lost," transforming the molecule into a *free radical.* An oxidant, which can be interchangeable with the term free radical, is a molecule capable of independently existing with one or more unpaired electrons in its outer orbital. This ability causes great molecular instability and gives the oxidant its high reactive character, which is extremely useful to the body in cases of immunological activation, drug detoxification, and proper relaxation function of the blood vessels. Despite their role in regular metabolic processes, these molecules provoke a cause for concern because an overwhelming presence can promote oxidation-induced damage to important macromolecules such as lipids, proteins, and nucleic acids.

The diradical characteristic of molecular oxygen presents a higher susceptibility to several highly reactive intermediates. The electron configuration of oxygen favors the tendency to receiving one electron at a given time. Sequential reduction of molecular oxygen (equivalent to sequential addition to electrons) leads to the formation of superoxide anion, peroxide (specifically hydrogen peroxide) (H_2O_2), and hydroxyl radicals (HO) (Figure 2). Reactive oxygen species (ROS) is a more commonly used term in regard to collectively addressing these 3 main intermediates generated from the complete reduction of oxygen to water (H_2O). Although superoxide anions and H_2O_2 are not highly reactive species, their activity as active oxygen species comes from their potential to produce extremely highly reactive HO through the Fenton reaction (I) and Haber-Weiss reaction (II). HO reacts with all biological materials, oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation. The overwhelming production of reactive molecules either weak or strong, could set off a cascade of reactions with important macromolecules, putting nucleic acids, lipids, and proteins at stake for oxidative damage.

1.	O_2	+	e^-	→	**$O_2^{\cdot-}$**			**Superoxide radical**
2.	$O_2^{\cdot-}$	+	H_2O	→	**$HO_2^{\cdot}$**	+	OH^-	**Hydroperoxyl radical**
3.	$HO_2^{\cdot}$	+	e^-	+	H	→	**H_2O_2**	**Hydrogen peroxide**
4.	H_2O_2	+	e^-	→	**HO**	+	OH^-	**Hydroxyl radical**

Figure 2. Complete reduction of oxygen and bolded are the oxygen-derived intermediates (Adapted from Clarkson et al 2000).

The most common location for radical attack is at the cell membranes, where polyunsaturated fatty acids (PUFAs) become victim to lipid peroxidation. Detrimental

change to the organization of cellular membrane could cause increased membrane rigidity, decreased activity of membrane-bound enzymes (i.e. sodium pumps), and altered membrane receptors and permeability. This results in an influx of Ca^{2+}, a loss of intracellular enzymes, and an influx of lysosomal (destructive) enzymes. Besides the basal level of oxygen needed in regular metabolic functions, there is no circumstance other than physical exercise that demands an exceedingly high level of oxygen consumption.

Oxidative Stress Induced by Exercise

Ignited by early studies of skeletal muscle production of free radicals during contractile activity, overwhelming evidence has confirmed exercise-induced oxidant production (Dillard et al 1978, Davies et al 1982, Criswell et al 1993, Venditti et al 1997, Child et al 1997, 1998, Liu et al 1997, Sacheck et al 2000, Ji 2002, Powers et al 2004). The term oxidative stress is used in circumstances where an overwhelming amount of oxygen radicals are produced, exceeding the cellular antioxidant defense system (Figure 3). The magnitude of the exercise-induced oxidative damage is dependant upon the rate of oxygen consumption and the presence of cellular antioxidant systems (Schneider et al 2004). Current evidence suggests the following sources of skeletal muscle radical production: 1) the mitochondria, where ROS that have escaped scavenging enzymes present in the mitochondria may leak into the sarcoplasm, 2) the capillary endothelium, where hypoxia or reoxygenation process is created during exercise, and 3) an oxidative explosion due to inflammatory cells mobilized as a result of muscle and tissue damage (Figure 1) (Evans et al 2000).

Although the mitochondria are often viewed to be the *power house for cells*, the "mitochondrial leak" accounts for the majority of free radicals produced. During exhausting exercise, an athlete's ability to approach maximum oxygen consumption (VO_{2max}) can cause the whole body to increase 10 to 20-fold above resting levels of oxygen consumption and potentially raise 200-fold in active muscle fibers (Child et al 1997, 1998, Sacheck et al 2001). While 85 to 90 percent of the skeletal muscle oxygen consumption in the mitochondria undergo the electron transport chain (ETC) to produce adenosine 5'-trisphosphate (ATP) and water, 10 to 15 percent is directed by oxidation chemical reactions. At the terminal part of ETC, the enzyme cytochrome oxidase oxidizes 4 cytochrome-reduced-molecules to form water (95 to 98 percent from the 85 to 90 percent mentioned above). The remaining 2 to 5 percent of this oxygen undergoes one electron reduction resulting in superoxide radical formation (equation 1 in Figure 2) (Schneider et al 2004). Elevated muscular activity and oxidative metabolism coupled by a proportional increase in ROS formation are the underlying means of offsetting the redox scale (Figure 3) (Child et al 1997, Schneider et al., 2004, Powers et al 2004).

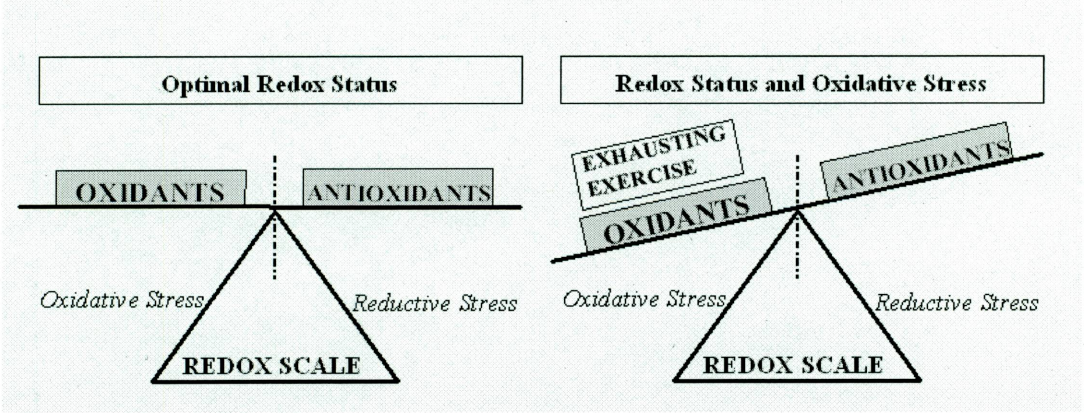


Figure 3. Relationship between oxidants and antioxidants (Modified from Powers et al 2004).

Measuring Oxidative Stress

Due to the short half-lives (typically 10^{-6} to 10^{-12}) of reactive intermediates, it is difficult to monitor oxidative stress *in vivo* (Leeuwenburgh et al 2001). Because it is not possible to directly measure free radicals in the body, scientists have approached this question by measuring the by-products that result from free radical reactions. If the generation of free radicals exceeds the antioxidant defenses then one would expect to see more of these by-products. These measurements have been performed in athletes under a variety of conditions.

Lipid Peroxidation By-Product

In the inner mitochondrial membrane, superoxide radicals are formed during the reduction of oxygen. These radicals can trigger a cascade of reactions in the fatty acids of phospholipids, resulting in membrane lipid peroxidation and disruption of the organization of the membrane bilayer. The integrity of the barrier such as fluidity and permeability is altered and compromised. Polyunsaturated fatty acids become victim to peroxidation and generate reactive free radicals and toxic aldehydes, which can hamper or completely inhibit the normal functionality of the components of the cell. Although harmful to cellular function, these by-products offer effective biomarkers for oxidative stress. While not limited to three, the following are three common biomarkers used for oxidative stress: 1) conjugated dienes, 2) lipid hydrocarbons, and 3) thiobarbituric acid-reactive substance (TBARS) such as malondialdehyde (MDA) (Clarkson et al 2000, Leeuwenburgh et al 2001),

Diene conjugation. One of the first products of unsaturated fatty acid peroxidation, conjugate dienes, absorbing ultraviolet light at 230 to 235 nm, has been commonly used to

infer oxidative stress, but previous studies noted its use with caution (Clarkson et al 2000). Due to the nature of conjugated dienes, they are useful to measure bulk lipids (LDL) and monitor the early stages of the peroxidative process. However, in the circumstances of measuring human fluid, this biomarker has its limitations. Even with the use of high-pressure-liquid-chromatography (HPLC), used to separate conjugated dienes from human fluid, the extraction were found to be unsuccessful, leaving non-oxygen-containing isomer of linoleic acid (Halliwell et al 1993).

Lipid hydrocarbons. During the decomposition of lipid peroxides, hydrocarbon products of ethane and pentane are also generated. Ethane and pentane have also been used to infer oxidative stress by the collection of these products through exhalation (Clarkson et al 2000). Its dependency on the presence of metal ions to decompose lipid peroxides may not give an adequate index of overall peroxidation (Halliwell et al 1993). Hydrocarbons are also produced by bacteria and are air pollutants and these confounding factors set another limitation to this approach.

