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The purpose of this study was to determine the effects of a combined antioxidant supplementation on myocardial protein carbonyls (PC) and malondialdehyde (MDA) in rats after aerobic downhill running. Sixty-six male Sprague-Dawley rats were randomly assigned to either a normal diet or antioxidant supplemented diet (2,000 mg vitamin C + 1,000 IU vitamin E/kg diet) for 2 weeks. Exercised rats (N=48) ran on a rodent treadmill for 90 min at a speed of 16 m/min and – 16 degrees downhill grade. Rats were killed either at rest, immediately, 2 hrs, or 48 hrs post exercise. Rested rats were killed at the same times as the exercised rats all between 0700-1000 hrs. Ventricular tissue was analyzed for two markers of oxidative stress, PC and MDA. Only exercised rats that completed the 90 min run were included in the PC and MDA data analysis. There was a significant time main effect for PC concentration ($p= 0.02$) but post-hoc analysis indicated no specific time differences. However, PC approached significance ($p=0.064$) for the 2 hr post exercise time. There were no significant treatment or time*treatment effects for PC. MDA results showed no significant time, treatment or time*treatment effect. These data indicate that downhill running with modest intensity does not significantly increase oxidative stress in hearts as indicated by PC and MDA but a larger sample size is needed to confirm these results.

EFFECT OF COMBINED ANTIOXIDANT SUPPLEMENTATION
ON OXIDATIVE STRESS IN MYOCARDIUM FROM RATS

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

The antioxidant capacity of the cell is important to maintain proper function. When the antioxidant capacity is unable to match the production of reactive oxygen species (ROS), there is a build up of ROS and their subsequent by-products. ROS are formed during normal cellular metabolism and are important in cell signaling and in breaking down cell structures (Bonifacic and Asmus, 1984). Unfortunately, these ROS can also react with and damage lipids, carbohydrates, proteins, and DNA. ROS are generated during oxidative metabolism in the mitochondria, along with various other processes (Jackson, 1994). It has been estimated that 2-5% of the total oxygen in the electron transport chain can escape to form oxygen radicals (Sen and Hanninen, 1994). Since aerobic exercise increases oxygen metabolism through the mitochondria, it is believed the amount of ROS produced during aerobic exercise is increased proportionately. This has been substantiated by the measurement of indirect markers of ROS. Numerous studies have reported increases in indirect markers of ROS and their interaction with lipids, thiols, proteins and DNA. The increase in these markers is an indication of oxidative stress.

Oxidative stress is a state where the ROS production overcomes the antioxidant system's ability to handle the ROS. Exercise-induced oxidative stress is a condition where the exercise has created a condition that produces ROS that cannot be adequately

handled by the antioxidant defense mechanisms of the system being measured. This exercise-induced oxidative stress can be produced through both aerobic exercise of sufficient intensity and duration, as well as high intensity exercise of short duration (Goldfarb et al., 1994).

The type of exercise, the intensity and duration of exercise, and the form of muscle contraction involved appear to be important factors for inducing oxidative stress. Concentric contractions (muscle actively shortening) during aerobic or intense short duration exercise has been reported to increase by-products of lipid breakdown, activate antioxidant defense mechanisms and decrease the amount of reduced thiols (Armstrong, 1990). In contrast, eccentric contractions (muscle actively lengthening) that can result in greater muscle damage utilizes less oxygen for the muscle contraction. In addition, there are fewer muscle motor units recruited for the same or greater force in an eccentric action compared to a concentric muscle action (Armstrong, et al. 1983). As a result there is a greater likelihood for muscle damage with eccentric types of contractions. The exact cause of the damage is still in question but seems to be related to the tension placed on the muscle fibers.

When muscle damage occurs, many processes are initiated, one of which is inflammation. During the inflammatory response, macrophages and neutrophils infiltrate the muscle and can activate ROS production (Jackson, 1998). Activities that have been used to induce muscle damage are downhill running and resistance exercise using a bar which forces the muscle to lengthen while exerting a force against the bar. Downhill running requires a large amount of eccentric contraction from the involved muscles as

well as oxygen delivery to the muscles. Therefore, downhill running can be used to induce damage to muscles (Armstrong et al., 1983) and also been reported to result in exercise-induced oxidative stress in rats (Cannon et al. 1990). Thus, downhill running has two potential mechanisms to induce increases in ROS.

Reperfusion injury has also been proposed as a source of ROS production (Di Meo et al., 2001). The sudden flux of blood to the area can lead to circumstances similar to those stated above in relation to oxidative metabolism in the mitochondria. This could also cause an increased production of ROS in skeletal muscle.

The heart is highly oxidative, extracting approximately 80% of oxygen from the blood. Because of this high oxidative capacity, when more oxygen is needed by the myocardium during exercise, the increase is achieved through an increase in blood flow (Di Meo et al., 2001). This increased blood flow to the myocardium could produce a situation similar to reperfusion injury and could lead to ROS production (Goldfarb et al., 1996; Kumar et al., 1992). Therefore, the heart may be considered another site of exercise-induced oxidative stress and has been shown to increase markers of oxidative stress following exercise (Goldfarb et al., 1996). However, the effects of downhill exercise on oxidative stress markers in the heart have not been investigated. In addition, the influence of antioxidant supplementation to this type of exercise on the heart has not been determined. Oxidative stress could lead to decreased cell membrane structure, damaged lipids, carbohydrates, proteins, and DNA. This is significant because oxidative stress in the heart may lead to a decreased functional ability of the myocardium. A

decrease in functionality of the heart could include decreased cardiac output, stroke volume, and filling capacity.

There are a number of endogenous enzymatic and non-enzymatic substances that can carry out antioxidant functions to help prevent or minimize the damage of ROS. The main contributors from the enzymatic group of physiological substances are superoxide dismutase (SOD), catalase (CAT) and the glutathione system enzymes, glutathione reductase (GR) and glutathione peroxidase (GPX). The primary non-enzymatic antioxidants are usually divided into two sub-categories: water-soluble and lipid-soluble. The main water-soluble antioxidants are vitamin C (ascorbate) and the thiol glutathione (GSH). The main lipid-soluble antioxidants are vitamin E (alpha-tocopherol) and beta-carotene. The main function of all of these antioxidant substances is to protect the cell from ROS-mediated damage by quenching or decreasing the activity of the ROS.

Non-enzymatic antioxidants such as vitamin C are important water-soluble antioxidants. Vitamin C can quench singlet oxygen as well as water-soluble ROS. Vitamin C also plays an important role in the regeneration of vitamin E (Packer, 1991). Supplementation of vitamin C has shown mixed results depending on the amount given and when it was given in human subjects. It has been shown to attenuate muscle leakage of proteins or reduce oxidative stress (Bryer and Goldfarb, 2001) at high levels, as measured by creatine kinase in blood and plasma protein carbonyls. However, it has also been shown to have little or no effect on oxidative stress, as measured by plasma thiobarbituric acid reactive substances (TBARS), at levels 1.0 gms and less (Alessio et al., 1997). The differences in the reported results might be due to dose and time of

treatment. Bryer and Goldfarb (2001) used a dose of 3.0 g/day over a period of two weeks. Alessio et al. (1997) used a dose of 1.0 g/day for only one week. Thompson et al (2001) gave 200 mg twice a day for two weeks. The results are equivocal and none of these studies examined the effects to downhill running and the potential to protect the heart.

Vitamin E, a lipid-soluble antioxidant, can inhibit lipid peroxidation, by acting as a chain breaker of lipid peroxidation, and stabilize membranes within the cell. Vitamin E has been shown to reduce oxidative stress markers in the heart in response to an aerobic exercise (Goldfarb et al., 1996). Kumar et al. (1992) also reported some beneficial effects of vitamin E in the heart in response to exercise. However, the effect of vitamin E in the prevention of exercise-induced oxidative stress in the heart in response to downhill aerobic running has yet to be determined.

The effect of aerobic exercise-induced oxidative stress has been shown to be reduced by vitamin E supplementation in both animal and human studies (Goldfarb et al., 1994; Reznick et al., 1992; Sen et al., 1997; Sumida et al., 1989). This decrease was dependent on the amount of vitamin E given, the form of the vitamin E and the tissue examined. In most cases, the vitamin E only demonstrated a partial protection to the exercise-induced oxidative stress. Beta-carotene, another lipid-soluble antioxidant, is a powerful quencher of singlet oxygen and may aid in the inhibition of lipid peroxidation. However, studies that gave beta-carotene seem to enhance the risk for certain chronic diseases as opposed to lowering the risk.

Antioxidant supplementation can be of value in helping to protect against exercise-induced oxidative stress. Several studies have already examined the effect of antioxidant supplementation on exercise-induced oxidative stress. Alessio et al. (1997) reported that both one day and two weeks of vitamin C supplementation (1 g/day) did not seem to reduce plasma TBARS. However, a more recent investigation noted that a higher-dose of vitamin C supplementation (3 g/day) for two weeks prior to the exercise helped reduce oxidative stress as indicated by protein carbonyls in the blood (Bryer & Goldfarb, 2001). Perhaps the aqueous phase components were protected by the vitamin C. These results suggest some protective effects of vitamin C supplementation on aqueous soluble molecules can occur. However, it is questionable if vitamin C alone can protect other aqueous substances.