Thiobarbituric Acid-Reactive Substance (TBARS) - Malondialdehyde (MDA). For its mere ease and cost effectiveness, the thiobarbituric acid-reactive substance (TBARS) is the most widely used technique to detect lipid peroxidation: malondialdehyde (MDA) is commonly used for human fluids. Sample material is heated at low pH with thiobarbituric acid (TBA) and the resulting chromogen is measured by absorbance at approximately 532nm or fluorescence at 553 (Halliwell et al 1993). A major setback to this approach is that TBARS is prone to artifacts because TBA can react with a wide variety of compounds that absorb at the same range, including sugars (Leeuwenburgh et al 2001). Thus TBARS has been subject to criticism; even when it does offer an empirical window on the complex

process of lipid peroxidation (Halliwell et al 1993, Clarkson et al 2000, Leeuwenburgh et al 2001).

In the case of oxidative stress, oxidant products are typically detected at low levels, which present a challenge in observing accurate results. Specifically for screening of human body fluid, recent improvements have been adapted to the TBARS assay: 1) amplification and 2) purification process. To hamper amplification of peroxidation and limit the variations in sample lipid content and/or antioxidant content, butylated hydroxytoluene (BHT) is added to the sample. To eliminate possible artifacts due to the reaction of TBA with other body-fluid content, a separation process is conducted through HPLC to separate the authentic $(TBA)_2$-MDA (Leeuwenburgh et al 2001). Indeed these 2 additional procedures do not eliminate the problem, but it does enhance the accuracy and allows for general measurement of peroxidation.

Antioxidant Activity

Since there is yet to be a "gold standard" in measuring oxidative stress, previous studies have recommended that at least 2 techniques be used for an accurate and consistent evaluation. Other than looking directly at the by-products of oxidation, antioxidant activity has been another way to express the degree of exercise-induced oxidative stress. Previous studies tended to use this approach because it required a small amount of human body fluid for such reliable and practical use (Clarkson et al 2000). Not limited to the following 3, these are common antioxidant activity assays with the latter specifically looking at total phenolic capacity: 1) Ferric Reducing Ability of Plasma (FRAP), 2) Trolox Equivalent Antioxidant Capacity (TEAC), and 3) Total Radical-Trapping Antioxidant Parameter (TRAP)

Ferric Reducing Ability of Plasma (FRAP). The time and cost efficiency of the Ferric Reducing Ability of Plasma (FRAP) assay makes it one of the commonly used tests for the *antioxidant power* of a given sample. The general concept of FRAP is through the reduction of ferric to ferrous ions at low pH, which cause a colored ferrous-tripyridyltriazine complex that can be compared in the test reaction mixture by absorbance change at 593nm (Benzie et al 1996). As in other test of oxidative stress and antioxidant defense, FRAP reaction conditions are far from physiological, and must be interpreted with caution for *in vitro* testing of human body fluid may not reflect *in vivo.* (Benzie et al 1999).

Trolox Equivalent Antioxidant Capacity (TEAC). Essentially, this method is an inhibition method, in which radical species is generated. There is an end point by which the presence of the radical is detected, and the antioxidant activity of the added sample inhibits the end point by scavenging the free radicals (Re et al 1999). TEAC is the capacity of an individual antioxidant to inhibit preformed radical monocation of 2', 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) at a defined time point relative to Trolox (6-hydroxy-2,5,7,8-tet-ramethychroman-2carboxlic acid). This decolorization assay screened for both lipophilic and hydrophilic antioxidants – flavonoids, hydroxycinnamates, carotenoids, and serum antioxidants (ascorbic acid, α-tocopherol, gluthathione, and uric acid).

Due to its common use to indirectly infer exercise-induced oxidative stress, there have been many developments to improve this approach (Re et al 1999, Art et al 2001, Arts et al 2002). One is the direct production of the blue/green $ABTS^{\cdot+}$ chromopore through the reaction between ABTS and potassium persulfate, which broadens the range

in which absorption can be read (Re et al 1999). Another is to add a deprotenation process to render the effects of protein masking actual antioxidant capacity (Arts et al 2001, 2002). The practical implication of this assay justifies its frequent use and reference, but should not be the sole determinant of oxidation-reduction status.

Total Radical-Trapping Antioxidant Parameter (TRAP). The basic concept of the Total Radical-Trapping Antioxidant Parameter (TRAP) assay uses Folin-Ciocalteau regent to reaction with present polyphenols through an extraction/hydrolysis and protein precipitating step. Often repeated, the polyphenol extraction involves a hydrolysis step, which breaks the links of polyphenols with lipids and final precipitation of the supernatant to be filtered and assayed with Folin-Ciocalteau reagent (Serafini et al 1998, O'Byrne et al 2002). A direct relationship between total phenolic content and total antioxidant activity in phytochemical extract from dried fruits has been demonstrated in previous studies (Sun et al 2002). With this premise, the use of TRAP assay has become more common.

Antioxidant Network: The Endogenous Protective Mechanism

The question that arises now is, how effectively can athletes defend against the increased free radicals resulting from exercise? Do athletes need to take extra antioxidants or does a training adaptation exist? The human body maintains an elaborate antioxidant system that is extremely effective at counteracting oxidative damage under normal metabolism. This system is composed of several enzymes, vitamins, and minerals acting as: radical scavenger, hydrogen donors, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents.

In both the intra- and extracellular environment, there are 2 broad classes of endogenous antioxidant defenses that inhibit or react with radicals and radical intermediates: 1) enzymatic and 2) non-enzymatic (Table 1). Within these 2 divisions, antioxidant can be further classified into four categories based on function (Noguchi et al 2000). Comprised mostly of enzymatic antioxidants, the *preventive antioxidants* work to suppress the formation of free radicals. The *radical scavenging antioxidants* work to suppress chain initiation and/or breaking chain propagation reactions. The third category, *de novo antioxidant's*, main function is to simply repair compromised enzymes. Lastly, the fourth function responds to adaptation where the signal for the production and reaction of free radicals induce formation and transport of the appropriate antioxidant to the right site.

Table 1

Important enzymatic and non-enzymatic physiological antioxidants (Derived from Powers et al 2004).

Enzymatic antioxidants	**Location**	**Main Properties**
Superoxide dismutase (SOD)	Mitochondria & cytosol	Dismutase superoxide radicals
Glutathione peroxidase (GSH-Px)	Mitochondria & cytosol	Removes H_2O_2 and organic Hydroperoxide
Catalase (CAT)	Mitochondria & cytosol	Removes H_2O_2
Non-enzymatic antioxidants	**Location**	**Main Properties**
Vitamin C (Ascorbic acid)	Aqueous phase of cell	Acts as free radical scavenger and recycles vitamin E
Vitamin E (α-tocopherol)	Cell membrane	Major chain-breaking antioxidant in cell membrane
Glutathione	Non-protein thiol in cell	Serves multiple roles in the cellular antioxidant defense
α-lipoic acid	Endogenous thiol	Effective in recycling vitamin C, may also be an effective glutathione substitute
Uric acid	Purine metabolism product	Scavenger of OH radicals
Carotenoids	Membrane tissue	Scavengers of ROS, singlet oxygen quencher
Bilirubin	Product of heme metabolism in blood	Extracellular antioxidant
Ubiquinone	Mitochondria	Reduced form are efficient antioxidants

Enzymatic Antioxidants

Superoxide Dismutase (SOD)

Three groups of enzymes play significant roles in protecting cells from oxidant stress. The first, located both in the mitochondria and cytosol, superoxide dismutase (SOD) is the primary defense against superoxide radicals. This is an antioxidant that is poorly understood as it catalyzes to form a weak oxidant. However the benefit is that this oxidant is less toxic than superoxide anions. Acting as a Bronsted base in aqueous solutions, superoxide radicals ($O_2^{\cdot-}$) shift the acid-base equilibrium to form a

hydroperoxyl radical ($HO_2^{\cdot}$) and in low pH conditions form hydrogen peroxide (H_2O_2) (equation 1 Figure 2). This reaction can accelerate up to 10^4 times the frequency for spontaneous dismutation in physiological pH with the presence of SOD (Schneider et al 2004). SODs are metal-containing enzymes that depend on cofactors of bound manganese, copper, or zinc for their antioxidant activity. The manganese-containing enzyme is most abundant in mitochondria matrix, while the zinc or copper forms are predominant in cytoplasm. Interestingly, SODs are inducible enzymes – exposure of cells to higher concentrations of oxygen results in rapid increases in the concentration of SOD. (Williams et al 1998, Powers et al 1999)

Catalase (CAT) and Glutathione Peroxidase (GSH-Px)

As discussed earlier, H2O2 is not a radical, but because it is highly likely to produce hydroxyl radicals, which presence in the body is detrimental. To hamper any formation of hydroxyl radicals, catalase (CAT) and glutathione peroxidase (GSH-Px) are equally active in the blood to detoxify and/or reduce H2O2. Found mostly in the cytosol or peroxisomes and only in the mitochondria of the heart, CAT is a protein that contains iron in the form of heme and reduces H2O2 to water and oxygen hence finishes the detoxification reaction started by SOD. On the other hand, located in both cytosol and mitochondria, glutathione peroxidase reduces H2O2 to water by means of lipid peroxides metabolism. GSH-Px is a group of enzymes, the most abundant of which contain selenium. They also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants. In addition to these enzymes, glutathione transferase, ceruloplasmin, hemoxygenase and possibly several other enzymes may participate in

enzymatic control of oxygen radicals and their products. (Williams et al 1998, Power et al 1999)

Especially studies investigating exercise-induced oxidative stress, SOD, GSH-Px, CAT are common markers for antioxidant status in human studies. An early study by Robertson et al examining the response of runners to training load reported a positive correlation with the weekly training of low range (16 to 43km) to high load range (80 to 147km) to erythrocyte activity of enzymatic antioxidants (1991). However, subsequent studies showed varying results, which may be due primarily, but not limited to study design. Surmen-Gur et al. examined acute exercise on young male smokers and non-smokers at a load of 60 percent VO2max (2003). Their study suggests that SOD and GSH-Px are more prone to oxidative damage with acute exercise. However, another fairly recent study investigating the effect of exercise training on SOD mRNA expression, found no significant difference before or after training (Morikawa et al 2004). There may be an increase in these enzymes and other common enzymatic antioxidants in response to increased exercise-induce oxidant production, yet in terms of adaptation response to exercise, it is still unclear (Aslan et al 1998, Surmen-Gur et al 2004, Morikawa et al 2004). Since there is a strong association between intense exercise and oxidative stress, one can assume under these circumstances, sources of enzymatic antioxidants are exhausted, which leads to another line of defense: the non-enzymatic and dietary antioxidants.

Non-enzymatic and Dietary Antioxidants

Non-enzymatic antioxidants are another line of defense against radicals. These are found primarily within the lipid (fatty) and aqueous (watery) portions of the body.

The major aqueous-based antioxidants are reduced glutathione (GSH) and vitamin C, while the major lipid antioxidants are vitamin E, ubiquinol (coenzyme Q10) and β-carotene. Certain phytochemicals, like flavonoids also possess antioxidant ability.

Glutathione (GSH)

Primarily synthesized in the liver (approximately 90 percent) and transported to tissue via circulation, GSH is the main source of non-protein thiol in muscle cells (Ji et al 1995). It is a tripeptide (glutamyl-cysteinyl-glycine) and because of its structural composition, GSH is highly susceptible to degradation in the small intestine. Therefore, GSH cellular concentrations are not directly influenced by diet. However, based on several independent experimental studies, it appears that GSH content and GSH enzyme activities respond to training (Sen et al 2000). Animal models have demonstrated adaptation possibility due to training exercise with skeletal muscle GSH concentrations, where there is 600 percent more GSH and corresponding enzymes as well as catalase in (slow) type I fibers than (fast) type IIb fibers (Ji et al 1995). Perhaps this is due to greater oxygen utilization by the Type I fibers and GSH ability for compensatory response to acute exercise and training. Balakrishnan et al conducted a study comparing depletion of antioxidants among trained and untrained males and found that the basal GSH was negatively correlated with conjugated diene, a lipid peroxidation by-product, and VO2max, demonstrating the possible strain exercise has on the GSH basal source (1998). In terms of acute, chronic, and training response to exercise, GSH has been seen to modulate accordingly, thus demonstrating the active role GHS plays in defense against oxidant production (Ji et al 1995, Clarkson et al 2000, Sacheck et al 2001). (Williams et al 1998, Powers et al 2004)

During exercise, these defense systems become more active, although there is no clear explanation for the increase. Antioxidants have been observed to have a marginal adaptation. Such rapid up-regulation of gene expression of antioxidant enzymes in response to acute oxidative stress is not likely. Studies have observed exercise training to promote adaptation by the antioxidant defenses as antioxidant enzymes are increased as well as glutathione (Ji et al 1995).

Glutathione is a major non-enzymatic antioxidant and has several important functions: detoxification and recycling antioxidants (Table 1). Detoxification of ROS is accomplished through 2 general mechanisms: 1) direct or spontaneous reaction with ROS and 2) GSH-Px catalyzing ROS decomposition. During a reaction catalyzed by glutathione peroxidase, GSH donates a hydrogen pair in the removal of hydrogen and organic peroxides (i.e. lipid peroxide) by oxidizing 2 GSH to form glutathione disulphide. The cysteine from its tripeptide composition provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack, thus allowing for the second role as an effective and direct scavenger to a variety of strong radicals like hydroxyls and carbon centered radicals (Sen et al 2000). Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH (Figure 4). This same redox cycle and that of α-lipoate redox cycle demonstrates the strong interwoven link to the antioxidant defense system, where GSH acts as an effective reducing agent to support the recycling of vitamin C, ubiquinol, and vitamin E (Figure 4) (Sen et al 2000). (Williams et al 1998, Powers et al 2004)

Vitamin E: Tocopherols or Tocotrienols

Vitamin E is by far the most important lipid-soluble antioxidant and most commonly present in nuts (especially almonds and hazelnuts), seeds, vegetable and fish oils, whole grains (especially wheat germ), fortified cereals, and apricots. Vitamin E is a generic term to represent at least 8 isomers of tocopherol or tocotrienols. There are 4 forms of tocopherols and tocotrienols: α, β, γ, and δ, which differ in the position of methyl groups on the aromatic ring. Most studies examine α-tocopherol because is the most biologically active, potent in antioxidant ability, and the most widely distributed in food (Powers et al 2004). Traditionally, tocopherol and tocotrienols have been expressed in terms of international units (IU), where 1 IU is equal to 1mg of d-α-tocopherol acetate. The current recommended daily allowance (RDA) is 15 IU per day for men and 12 IU per day for women. The biological activity of tocopherol and tocotrienols recently has been expressed in d-α-tocopherol equivalent (α-TE). (Tidus et al 1995).

The primary activity of tocopherol is to trap superoxide, hydroxyl, and lipid peroxyl radicals to less reactive forms in the cellular membranes. Lipid hydroperoxides are oxidized to peroxyl radicals (ROO), which can react with fatty acids in the membranes, but if vitamin E is present, these radicals react with vitamin E to form ROOH. Embedded strategically within the various lipid-rich membranes (i.e. sarcolemma, inner mitochondrial, and plasma membrane) and due to its lipid-soluble character, its major function is to protect membranes and lipoproteins from oxidative damage. (Tidus et al 1995, Williams et al 1998)

Although vitamin E is a chain-breaking antioxidant which reacts rapidly with fatty acid radicals to prevent further propagation, during the course of such reactions the

vitamin can itself be oxidized to tocopheroxyl radicals. Pincemail et al looked at tocopherol mobilization during bouts of intense exercise, and found a significant increase during the exercise and a return to baseline post-exercise (1988). Robertson et al found similar response and reported that in response to training load, levels of vitamin E were elevated in erythrocyte among running groups compared to sedentary subjects (Robertson et al 1991). Similar reports were found in single bouts of endurance exercise (Hutler et al 2001). Deuterated α-tocopherol was observed to deplete faster during an extreme ultramarathon (50km) than that of sedentary subject (Mastaloudis et al 2001). An explanation of this disappearance is due to increased levels of vitamin E committed to respond to the exercise-induced lipid peroxidation. Therefore, these radicals must be reduced back after each reaction by glutathione or vitamin C (Figure 4). The remarkable ability of vitamin E to continuously work against lipid peroxyl radicals in the membrane can be explained by the continuous recycling of this vitamin and explains why vitamin E has the reputation of being one of the most effective antioxidants (Sen et al 2000).

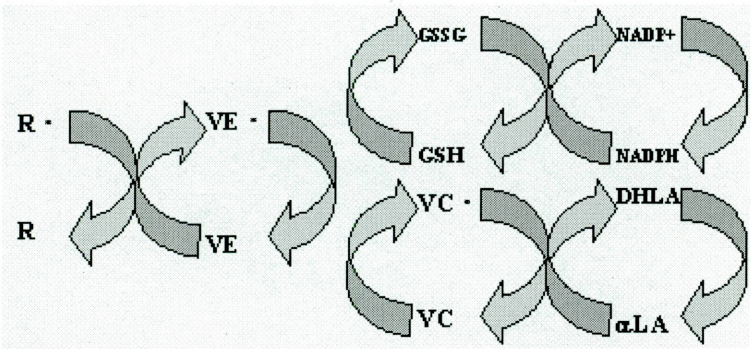


Figure 4. Interaction between non-enzymatic antioxidants. α-lipoic acid (αLA), glutathione (GSH), and vitamin C (VC) in the recycling of vitamin E (VE). $VC^{\cdot}$ = ascorbate radical; VE · = vitamin E, DHLA = dihydrolipoic acid; GSSG = oxidized glutathione (Adapted from Ji 1995).

Vitamin C: Ascorbic Acid

Vitamin C (ascorbic acid) is a water soluble vitamin present in citrus fruits and juices, green peppers, spinach, broccoli, kale, cantaloupe, kiwi, and strawberries. As opposed to vitamin E, its water-soluble character allows for antioxidant activity to be prominent in aqueous environments. These chemical properties allow it to interact directly with superoxide and hydroxyl in the aqueous phase such as plasma thus preventing damage to erythrocyte membrane (Ji et al 1999). The RDA is 90 mg/day for men, 75 mg/day for women, with an additional 35 mg/day for smokers. Intake above 2000 mg may be associated with adverse side effects in some individuals (Powers et al 2004).