There are relatively few studies that have examined the effect of combined antioxidant supplementation and exercise induced oxidative stress. Rokitski et al. (1994) did not find any changes in plasma TBARS following aerobic exercise, utilizing a combination of vitamin C and E supplementation for four and a half weeks. The use of TBARS is an indirect and rather insensitive marker for lipid peroxidation. However, Kanter et al. (1993) reported a decrease in both resting plasma MDA and pentane following six weeks of combined vitamin C, E, and beta-carotene supplementation. Another study found that the use of a combined vitamin C, E and beta-carotene supplementation diet attenuated the glutathione changes following 60% VO_2 max aerobic exercise and CK (Viguie et al., 1989). However, they did not measure any other oxidative stress markers. Little information is currently available on the protective effect of dietary

antioxidant pretreatment in skeletal muscle (You et al., 2005). These authors recently reported that the combined pretreatment of vitamin C, E and selenium could protect only certain muscles from an increase in oxidation of proteins.

As previously indicated, there is no information concerning the combined antioxidant treatment on the heart with exercise induced oxidative stress. Previous studies using aerobic exercise have only used vitamin E. Goldfarb (1996) and Kumar (1992) both showed a reduction in oxidative stress in myocardium using vitamin E. Therefore, it was hypothesized that the combination of both vitamin E and vitamin C in the diet may act to protect the heart to a greater extent than vitamin E alone from exercise induced oxidative stress. This may protect the heart from decreased function due to the effects of oxidative stress.

Problem Statement

The heart is the most oxidative muscle in the body, extracting approximately 80% of oxygen from the blood (Di Meo et al., 2001). During exercise, blood flow to the myocardium is increased to meet oxygen demands. This condition could lead to exercise-induced oxidative stress in the heart. Although combined antioxidant supplementation has been shown to have protective effects in skeletal muscle and blood, it has not been examined in the myocardium.

Purpose of the Study

The purposes of this study were to determine: (1) if acute (90 minute duration) downhill running compared with no exercise could induce oxidative stress in the heart during the forty-eight hour period following exercise in male Sprague-Dawley rats; and

(2) if two weeks of combined antioxidant supplementation (vitamin E and C) could diminish the amount of oxidative stress in the heart during the forty-eight hour period following acute downhill running compared to a normal diet.

Hypotheses

1. Downhill running will result in oxidative stress in the heart as measured by MDA.
2. Downhill running will result in oxidative stress in the heart as measured by protein carbonyls.
3. Antioxidant supplementation will reduce the extent of oxidative stress, as indicated by MDA in the heart, compared to standard diet.
4. Antioxidant supplementation will reduce the extent of oxidative stress as indicated by protein carbonyls in the heart, compared to standard diet.

Limitations

1. Some rats might be better runners than others.
2. Not all rats ran the entire time. If they did not run at least 90 minutes of the run they were not included in the results section.
3. Food intake for each rat was matched for cage but not for each individual rat.
4. Antioxidant (vitamin C + E) supplements for each rat were not determined. The amount of antioxidant intake for each rat was estimated at 60 mg vitamin C and 30 IU vitamin E/day based on the food intake.
5. Not all of the food given was eaten by the rats, since some part of the food fell into the cage.

6. Only ventricular tissue was examined so this may not indicate all of the heart's changes.

Delimitations

1. Sixty-six apparently healthy male Sprague-Dawley rats approximately 6 weeks of age were chosen for this experiment.
2. The rats in the control group ate a standard diet, while the rats in the supplementation group ate the same diet with vitamin C + E supplemented (2,000 mg vitamin C and 1,000 IU of vitamin E/kg diet).
3. All rats were exercised for 90 minutes (-16 degrees) at 16 m/min between the hours of 07:00 and 10:00 and sacrificed at the designated times to avoid diurnal variations (Armstrong et al., 1983).
4. Each day only two rats in each treatment group were sacrificed.
5. Only partial ventricles from the heart tissue were examined within each homogenate.
6. Samples were taken immediately after exercise, two hours post-exercise, and 48 hours post-exercise.

Significance of the Study

The significance of this study was to determine if a combined antioxidant (vitamin C and E) supplementation could attenuate exercise induced oxidative stress in the ventricles from rats. This study determined if several markers of oxidative

stress are influenced by combined antioxidant supplementation in response to a 90-minute downhill run, compared to standard diet. If a protective effect was found, then it may be assumed that the heart can be protected from exercise-induced oxidative stress through antioxidant supplementation. This could help to ensure proper heart function.