There are basically 3 main functions that vitamin C plays, 2 of which are linked to the body's antioxidant defense mechanism (Table 1). Taking on the role first as a free radical scavenger, ascorbate can react with superoxide, hydroxyl, and lipid hydroperoxides for their removal. Secondly, vitamin C is a major contributor to recycling vitamin E radicals by converting reduced vitamin C to a semiascorbyl radical, which is later reduced back into its reduced state through NADH semiascorbyl reductase, GPH, or dihydrolipoic acid (Figure 4). These 2 functions play a vital role in the antioxidant defense network against oxidative stress. Thus, it is no surprise that ascorbate acid has been observed to respond to extreme bouts of exercise. Gleeson et al, investigating endurance runners, reported that the plasma concentration of ascorbic acid increased significantly in response to a 21-km run, however at 24 hours post-exercise, baseline levels of ascorbic acid were reduced to 20 percent and remained that low for another 48 hours (1987). Subsequent studies found similar response to concentration of vitamin C during and post-exercise (Clarkson et al 2000). Although the vitamin C antioxidant mechanism is well established,

the importance of vitamin C in protecting against exercise-induced oxidative stress is not clear.

Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances, especially in the presence of transition metals such as Fe^{3+} or Cu^{2+}. The ability for vitamin C to convert reduced iron (Fe^{3+}) to the ferrous state (Fe^{2+}) would ignite a cascade of further production of free radicals (Ji et al 1995). The notion to mega-dose vitamin C supplementation to overcompensate loses is still unclear and avoided due to its potential ability to be a pro-oxidant.

Flavonoids

Flavonoids are not part of the endogenous antioxidant family, but rather a larger family, the diphenolpropanes (over 4,000 members), which contain antioxidant properties and ability to reduce oxidative stress (O'Byrne et al 2002). They are commonly found in everyday foods, like fruits (especially grapes and raisins), vegetables, chocolate, tea, and wine.

In addition to the three main endogenous non-enzymatic antioxidants and the fourth as a phytochemical, there are numerous small molecules that function as antioxidants: dihydrolipoic acid (active form of α-lipoic acid), carotenoids, ubiquinone, bilirubin, uric acid, and certain trace minerals. Their main properties and location is briefly summarized in Table 1.

Combating Oxidative Stress: Nutrition Manipulation

The Supplementation Approach

Table 2 summarizes the studies that have investigated the effect of antioxidant supplementation use prior to an intense bout of exercise on exercise-induced oxidative

stress. A randomized, single blinded study of 20 non-smoking males (mean age of 25.0±5.6 years) with varying levels of training compared the effect of a daily dose of vitamin supplement mixture (592mg α-TE, 1000mg ascorbate acid, and 30mg β-carotene) to a placebo for 6 weeks prior to a exercise run (Kanter et al 1993). The exercise test consisted of a 30 minute treadmill run at 60 percent VO_{2max}, followed by a 5 minute run at a pace that elicited approximately 90 percent VO_{2max}. Results reported a significant lower rate of pentane production and MDA at rest after the vitamin supplement mixture group (significant values at $P<0.05$) and no significant changes with the placebo group.

Table 2
Human studies on the effect of antioxidant supplementation use prior to an intense about of exercise on exercise-induced oxidative stress

Reference	Subjects	Study Design	Length	Treatment	Factors Studied	Results
Kanter et al 1993	20 non-smoking males, (25.0 ± 2.9 yrs)	Randomized, single-blind	6 wks	Daily dose of 1 of the following: • ***Vitamin supplements (VS)*** (592mg-αTE 1000mg-ascorbate acid, 30mg-βcarotene) • ***Placebo(P)*** (saturated medium-chain Triglyceride gelatin)	Pentane TBARS - MDA	VS vs. P: ↓ Absolute pentane, ↓ MDA
Vasankari et al 1997	8 endurance male runners, (25 – 39 yrs)	Randomized, single-blind crossover	8wks + 4wk interval	Daily dose of 1 of the following 4wks prior to each exercise test (2): • ***Vitamin supplements (VS)*** (294mg vitamin E, 1000mg vitamin C, 60mg ubiquinone) • ***Placebo(P)***	Antioxidant potential (TRAP), Serum α-tocopherol, Diene conjugates (DC)	VS vs. P: ↑ LDL- & serum TRAP ↑ Serum α-tocopherol, No sig. difference on DC
Vasankari et al 1998	17 endurance athletes: Study I – 9 (20-37yrs) & Study II – 8 (24-34 yrs)	Randomized, single-blind crossover	2 bouts of exercise test + 7d interval	• ***Study I*** (Ingested 1 of the following prior to each exercise test)*:* vitamin C solution (VC): 2.0g ascorbic acid or placebo (P) • ***Study II*** (Ingested 1 of the following prior to each exercise test)*:* carbohydrate solution (C): 5g glucose + 10g maltodextrin or placebo (P)	Diene conjugates (DC)	Study I: VC vs. P No sig. difference during test, ↓DC @ recovery; Study II: No sig. difference between C vs. P

Table 2 (continued)

Reference	Subjects	Study Design	Length	Treatment	Factors Studied	Results
Buchman et al 1999	26 marathon male (24) & female (2) runners (18-55yrs)	Randomized, single-blind, placebo controlled	2wks pre-test + 1 bout of exercise test	Daily dose of 1 of the following 14d prior to exercise test: • ***Vitamin supplement (VS)*** (1000IU d-α-tocopherol) • ***Placebo(P):*** (soya lecithin + 0.02mg α-tocopherol)	Salicylate, Plasma Vitamin E (VE), total lipid	VS vs. P: No sig. difference in Salicylate & plasma VE, ↑ VE:total lipid ratio
Sacheck et al 2000	22 females: 11(19.6±0.9), 11(20.5±1.7)	Ancillary, unblind parallel	3month (1 bouts of exercise test during early follicular phase	Daily consumption of 1 week prior to exercise test (at least 3-days (1 weekend + 2 weekdays) was confirmed): • ***Low fat diet -LF (<40g fat/d):*** (2.9mg vitamin E/d) • ***High fat diet -HF (>60g fat/d)t:*** (9.8mg vitamin E/d)	VE, MDA, DC	LF vs. HF: No sig. difference
Mastaloudis et al 2001	11 athletes: 8 males (46±3yrs) & 11 females (44±3yrs)	Unblind crossover	2 wks pre-race + 1 bout of exercise test (Trial 1) & 1month post-race (Trial 2)	14hrs prior to each trial (2) consumed both of the following: 75mg d_3-RRR & d_6-all-rac-α-tocopherol	Ascorbic acid (VC), d-tocopherol (d-VE), Plasma F_2-isoprostanes	Trial 1 vs. 2: No VC comparison between trials due to lost of samples, ↑ rate d-VE depletion, Plasma F_2-isoprostane no sig. difference
Palazzetti et al 2004	17 male triathletes, (32.9±9.9)	Randomized, double-blind	4wk overload training - OT & 4wks normal training-NT)	Daily dose of 1 of the following prior to training: • ***Vitamin supplement (S)*** (75μg Se + 75μg retinyl acetate, 60mg ascorbic acid and 60mg d-α-tocopherol) • ***Placebo(P)***	Gluthathione(GSH), Glutathione peroxidase (GSH-Px), Superoxide dismutase (SOD), Creatine kinase (CK)	OT vs. NT: ↑ GSH S vs. P: GSH-Px higher in both OT & NT, ↑ SOD, ↓CK

Another randomized, single-blind, however in a crossover fashion of 8 endurance male runners ages ranging from 25 to 39 years compared the effects of a daily dose of a combination of antioxidants mixture (294mg vitamin E, 1000mg vitamin C, and 60mg ubiquinone) to a placebo for 4 weeks, prior to each exercise test (Vasankari et al 1998). The exercise test consisted of a repeated 31km running exercise twice with a washout period of 4 weeks. Among the vitamin treated group, levels of LDL-TRAP, serum TRAP, and serum α-tocopherol inferring antioxidant potential were significantly increased compared to the placebo group ($P = 0.0031$, $P = 0.0037$, and $P = 0.0031$ respectively). There was no significant difference found between the groups for diene conjugates.

Vasankari et al conducted another randomized, single-blind crossover study on 17 endurance athletes on 2 bouts of exercise tests: Study I: 9 athletes (ages ranging from 20 to 37 years) and Study II: 8 athletes (ages ranging from 24 to 34 years) (1998). Study I compared the effects of a vitamin C solution (2.0g ascorbate acid) to a placebo alternate. The washout period was 7 days. There was no significant different during the exercise, but among the vitamin C group, there was a significant decrease of conjugate dienes at recovery compared to the placebo group. Study II compared the effect of ingesting a carbohydrate solution to a placebo with a washout period of 7 days too. There was no significant difference between the carbohydrate group and that of the placebo group.

In another randomized, single-blind placebo controlled study, 26 marathon runners consisting of 24 males and 2 females (ages ranging 18 to 55 years) compared the daily dose of vitamin supplement of 1000IU of d-α-tocopherol to a placebo (soya lecithin and 0.02mg-α-tocopherol) for 14 days prior to the 1996 Houston-Tennaco Marathon run

(Buchman et al 1999). Subjects were included if they had no use of non-steroidal anti-inflammatory drugs (NSAIDS) within 24 days of the race or vitamin or mineral supplements containing vitamin C or E or selenium within 30 days of the race. Runners were studies 2 weeks prior to the race. The vitamin E:total lipid ratio had a significant increase in the supplemental group ($P = 0.02$), but not in the placebo group ($P = 0.25$). There was no significant difference in salicylate and plasma vitamin E.