Definition of Terms

1. Oxidative Stress - involves the state that when cellular production of oxidants overwhelms its antioxidant macromolecules and thiols, lipids, protein, and DNA can demonstrate oxidation.
2. Reactive Oxygen Species (ROS) - are defined as by-products of cell metabolism by successive electron additions to oxygen and include singlet oxygen ($O^{\cdot-}$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}).
3. Free Radical - is defined as a highly reactive molecule with unpaired electrons that seek to accept an electron from another molecule.
4. Antioxidants - are defined as the substances that help to reduce the severity of the oxidative stress by donating an electron to a free radical to form a lesser active radical or to quench the reaction.
5. Glutathione - is defined as an endogenous water-soluble antioxidant which is a tripeptide containing a thiol group that can donate hydrogen ions to free radicals and reduce oxidative stress.

6. Malondialdehyde - is defined as a stable end product of the radical-lipid chain reaction and is used as a marker of lipid peroxidation.
7. Protein Carbonyls - are defined as the products of metal-catalyzed oxidation of amino acid residues of proteins and are used as a marker of protein oxidation.

CHAPTER II

REVIEW OF LITERATURE

Introduction

The antioxidant capacity of the cell is important in maintaining proper cell function, so that cellular balance is maintained with regard to oxidants and prooxidants do not damage the cell. When the antioxidant capacity cannot match the production of prooxidants such as reactive oxygen species (ROS), a condition known as oxidative stress occurs. ROS production increases when oxidative metabolism increases. Aerobic exercise increases oxidative usage through the mitochondria and a small portion of the oxygen leaks out of the mitochondria (2-5%); therefore, increasing the amount of ROS produced in proportion to the oxygen consumption which is based on the intensity and duration of aerobic exercise. The increase in the build up of ROS or by products with cell substances in response to exercise produces a state termed exercise-induced oxidative stress. Previous studies have reported increases in ROS or molecules related to ROS as a result of exercise in muscle (Jackson, 1998; Hanninen et al., 1998). This is in part related to the increased blood flow and oxygen consumption of the muscle. In contrast, the heart is a highly oxidative tissue, where the increase in oxygen utilization is almost exclusively dependent on blood flow. The increased blood flow through the cardiac muscle may only be 3-4 fold compared to skeletal muscle which can increase 20-100 fold. However, there

is some evidence that cardiac muscle may be susceptible to exercise-induced oxidative stress. Previous studies have reported that exercise can induce an increase in the markers of oxidative stress within the heart tissue of rats (Goldfarb et al., 1996; Kumar et al., 1992). Combined antioxidant supplementation has been shown to attenuate oxidative stress in skeletal muscle (You et al., 2005). It results in the decrease of exercise-induced oxidative stress. The protective effect appears to be dependent on the type and amount of antioxidant given as well as the type of exercise stress. Since antioxidant supplementation has been shown to provide partial protection against exercise-induced oxidative stress in skeletal muscle, it may also help to protect the myocardium. Several studies have examined the protective effect of vitamin E supplementation in the myocardium in response to aerobic exercise (Goldfarb et al., 1996; Kumar et al., 1992). These studies reported that vitamin E supplementation can partially protect the heart from exercise-induced oxidative stress. It is unclear if the combination of vitamin E and vitamin C would give additional protection to the heart.

Oxidative Stress

Prooxidants are reactive intermediates that organisms are typically exposed to and there is an antioxidant defense that tends to protect the organism from these prooxidants (Bonifacic and Asmus, 1984). When the antioxidant capacity is unable to match the production of ROS, there is a build up of ROS and their subsequent by-products. This state is referred to as oxidative stress. Exercise-induced oxidative stress occurs when the exercise has created a condition that produces ROS and their by-products that cannot be adequately handled by the antioxidant defense of the system measured. Under this

condition, damage may occur to cellular macromolecules such as lipids, proteins, carbohydrates, and DNA. Oxidative stress may influence the normal state of the system and has been implicated in several disease processes as well as in the aging process (Jackson, 1994). During muscular damage there is an increase in ROS production through the repair process and by activation of proteases (Jackson, 1998). The increase in the ROS from macrophages and neutrophils (neutrophil burst activity) in the repair helps degrade injured tissue.

Reactive oxygen species

Free radicals are highly reactive molecules that have an unpaired electron. These molecules seek out an electron from another molecule to increase stability. If a molecule donates an electron or electrons, it will then become a reactive molecule until its unpaired electron is joined by another electron from a different molecule or a hydrogen ion. This chain reaction of electron giving can continue until a termination reaction occurs such as when a hydrogen ion is donated to the molecule by a reducing agent. An oxygen free radical is a molecule in which the unpaired electron(s) exists in the outer orbit of molecular oxygen or an oxygen centered molecule (Halliwell, 1998).

Oxygen free radicals or other oxygen-derived molecules that are easily converted to oxygen free radicals are known as ROS (Radak, 2000). ROS are important in cell signaling and in breaking down cell structures (Bonifacic and Asmus, 1984). The most notable ROS are singlet oxygen (^1O), superoxide radical (O_2^-), hydroxyl radical (HO^\bullet), peroxyxynitrate (OONO^\bullet) and hydrogen peroxide (H_2O_2). The two most reactive oxygen species are the superoxide and hydroxyl radicals. Although it is not really viewed as an

oxygen free radical, hydrogen peroxide can be quickly converted to reactive oxygen molecules (Bonifacic and Asmus, 1984).

Multiple sources, both primary and secondary, have been implicated in the production of ROS or reactive nitrogen species (RON). One of the primary sources for the formation of ROS during oxidative metabolism is the processing of oxygen in the mitochondria. During metabolism, oxygen undergoes a series of one-electron reductions in the mitochondrial respiratory chain for substrate metabolism and ATP production. It has been estimated that 2-5% of the total oxygen passing through the electron transport chain can escape to form oxygen radicals, specifically the superoxide radical (Sen and Hanninen, 1994). Sjodin et al. (1990) proposed that coenzyme Q was the site for this apparent loss of singlet oxygen. The amount of ROS produced during aerobic exercise is believed to increase due to the increased oxygen flux through the mitochondria during aerobic exercise. The heart is the most oxidative muscle tissue in the body. Typically, the heart increases its oxygen utilization by increasing blood flow (DiMeo et al., 2001). This is different than skeletal muscle, which increases both blood flow and oxygen extraction. This increase in blood flow leads to an increase in the amount of oxygen metabolism in the mitochondria of the heart. This increase in blood flow to the heart appears to increase oxygen radical formation as shown with exercise (Goldfarb et al., 1996; Kumar et al., 1992).

Ischemic- reperfusion and nitric oxide have been shown to increase the production of ROS and RONS and markers of oxidative-stress. The increase in the blood flow to the heart and activation of vasodilator molecules like nitric oxide may also

contribute to the production of reactive oxygen centered molecules during strenuous exercise. Xanthine oxidase has been implicated as another primary source of ROS (McCord, 1985). The formation of xanthine oxidase begins a chain reaction of activities that eventually leads to the formation of xanthine (uric acid) and the superoxide radical. Prostanoids, which can also increase in the circulation during exercise and in response to inflammation injury, are also thought to be another factor that might increase ROS (Halliwell, 1998). It has been further proposed that neutrophils and other inflammatory cell types, and NAD(P)H oxidase may produce ROS (Babior, 1984).

Jackson (1998) has proposed a number of secondary sources of ROS that include phagocytic white cells, muscle calcium accumulation, and disruption of iron-containing proteins. The free iron released from iron-containing tissues such as hemoglobin and myoglobin may stimulate ROS production. ROS can be produced after acute muscle damage due to the accumulation of macrophages and other phagocytic cells in muscle fibers (Jackson, 1998). The calcium-mediated protease mechanism has been implicated in the breakdown of proteins and stimulation of ROS in ischemic tissue. DiMeo et al. (2001) have proposed an increase in ROS production with the ischemic-reperfusion process. This may be important when considering that increased blood flow to the myocardium is transiently altered during the cardiac cycle and could mimic the reperfusion injury model and lead to ROS production (Goldfarb et al., 1996; Kumar et al., 1992).

Exercise and oxidative stress

ROS can be generated through different pathways and from different forms of exercise. The balance between ROS production and the capacity of the antioxidant

defense determines the extent of oxidative stress within the system. Jackson (1994) suggested that the title of exercise is an umbrella term. Since there are different forms of exercise, exercise induced oxidative stress must be considered based on the type of exercise performed.

During aerobic exercise, there is an increased need for ATP production in the muscle cells. This production occurs through the reduction of molecular oxygen in the mitochondria. With increased aerobic metabolism comes an increase in the electron flux through the mitochondrial electron transport chain. The increased oxygen metabolism can increase the incidence of oxygen radical formation through the mitochondrial respiratory chain. In addition, if the exercise has a high mechanical impact, there may be destruction of erythrocytes, which could release iron into the circulation. Myoglobin may also be released into the circulation if there is damage to skeletal muscle. Hydroxyl radical production can be stimulated by these free iron ions by catalyzing the “Fenton reaction” (Halliwell et al., 1989).

It has been reported that acute aerobic exercise of sufficient intensity and duration can induce oxidative stress (Goldfarb et al., 1994; Bejma et al., 1999; Liu et al., 2000). Goldfarb et al. (1994) showed increased thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LH) in plasma and TBARS in red slow-twitch and white fast-twitch muscles from rats following one-hour treadmill exercise. Malondialdehyde (MDA) an end product of lipid peroxidation was shown to increase in the mitochondria from rat liver in response to exhaustive treadmill running in rats (Liu et al., 2000).