Sacheck et al conducted an ancillary, unblind, parallel study on 22 female rowers (11 of whom were ages 19.6±0.9 years and 11 of whom were ages 20.5±1.7 years) comparing a daily consumption of a low fat (LF) diet (<40% in fat/d) to a high fat (HF) diet (>60% in fat/p) with the premise that the LF would have a significantly low amount of vitamin E (2.9mg vitamin E per day) in respects to the RDA than that of the HF (9.8mg vitamin E per day) (2000). The pilot study spanned 3 months until a single exercise test was performed during the early follicular phase to see the effect of the treatments. The exercise consisted of running on a motorized treadmill for 5 minutes (0 percent grade) and then another 45 minutes at a downhill grade of -10° at 75% of each subject's age-predicted maximum heart rate (220 – subject's age). The diet was maintained for at least 1 week prior to exercise (at least 3-days were confirmed through a dietary recall. No significant difference for vitamin E, MDA, and DC were seen between the 2 groups.

Another unblind, crossover study on 19 athletes, 8 males (ages 46± 3 years) and 11 females (ages 44±3 years), compared the effects of consuming both 75mg d_3-RRR and d_6-all-rac-α-tocopherol 14 hours prior to a single bout exercise trial and a sedentary trial (Mastaloudis et al 2001). Trial 1 consisted of a 5km ultramarathon run with a 1 month washout period and trial 2 was no physical exercise. The baseline of this study was 2

weeks prior to the race. Due to loss of samples from trial 2 because of technical reasons, there was no comparison test run between the 2 groups. There was no significant difference in F_2-isoprostene between groups. However, there was a significant increase in the rate of d-tocopherol disappearance in trial 1 compared to trial 2 ($P<0.03$).

In a more recent randomized, double-blind study (Palazzetti et al 2004), 17 male triathletes (32.9 ± 9.9) compared the daily dose of either a vitamin supplement mixture (75μg Se + 75μg retinyl acetate, 60mg ascorbic acid and 60mg d-α-tocopherol) to a placebo for 4 weeks of either overload training (OT) or normal training (NT). Training loads (NT) were individualized for each subject, quantitatively by collecting personal data in regard to past training and qualitatively by functional assessments. OT was defined as 42 percent increase from NT. There was significant increase in GSH in response to supplementation and remain elevated in the OT group compared to the NT group ($P<0.05$). GSH-Px was significantly higher in the supplement group in all cases after NT and OT ($P<0.01$). In response to OT, supplementation increased SOD significantly ($P<0.05$) and significantly decreased the magnitude of creatine kinase ($P<0.05$).

The Whole Foods Approach

Plants are especially susceptible to damage by active oxygen (exposed to radiation UV light) and it is no surprise that plants have their own antioxidant defense systems that can act as very potent antioxidants. (Bruce et al 2000, Karadeniz et al 2000, Sun et al 2002, O'Byrne et al 2002). Daily foods contain a wide variety of free radicals scavenging molecules, such as nuts, fruits, vegetables, teas, wines, and a variety of other foods are product rich in natural antioxidant compounds.

In a randomized, crossover study on 12 hyperlipidemic women 2 diets were compared (refined-food diet and phytochemically-rich food diet), where the phytochemically-rich diet intake was increased in dietary fiber, vitamin E, vitamin C, and carotene (respectively 160, 145, 160, and 500 percent increase) compared to that of the refined-food diet (Bruce et al 2000). Not only was the lipid profile improved by a decrease of 13 percent in total cholesterol ($P<0.05$) and 16 percent decrease in low-density-lipoprotein-cholesterol ($P<0.001$), but also blood SOD decreased by 69 percent ($P<0.01$) and GSH-Px by 35 percent ($P<0.01$) on the phytochemical-rich diet. These results suggest that phytochemical-rich foods may offer a beneficial effect on lipid profile and possibly decrease the need for oxidative defense mechanisms.

Another randomized study on 17 healthy adults compared the antioxidant effects of Concord grape juice (10ml) and α-tocopherol supplementation (400 IU RRR-α-tocopherol) on biomarkers of oxidative stress (O'Byrne et al 2002). Both regimes significantly increased serum oxygen radical absorbance capacity (ORAC) ($P<0.001$) and LDL lag time ($P<0.001$), suggesting the Concord grape juice as an equal alternative to vitamin E supplementation. In normal metabolic circumstances, the whole food approach does demonstrate merit for recommendation purposes. However, it would also be of interest to see if this approach holds up in conditions inducing oxidative stress, like that of intense physical exercise.

Research Gap

Form of Antioxidant Delivered

All of the studies thus far have assessed the effects of dietary antioxidants on exercise-induced oxidative stress by using dietary supplements instead of considering the

possible additive or synergistic effects of nutritious whole food sources. No studies have yet to standardize a pre-exercise beverage in respect to taste, energy, consistency, and antioxidant composition requirements like that created in the Food Laboratory at Loma Linda University. This study will be the first to systematically explore the effects of whole food pre-exercise snacks (almond-raisin beverage versus a commercialized sports beverage (Gatorade ®)) on antioxidant activity and lipid peroxidation among endurance male runners.

Methodology

Over the last decade of studies examining the effect of dietary antioxidants on exercise-induced oxidative stress, common biomarkers used to monitor antioxidant activity and lipid peroxidation were reported to have inconsistent results. The explanation to this variability range is a number of possibilities including the differences in the mode of exercise used, the time points examined, the level of training of subjects, environmental factors (i.e. altitude), and/or the lack of control for changes in the plasma/serum volume (Clarkson et al 2000). Furthermore, few studies have sought to determine and utilize optimal dosing strategies in regard to both dosing and delivery. These studies often neglect the possibility that mega-dosing may overcompensate the shift in the redox balance. Aside from study design limitation, assay protocols, sample size, and possible training adaptation effect, other confounding factors may be contributing to the variability.

Clearly, additional research is necessary before it can be stated with certainty that dietary antioxidant supplements in athletes are effective in combating exercise-induced oxidative stress. Furthermore, studies paving the way to investigate whole food sources may offer a more conducive, convenient, and natural alternative for athletes concerned with

this arena. The "Almond-raisin Beverage versus Commercial Sports Beverage Study" provides an ideal study design and data set that may fill some gaps in research concerning antioxidant activity and lipid peroxidation among endurance athletes.

Pre-exercise Beverages

Commercial Sports Beverage (Gatorade ®)

Commercial sports beverages (like Gatorade®) have become a very important part of the current sports market. Research has confirmed that, for the most part, the claims about the effectiveness in endurance events and athletic competitions may hold truth (Coombes et al 2000). Gatorade ® and other commercial sports beverages have been reported to be effective in preventing dehydration, providing carbohydrate, stimulating rapid rehydration, and in most cases encouraging athletes to drink enough fluid to avoid dehydration without any adverse side effects (Davis 1990, Ryan 1991, Burke 1993). Although commercial sports beverages encompass a number of vital factors in physical exercise (energy and hydration), it is lacking in response to the last decade of studies indicating a need to combat exercise-induced oxidative stress.

The Almond-Raisin Combination

Almonds

Over the past few years there have been a growing number of new findings on the nutritional benefits of nuts, especially almonds. Almonds are unique in composition because they are an excellent source of plant protein, fiber, and vitamin E. Of all the nuts, almonds have the highest concentration of natural α-tocopherol, the most biologically active form of the vitamin E compounds (Kris-Etherton et al 1999, Sabate et al 1996, 2001, 2003). Investigating an almond-rich diet, Sabate et al reported that on average men needed

5mg of α-tocopherol and women 8mg to meet the daily vitamin E requirement (2001). Furthermore, the study demonstrated that just one ounce of almonds a day almost bridges the gap for the daily requirement (Sabate et al 2001).

The source of almond's antioxidant potency comes not only from vitamin E, but also from manganese and copper. These 2 trace minerals are essential cofactors for proper scavenging of the non-enzymatic antioxidant: superoxide dismutase (SOD).

Raisins

According to the USDA, raisins rank among the top antioxidant foods. Not necessarily in terms of antioxidant vitamins, but rather in a high composition of flavonol glycosides and phenolic acids (Karadeniz et al 2000). A direct relationship between total phenolic content and total antioxidant activity in phytochemicals extracts in dried fruits has been demonstrated in previous studies (Sun et al 2002). Only in the past few years have studies examined whole foods promoting optimal health, so the area of research of raisin and health is in its early stages. Raisins have been the object of research primarily for their unique phenol content. There are less studies in comparison with almonds on the effect of raisins and antioxidant status and no study to date that associate the consumption of them to protect exercise-induced oxidant production.

The Almond-raisin Beverage

Although incorporating almonds and raisins in the diet to improve oxidative status to meet the needs of normal metabolic processes has been studied, there has not yet been a study that has considered almonds and raisins as an antioxidant source to combat against exercise-induced oxidant production. This study has opted to combined almonds and raisins in the form of a liquid as a pre-exercise beverage due to its ease of consumption and

consistency in studying its effect as an antioxidant source versus a commercial sport beverage (Gatorade ®) among endurance male runners. The beverage was prepared by toasting the almonds and soaking the raisin in water overnight before blending them in a high-speed Food Processor. The amount of water used to prepare the almond-raisin beverage was accounted for to ensure equal water consumption between the 2 beverages. Isocaloric conditions between the 2 beverages assured equal energy comparisons. The primary reason for combining almonds and raisins is the nutrient composition – especially the antioxidant potency that each possesses, and its potential combined antioxidant power. (Bazilian et al 2003).

Clearly the consumption of natural whole food combinations rich in antioxidant potency has the potential to improve antioxidant status. The degree of that improvement among male endurance runners is the primary objective of this study. By building on the much-needed data, the long-term objectives of the "Almond-raisin Beverage Versus Commercial Sports Beverage Study" is to pave the way for further studies examining the role of combining different types of whole food sources such as nuts and dried fruit as pre-exercise, during exercise, and post-exercise snacks to combat exercise-induced oxidative stress.

CHAPTER THREE

OUTLINE OF THE RESEARCH

The *Effect of a Pre-exercise Almond-Raisin Beverage versus a Commercial Sports Beverage on Endurance in Male Runners* was conducted in 2002 with the support of the Nutrition Department and the Center for Health Promotion of Loma Linda University with Sujatha Rajaram, PhD, as the primary investigator (Rajaram et al 2003 submitted). The primary objective of the nutrition-exercise study was to determine the effects of fat and carbohydrate on fuel utilization and endurance performance in trained male runners. In the course of this study, extra serum samples were stored for further testing of an ancillary hypothesis such as the current study that examined the effect of an almond-raisin beverage on antioxidant activity and lipid-peroxidation.

Subjects

Male endurance runners were recruited from Loma Linda University, California State University San Bernardino, University of California Riverside, and the surrounding communities. Representation of the major ethnic groups in Southern California was considered: Caucasians, Asians, African Americans, and Hispanics.

Inclusion Criteria

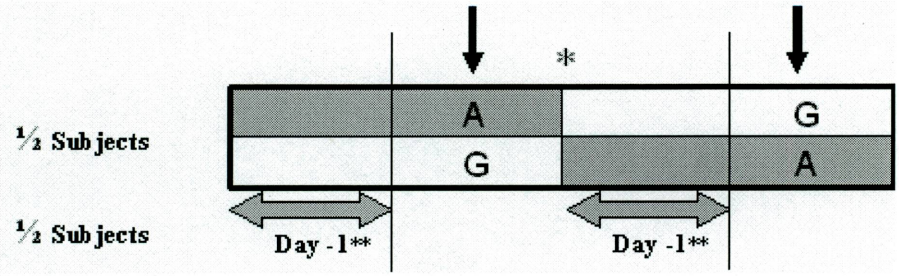
Subjects qualified to participate in the study were healthy males between the ages of 20 to 35 (age 28$\pm$1.2) years, habitual endurance runners (running at least 30 miles per week), VO_2max between 56 to 65 ml/kg/min (VO_2max 61.9$\pm$2.1 ml/kg/min), had no adversities to nuts or dried fruit (specifically almonds and raisins), maintained the same training schedule throughout the study period, and adhere to the diet and exercise protocol outlined in this study.

Exclusion Criteria

Based on a two-stage screening process, recruited individuals were excluded if they were not male, fell outside the age range of 20 to 35 years, have any medical conditions (cardiovascular disease, diabetes and/or other metabolic disorders) deeming unhealthy status based on brief medical history questionnaire, have adversities to nuts or dried fruit, and did not run at least 30 miles per week. Significant weight change (>2.27 kg) in the last year and unwillingness to maintain training status and adhere to the diet and exercise protocol for the duration of the study were excluded. Those who met this initial screening were then invited to review and sign the inform consent, complete a treadmill test to determine their maximal oxygen consumption (VO_2max), and provide a blood sample for a basic chemical profile. Contingent on the normative data report for the level of fitness for male runners by the American College Sports Medicine, VO_2max below 50 ml/kg/min were excluded. Those who donated blood within two months prior to signing informed consent, had medical conditions, or sub-normal nutritional status identified by the chemical profile were also excluded.

Study Design

This study was a randomized, single-blind, crossover experimental design (Figure 5) based on the Rajaram et al. 2003. In Rajaram et al. 2003, 10 subjects completed the study. Only viable serum samples were assayed and missing data was accounted for in the statistical analysis. Subjects were randomly assigned to one of the two treatment groups: (1) Almond-raisin beverage or (2) Commercial sports beverage (Gatorade®) as a pre-exercise snack before they ran to exhaustion on a treadmill at 70% VO_2max twice in a crossover fashion separated by at least 2 weeks.



A = Almond-Raisin Beverage
G = Commercial Sports Beverage – Gatorade

* Trials separated by at least 2 weeks
** 1 day standardized carbohydrate

Figure 5. Study design

Data Collection and Analysis

Serial blood measures were collected 60 minutes prior to treadmill test fasting (T_{-60}), prior to test (T_0), 20 min intervals from start (T_0) until exhaustion (T_{end}), and 30 minutes post-exercise (T_{+30}). Blood draws were performed at the Center for Health Promotion, Loma Linda University, centrifuged within 20 minutes, serum aliquoted immediately, and stored at -85 degrees Celsius until further analysis. Samples were then analyzed at the Nutrition Research Laboratory, Department of Nutrition, Loma Linda University.

Rationale for Selection

The methods employed to observe oxidative stress was based on cost, availability of viable serum, and most commonly used biological markers used in recent discussions of studying antioxidant activity and lipid peroxidation. Lack of accuracy, validity, or both of current methods to assess oxidative stress in humans was acknowledged and handled by conducting more then 1 technique to provide a better estimate. Due to these limitations of each biological assay alone, 3 assays were considered to study both sides of the Redox scale.

Total Antioxidant Activity: Trolox Equivalent Antioxidant Capacity (TEAC)

Serum TEAC was determined following an adapted and improved method of an assay described previously (Re et al, 1999). TEAC is the capacity of an individual antioxidant to inhibit preformed radical monocation of 2', 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) at a defined time point relative to Trolox (6-hydroxy-2,5,7,8-tet-ramethychroman-2carboxlic acid). This decolorization assay screened for both lipophilic and hydrophilic antioxidants – Flavonoids, hydroxycinnamates, carotenoids, and serum antioxidants (ascorbic acid, α-tocopherol, gluthathione, and uric acid). The minimum sample volume used for TEAC was 10μl for each serum sample and working Trolox standards (1, 0.5, and 0.25 mmolar). Each sample was read at the absorbance of 734 nm in the spectrophotometer within 1 minute after sample was exposed to $ABTS^{\cdot+}$.

Total Radical-Trapping Antioxidant Parameter (TRAP)

Serum levels of total polyphenols were measured by Total Radical-Trapping Antioxidant Parameter (TRAP) assay using Folin-Ciocalteau reagent and expressed as Gallic Acid Equivalents (GAE) described and adapted by previous studies (Singleton et al 1999). Originated in the late 1950s, by Swain and Hills, the modified TRAP method used adapted deprotenation of the sample prior to remove masking of phenolic compounds by proteins (Serfini et al 1998). The minimum volume of samples used was 500μl for each serum sample and working Gallic Acid Standards (500mg GAE/L): 1:10, 1:25, and 1:50. After 6 hours samples were read in the spectrophotometer at absorbance of 765 nm.

Lipid-Peroxidation By-Product – Malondialdehyde (MDA):

Thiobarbituric Acid Reaction (TBARS)

Serum malondialdehyde was monitored by thiobarbituric acid reaction (TBARS) using high-pressure-liquid-chromatography (HPLC). Although the TBARS is a non-specific technique, it is widely used in the discussion of lipid peroxidation because it does offer an empirical window on the complex process of lipid peroxidation. The improve method was used to enhance accuracy and reliability by a series of amplification and purification steps (with the use of HPLC) (Furkunaga et al., 1998, Clarkson et al., 2000). The minimum volume of samples used was 50μl for each serum sample and working MDA 5μmol/mL) standards with 1ml TBA-buffer solution and injections of 10μl was done thereafter into the HPLC.

Statistical Analysis

Statistical results were based on analysis carried out by the Statistical Analysis System, version 8.0 (SAS Institute Inc., Cary, NC). For each of 3 assays, TEAC, FC, and TBARS, the following analyses were conducted: (1) paired t-test on the change T_{-60} to exhaustion, (2) paired t-test on the change from $T_{-60\ to}\ T_0$, (3) longitudinal analysis using all time points from T_{-60} through T_{80}, and (4) paired t-test for the area under the curve (AUC) formed by readings at all time points from T_{-60} through T_{80}. Descriptive statistics was reported as means $\pm$ SD and the results with a P value of ≤ 0.05 were considered statistically significant. The longitudinal analyses were conducting using mixed linear models that included time and treatment as fixed factors, and subject as a random factor. This variance components covariance structure was to found to provide a better model fit than unstructured or autoregression covariance structures, either with or without a subjects

variance component. As an additional fixed factor, the models ran with hemolytic status. Missing data was handled by a test-by-test basis.