Anaerobic exercise of high intensity and short duration has also been reported to generate ROS (Bloomer et al., 2005). Jackson (1998) suggested that the process of transient ischemia during anaerobic muscle contraction might lead to the formation of xanthine oxidase and AMP. These substances can then be further metabolized into hypoxanthine. This is important because hypoxanthine and molecular oxygen can react and form xanthine and the superoxide radical. It has been reported that hypoxanthine can be released into the blood after ischemic exercise (Sutton et al., 1980). Therefore, superoxide radical formation can occur during and after anaerobic exercise in the presence of ischemia. It has also been shown that xanthine oxidase activity increases with acute anaerobic exercise in the blood (Sutton et al., 1980). Therefore, there are a number of processes that can stimulate the production of ROS and RON in response to exercise.

Eccentric exercise and oxidative stress

Mechanical trauma to the muscle and connective tissue has been shown to occur as a result of eccentric muscle contraction (Armstrong et al., 1983). Other symptoms that can result from eccentric exercise are loss of muscle strength, decreased range of motion, muscle soreness, micro-fiber insult, and a progressive secondary inflammatory response within the muscle.

Neutrophils, macrophages and other phagocytic cells may accumulate within the muscle in response to the inflammation caused by eccentric muscle contraction (Jackson, 1994). During the phagocytosis of tissue debris, these cells release both elastase and superoxide radicals, both of which can lead to the degradation of injured tissues (Tidball, 1995). However, this increase in radical production will occur regardless of the

mechanism of muscle damage. A second proposed source of ROS production during eccentric exercise is calcium imbalance. Following eccentric muscle contraction, there can be a failure to maintain calcium homeostasis. This will lead to the activation of calcium-dependent protease and phospholipase A₂, which can degrade the myofibrils and muscle membrane (Armstrong, 1990). ATP production in the mitochondria can also be influenced by an accumulation of free intracellular calcium (Kuipers, 1994). These processes will lead to an increase in the generation of the superoxide radical and hydrogen peroxide.

Armstrong et al. (1983) reported that downhill running results in muscle damage. This is due to the fact that downhill running requires large amounts of eccentric contraction from the muscles involved as well as oxygen delivery to these muscles. This, combined with evidence that downhill running can result in exercise induced oxidative stress (Cannon et al., 1990), shows that two potential components of downhill running can induce increases in ROS.

Exercise induced oxidative stress in the myocardium

The heart is a highly oxidative organ, extracting approximately 80% of the oxygen carried to it in the blood. This elevated rate of oxygen consumption (compared to skeletal muscle) favors a constant production of ROS due to the electron flux through the mitochondrial electron transport chain. Also, due to this high oxidative capacity, the increased energy demands of exercise must be met by an increase in blood flow to the myocardium. Myocardial blood flow and oxygen consumption increases 4-5 fold during maximal exercise (DiMeo et al., 2001). This increased blood flow and oxygen

consumption further increases the electron flux through the mitochondrial electron transport chain and is a potential site for exercise induced oxidative stress observed in the myocardium.

It has also been suggested that myocardium may be susceptible to exercise induced oxidative stress due to a moderate level of antioxidant enzymes when compared to other aerobic tissues (Ji, 1995). The heart antioxidant capacity is typically higher than that of aerobic skeletal muscle but lower than liver. This higher antioxidant capacity is necessary due to the constant aerobic demands of the heart. Although the heart has higher activities of all antioxidant enzymes when compared to skeletal muscle, these activities are substantially lower than in the liver. Ji (1995) suggested that considering the high rates of oxygen consumption and ROS production, the antioxidant capacity of the myocardium is limited compared to other tissues.

Although there has not been a lot of research on oxidative stress in the myocardium, there have been some studies that suggest exercise induced oxidative stress does occur in the heart. Kumar et al. (1992) found that myocardial MDA levels increased after 60 days of exhaustive swimming in rats. Lipid peroxidation, as measured by TBARS, was also found in the myocardium of rats after an acute bout of exhaustive exercise (Goldfarb et al., 1996). These results suggest that lipid peroxidation occurred in the myocardium as a result of exercise. Leeuwenburgh et al. (1996) also reported decreases in GSH levels in the myocardium following a swim to exhaustion. These studies suggest that it is possible to induce oxidative stress in the myocardium through exercise and that there is need for further research in the area.

Indicators of Oxidative Stress

There are several ways in which the presence of oxidative stress can be determined. One way is through the measurement of the activity of antioxidant enzymes. These enzymes can be tested for and levels measured to determine if oxidative stress has occurred. It has been suggested that an increase in the activity of the antioxidant enzymes is a response to the increase in ROS. Similarly, levels of antioxidant vitamins can be measured to determine the presence of oxidative stress. The amount of biomarkers that have been modified by reactive oxygen species can also be measured to determine oxidative stress. These biomarkers are modified lipids, proteins, DNA and carbohydrates as well as alterations in the reduced form of thiols such as glutathione (GSH) to its oxidized form (GSSG).