CHAPTER FOUR

RESULTS

The subject baseline characteristics are shown in Table 3. The subjects were healthy, male endurance runners that had a mean maximal oxygen capacity of 62±2.

Figures 6-8 shows the differences between almond-raisin beverage and commercial sports drink with respect to the markers of antioxidant status before, during and after running to exhaustion by the study runners. Figure 6 depicts the results from plasma antioxidant capacity (TEAC), Figure 7 that of serum total phenols measured as gallic acid equivalent (GAE) and Figure 8 that of serum malondialdehyde measured as thiobarbituric acid reactive substance (TBARS). Paired t-test on the change from 60 minutes prior to start of exercise (T-60) to exhaustion and the change from T-60 to start of exercise (T0) demonstrated no significant differences for any of the three antioxidant status markers. However, including all time points from T-60 through 80 minutes into the run (T80) in the longitudinal analysis, there was a significant main treatment effect with the almond-raisin beverage showing higher values than the commercial sports drink for total antioxidant capacity ($p=0.0002$) and total phenols ($p=0.002$). Serum malondialdehyde as TBARS showed no significant treatment effect ($p=0.08$).

For the plasma total antioxidant capacity analysis, the area under the curve for almond-raisin beverage was significantly greater than for the commercial drink ($p=0.016$). For serum total phenols (GAE), the area under the curve was also greater for the almond-raisin beverage compared to the commercial sports drink, but the difference was only borderline significant ($p=0.068$). The area under the curve for serum TBARS was not significantly different for the two pre-event beverages.

Table 3
Subject's baseline characteristics

Characteristics	Mean ± SEM
Age (yr)	28±1
Height (cm)	177±2
Weight (kg)	67±2
BMI	21.5±0.3
VO_{2max} (ml•kg-1•min -1)	62±2
Resting heart rate	61±2
Max heart rate	193±1

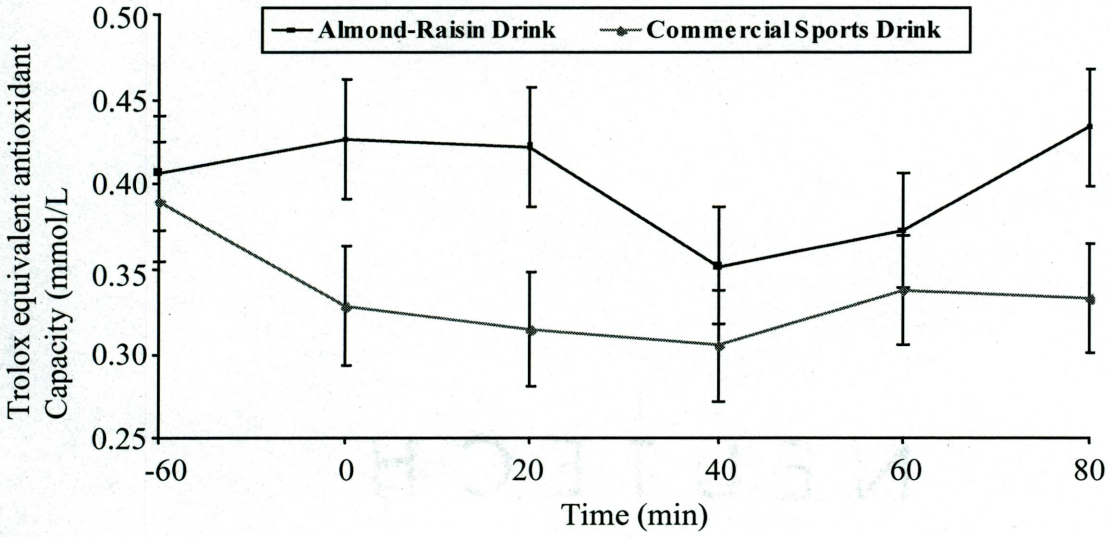


Figure 6. Plasma antioxidant capacity in subjects following the ingestion of the two test beverages (Means ± SD).

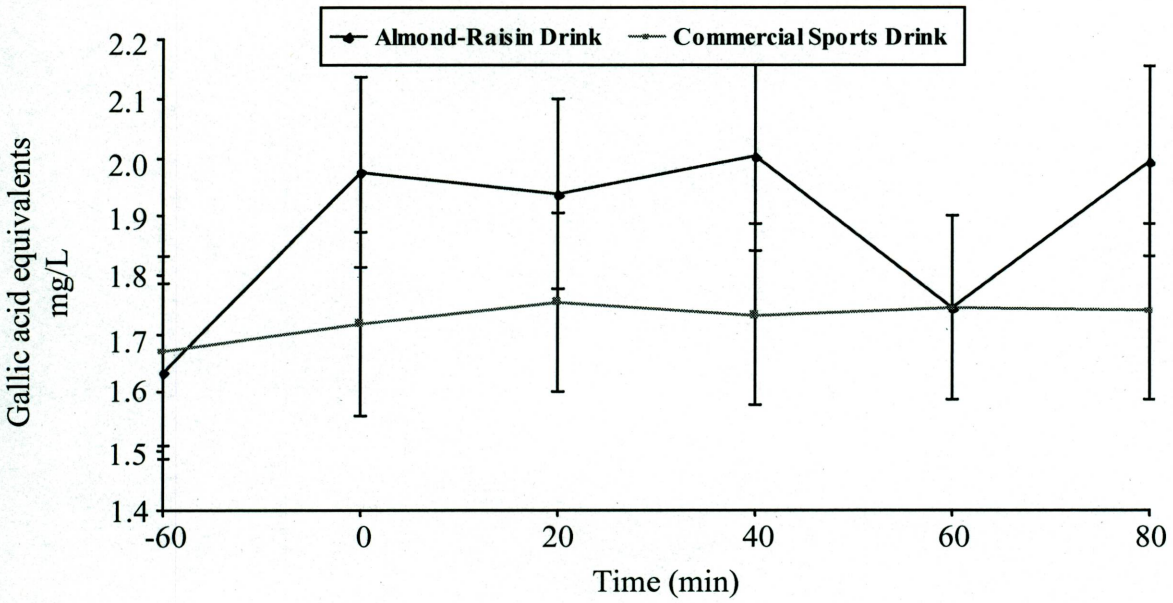


Figure 7. Serum total phenol in subjects following the ingestion of the two test beverages (Mean ± SD).

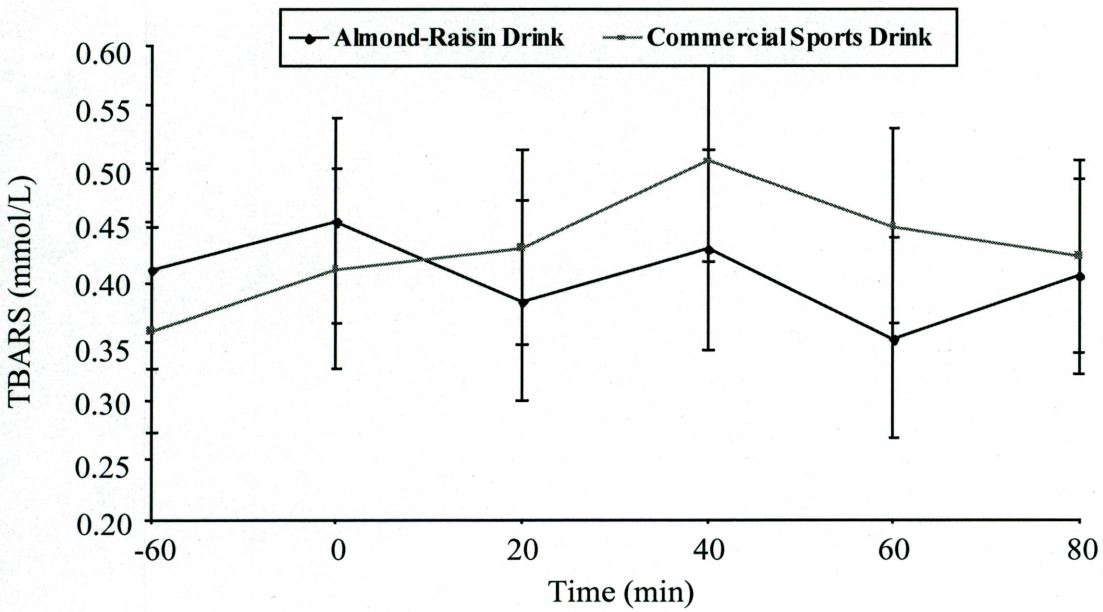


Figure 8. Serum malondialdehyde as thiobarbituric acid reactive substances following the ingestion of the two test beverages (Mean ± SD).

CHAPTER FIVE

DISCUSSION

This study sought to explore the effects of a whole food combination (almond-raisin) versus a commercial sports beverage (Gatorade ®) as a pre-exercise snack on lipid peroxidation and antioxidant activity before, during, and after a time-to-exhaustion treadmill run performed at 70 percent VO_{2max}. This study is a sub-study of a main study the objective of which was to determine if the performance time and fuel use by runners differed following the ingestion of the above mentioned pre-event beverages. The primary study results showed that performance time were similar following the ingestion of both the beverages; however fuel use was different in that the plasma fatty acid levels were higher following the consumption of the almond-raisin beverage. The primary objective of this sub-study was to look at the antioxidant status following the consumption of the two test beverages. The overall results demonstrate a more favorable antioxidant status following the ingestion of almond-raisin beverage compared to the commercial sports drink.

This study is the first to look at an antioxidant rich whole food combination on exercise induced oxidative stress. Previously published studies on antioxidant rich whole foods have looked at antioxidant status of participants at sedentary states more as a health outcome (Bruce et al 2000, O' Bryne et al 2002). On the other hand, studies that have looked at exercise induced stress have only looked at synthetic vitamin supplementation as a way to combat the free radicals. Therefore, our study is unique as it simultaneously

studied both sides of the redox scale with regard to antioxidant protection in runners that experience exercise-induced oxidative stress.

Antioxidant rich whole food like Concord grape juice has shown to increase oxygen radical absorbance capacity to a similar extent as 400 IU of vitamin E supplement in healthy adults (O' Bryne 2002). Consistent to this, we showed in our study that some of the biomarkers of antioxidant status such as total antioxidant capacity and total phenols, were higher among runners following the ingestion of the whole food test beverage (almond-raisin beverage) compared to the commercial sports drink.

Previous studies using synthetic vitamins as mixtures have demonstrated that antioxidant supplementation during exercise can help combat the exercise induced stress. Following the ingestion of a combination of antioxidants (592 mg of α-tocopherol, 1000 mg ascorbic acid, 30 mg β-carotene) for 6 weeks, a significant reduction in pentane and MDA production was observed among runners compared to a placebo (Kanter et al 1993). Another similar antioxidant mixture increased serum antioxidant capacity and serum α-tocopherol levels compared to a placebo in endurance male runners during an exercise test (Vasankari et al 1997). Increases in serum antioxidant enzymes such as glutathione peroxidase and super oxide dismutase were also seen following the ingestion of similar antioxidant mixtures compared to placebo among male triathletes (Palazzati et al 2004).

In comparison, studies that tested isolated antioxidants such as 75 mg α-tocopherol (Mastaloudis et al 2001) or 2 g ascorbic acid (Vanasankari et al 1998) or 1000 IU α-tocopherol (Buchman et al 1999) in comparison to a placebo failed to show significant changes in antioxidant markers following exercise. These observations

suggest that there may be a favorable synergistic interaction that exists among antioxidants given in mixtures that makes it more effective in improving antioxidant status during exercise compared to isolated nutrient supplement. The advantage of whole foods is that they contain a matrix of nutrients and non-nutrients that may have synergistic effect on each other thus enhancing their function as an antioxidant. It is likely that antioxidant rich whole foods may have similar or better antioxidant effects than isolated antioxidant vitamins in supplemental form. Thus future studies comparing whole food antioxidants to isolated synthetic antioxidant supplements as a way to combat exercise induced stress becomes important and a necessary next step in this line of research.

While the plasma total antioxidant capacity and serum total phenols were higher following the almond-raisin beverage ingestion among our study runners, no differences were seen with the serum TBARS-MDA levels between the two test beverages. The serum TBARS assay although not considered as the best biomarker of antioxidant status (Halliwell et al 1993, Clarkson et al 2000), was used in this study because it has also been demonstrated to have some merit as a general inference for oxidative stress (Leeuwenburgh et al 2001). It is possible that the lack of significant results for this assay even after amplification and purification procedures could be due to the non-specificity of this assay and possible inter-subject variability in MDA levels.

Future research is necessary before any definite conclusions are made for athletes with respect to the consumption of antioxidant rich whole food beverage as an alternate to the traditional sports drink. However, our preliminary data showed higher levels of certain biomarkers of antioxidant status following the intake of almond-raisin beverage

compared to the commercial sports drink. Also, we know that almonds and raisins are a combination of nutrients and non-nutrient phytochemicals many of which are more potent antioxidants than vitamins E or C. If indeed this combination is able to achieve antioxidant protection during intense exercise that is better than what can be seen with carbohydrate-electrolyte based sports drinks, it would be prudent to consider this as a viable alternate as a pre-event snack for endurance athletes.

Prior to making recommendations, future studies need to investigate the effects of antioxidant rich whole foods in comparison to antioxidant vitamin supplementation to combat exercise induced stress. Also, studies on the optimal dose of whole foods to be used, the best form to deliver the nutrients/non- nutrients and the practical convenience of using such foods needs to be studied. Finally, it has still not been clearly documented what the significance of reducing exercise induced stress is for athletes in terms of performance and health outcomes. Thus future studies should look at these variables and not just limit to reporting the antioxidant status of athletes. However, based on previous studies that show that eating nuts (almonds) and dried fruits (raisins) may actually improve health outcomes (Kris-Etherton et al 1999, Sabate et al 1996, 2001, 2003, Sun et al 2002), it may be prudent for athletes also to include these foods as part of their daily diet.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

This study sought to explore the effects of a whole food combination (almond-raisin) versus a commercial sports beverage (Gatorade ®) as a pre-exercise beverage on lipid peroxidation and antioxidant activity before, during, and after a time-to-exhaustion treadmill run performed twice at 70 percent VO_{2max}. High compliance to the protocol and favoring results suggest that the primary objective of this study was met. The primary outcomes appear to favor the use of the almond-raisin beverage as a pre-exercise snack over the traditional sports beverage in terms of antioxidant protection, in which a significantly greater main treatment effect was seen with the whole food source.

This investigation originated from the primary study that compared the effects of these two beverages on fuel use and performance among male endurance runners, where endurance time (time-to-exhaustion) was used as the primary outcome, at an alpha of 0.05. Therefore, the sample size (n=10) and sample collection were based on these parameters instead of that of lipid peroxidation and antioxidant activity markers. It is possible that this factor alone may have a limited achievement of a minimum of 80 percent statistical power for certain biomarkers used in this study.

To date, there are a few published experimental investigations on the effects of whole food sources rich in natural antioxidants as a method to combat exercise-induced oxidative stress. Our study sought to simultaneously study both sides of redox scale and

it demonstrates antioxidant protection, following exercise-induced oxidative stress (O'Byrne et al 2002).

Although there was a significant antioxidant protective effect, it was surprising to see that there was no significant difference across the board between groups in regard to lipid peroxidation. The TBARS–MDA test has generated some controversy in its discrepant results from previous studies, but it was used in this study because it also has demonstrated merit in regard to a general inference of oxidative stress. Even after amplification and purification procedures the non-specificity of this assay and possible inter-subject variability in MDA could explain these results.

Missing samples due to difficulty in blood draws, hemolysis, or insufficient amount collected for both the primary study and this sub-study could also account for insignificant results in paired t-tests on the change from baseline to exhaustion and the change from baseline to start of test. Due to the conditions of the exercise run, blood draws could not be done during actual run, but rather, the subject stopped and within 30 seconds blood was drawn and then exercise test resumed. However, possibly due to degree of hydration and circulation under such intense physical exertion, blood flow was often partially shunted in the upper extremities making the conditions more susceptible to hemolysis. Consequently, an insufficient amount of blood was collected for all analysis. Assays run with slightly hemolyzed samples were statistically adjusted, but did not affect the results of any assay.

Future Directions

Future research is necessary before any definite conclusions are made for athletes to clearly understand the antioxidant defense role, if any, on the consumption of whole

foods as a pre-exercise snack. If indeed this combination of almonds and raisins is able to achieve antioxidant protection during the event of intense exercise, it would be advantageous to continue this line of research not only to confirm the impact it may play in oxidative stress, but also to explore the mechanism of action. In doing so, a systematic approach defining an optimal dose or formula for improving antioxidant protection for athletes can be accomplished.

Future studies should set precedence on lipid peroxidation and antioxidant activity biomarkers as the primary research outcome variables. In doing so, the study design should provide adequate sample size, rigorous control against confounding factors (such as any antioxidant supplementation and/or high or low amount of fat or antioxidants in the diet), and a defined treatment dose, delivery, and duration to be able to achieve significant changes in oxidation-reduction status. Sample collection should be specific for lipid peroxidation and antioxidant biomarkers. It would be interesting to determine different dose ranges of the pre-exercise food combination needed for varying duration, intensity, and level of training of the athletes.

It is especially important that future research also determine the most accurate and replicable method of monitoring lipid peroxidation and antioxidant activity. To confirm the total effect of such a whole food combination, it is also necessary to monitor oxidative stress on both sides of the redox scale. Thus, it is suggested that at least two techniques monitoring lipid peroxidation and antioxidant activity are considered. It is also imperative to ensure minimal hemolysis and an adequate amount of human body fluid collected for all analysis. Pre-event protocols should consider hydration or any other influence on the blood flow during intense exercise.

The research on whole food sources as a pre-exercise snack and biomarkers of lipid peroxidation and antioxidant activity requires confirmation and further research is warranted regarding the effects of the almond-raisin combination and exercise-induce oxidative stress. Numerous studies have demonstrated that intense exercise overwhelms our natural antioxidant capacity thus leading to increased levels of oxidative stress. There is some evidence that antioxidant combination sources delay or lower the risk of exercise-induced oxidative stress and speed the recovery process or lessen muscle damage. Therefore, the best recommendation to date is still to eat a nutritionally adequate diet with generous servings of antioxidant rich foods, including those that contain vitamin E and polyphenols.

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