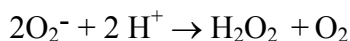
When polyunsaturated fatty acids in the cell membrane react with ROS, peroxy radicals are formed. These radicals initiate a chain reaction in the presence of oxygen. This chain reaction is called lipid peroxidation (Haramaki et al., 1994). This reaction can continue down the length of the cell membrane until a chain-terminating reaction occurs. Gerard-Monnier et al. (1997) recently developed an assay to determine if lipid peroxidation has occurred. This method measures a stable carbocyanine dye that is produced through the reaction of malondialdehyde (MDA) and a chromogenic reagent known as N-methyl-2-phenylindole (NMPI). MDA is one of the stable products of lipid peroxidation and makes up about 20% of the lipid molecules peroxidized. Therefore, MDA increases can be an indication of lipid peroxidation and in turn, oxidative stress.

Proteins are oxidized in the presence of ROS in biological systems. Several amino acid residues in protein are easily modified by ROS in the presence of transition metal ions. This oxidation leads to the formation of protein carbonyl groups (Stadtman and Oliver, 1991). The protein carbonyls assay is possibly the most widely used method for assessment of protein oxidation (Levine et al., 1990; Reznik and Packer 1994). A yellow adduct protein hydrazone that can be detected through a spectrophotometer is produced from the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH). Several studies have reported that protein carbonyls are elevated in response to different forms of exercise (Lee et. al., 2002; Radak, 2000; Bloomer et al., 2006). Protein carbonyls increases have been observed in plasma and skeletal muscle tissue. It was recently reported that eccentric exercise resulted in leakage of creatine kinase, increased muscle soreness and increased levels of protein carbonyls in plasma (Lee et. al., 2002). It is unclear if this increase would occur in the myocardium in response to eccentric exercise. Although it may not be a direct effect since the heart probably does not have eccentric contractions, the ischemic reperfusion model that occurs in skeletal muscle may contribute to the changes in biomarkers of oxidative stress within the circulation. These changes might influence the heart, specifically the possibility of the xanthine oxidase response in the circulation. Ischemic reperfusion studies with the heart have reported increases in markers of oxidative stress. When ischemia occurs there is also an increase in protein carbonyls that are released into the circulation (Goldfarb et al., 2006) in an isolated heart preparation in pigs.

Antioxidants

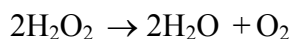
There are two major categories of antioxidants that help prevent or minimize the damage caused by ROS that are classified as the enzymatic and non-enzymatic antioxidants (Goldfarb, 1999). Superoxide dismutase (SOD), catalase (CAT), and the glutathione enzymes, glutathione reductase (GR) and glutathione peroxidase (GPX), are important antioxidants in the enzymatic group. These enzymes help to control ROS and work together to reduce these antioxidants to water or neutral molecules. There are two sub-categories of non-enzymatic antioxidants: water-soluble and lipid-soluble. Vitamin C (ascorbate) and the thiol glutathione (GSH) are the main water-soluble antioxidants. Vitamin E (alpha-tocopherol) and beta-carotene are the main lipid-soluble antioxidants. These antioxidants often work together to help control against ROS interaction with cellular components.

Superoxide dismutase, found in different sites in the cell (cytosolic compartment and mitochondrial matrix of eukaryotes) also varies in activity from tissue to tissue, and catalyzes a reaction with one-electron dismutation of the superoxide radical (Radak, 2000).

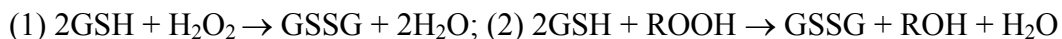


The SOD activity is higher in oxidative muscle tissue compared to muscle that is less aerobic (Jenkins and Goldfarb, 1993). This appears to help protect the cells that are more aerobic. SOD activity in heart is greater than that of aerobic muscle so the cardiac muscle should be well protected.

Catalase, located in the peroxisomes, mitochondria, and endoplasmic reticulum, has the main responsibility of decomposing hydrogen peroxide (Chance et al., 1979). It has been reported that catalase activity can increase in response to both acute and chronic exercise depending on the muscle tissue examined (Alessio et al., 1988).



Glutathione peroxidase works to reduce hydrogen peroxide in conjunction with glutathione reductase (Flohe, 1982). Glutathione peroxidase has both a selenium dependent and selenium independent form. They both help to take hydrogen peroxide to water by using the H^+ from reduced glutathione (GSH). Glutathione reductase helps to reduce the oxidized form of glutathione (GSSG) back to GSH by taking a hydrogen from NAD(P)H .



Vitamin C, found mostly in the cytosol and extracellular fluid, works as a scavenger of superoxide radicals and hydroxyl radicals (Rose et al., 1993). Vitamin C has also been shown to be important in the regeneration of reduced vitamin E (Chan et al., 1993). This is done through the donation of an electron to the vitamin E radical. Another chain of reactions will leave vitamin C in the form of dehydroascorbate, which can then be regenerated to vitamin C through the donation of H^+ from GSH (Winkler, 1994). Vitamin C is a water-soluble antioxidant, thus most of its protection is within the aqueous

phase. Frei et al. (1988) reported in vitro that vitamin C is the first antioxidant to be utilized in the aqueous phase.

Few studies have examined the role of vitamin C in controlling exercise-induced oxidative stress. Alessio et al. (1997) reported that 1 gm/ day for one week had minimal effects on plasma levels of markers of oxidative stress in response to 30 minutes of running at 70% VO_2 max. Kaminski and Boal (1992) reported that 3 gm/day of vitamin C could attenuate the pain associated with eccentric exercise. Recently, Bryer and Goldfarb (2006) reported that this same dose of vitamin C could not only attenuate the delayed soreness to eccentric exercise but could also attenuate the increase in glutathione ratio but did not alter the leakage of proteins out of muscles as indicated by blood creatine kinase. More work is needed to determine if vitamin C can give protective effects to ROS damage of cardiac muscle.

Vitamin E is a lipid-soluble antioxidant; therefore, it is found mostly in membrane structures within the cell, especially the plasma and mitochondrial membranes. Vitamin E is important in the inhibition of lipid peroxidation because it scavenges superoxide radical and hydroxyl radicals in the lipid phase (Liebler, 1993). Because it is lipid-soluble and works to inhibit lipid peroxidation, vitamin E also acts as a membrane stabilizer. Beta-carotene is also a lipid-soluble antioxidant that can be found in the cellular membrane structures and has a main function of quenching singlet oxygen. It has also been implicated in the inhibition of oxygen or carbon-centered free radicals (Liebler, 1993). Many studies investigating vitamin E supplementation and exercise-induced oxidative stress have been published (Goldfarb et al., 1994; Reznick et al., 1992; Sen et

al., 1997; Sumida et al., 1989). Not all studies have shown beneficial effects in part due to the dose of vitamin E, the form of vitamin E given, and the nature of the exercise.

Antioxidant Supplementation

The role of endogenous antioxidants in the cell is very important. They help to reduce the amount of reactive oxygen species and the extent of oxidative stress. There are a number of roles that these antioxidants can play. They include; preventing free radical formation, scavenging reactive oxygen species and converting them to a less active molecule, helping to repair the free radical-induced damage, and assisting other agents to supply reducing power (Goldfarb, 1999). These antioxidants can be supplemented through diet and have been used in a prophylactic manner for exercise induced oxidative stress. Ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), beta-carotene, and ubiquinone are examples of endogenous antioxidants.

In studies dealing with vitamin C supplementation in humans, the doses have varied considerably. Alessio et al. (1997) used acute (1 and 7 days) (1.0 g/day) vitamin C supplementation and did not observe a reduction in exercise induced oxidative stress. Conversely, a higher dose (3.0 g/day) vitamin C supplementation for two weeks was shown to reduce plasma protein carbonyls (Bryer and Goldfarb, 2002). Goldfarb and Patrick (2005) reported that both 500 mg and 1 gm of vitamin C given for two weeks prior to exercise could attenuate the exercise induced oxidative stress as indicated by a reduction in protein carbonyls. The results showed a dose dependent reduction. Interestingly, there was no change in glutathione status with the exercise or the treatment. Muscle soreness and oxidative stress have both been reported to be reduced with high

dose vitamin C supplementation following eccentric exercise (Kaminski et al., 1992; Bryer and Goldfarb, 2006). It seems that high dose vitamin C supplementation can have beneficial effects when dealing with exercise induced oxidative stress. The role of vitamin C supplementation has not been studied in exercise induced oxidative stress in myocardium.

A beneficial effect of vitamin E supplementation on exercise induced oxidative stress seems to be supported by the current literature (Goldfarb, 1999). Exercise induced oxidative stress has been shown to be reduced in both humans and animals as a result of vitamin E supplementation (Sumida et al., 1989; Reznick et al., 1992; Goldfarb et al., 1994; Sen et al., 1997). Conversely, this beneficial effect has not been found in other studies (Boyer et al., 1996; Siciliano et al., 1997). Although it has not been studied extensively, some investigators have shown vitamin E supplementation to have beneficial effects on exercise induced oxidative stress in the myocardium. Goldfarb et al. (1996) reported that vitamin E supplementation (250 IU Vitamin E/kg of diet) reduced exercise induced oxidative stress in rat myocardium as measured by TBARS. Long term (60 days) vitamin E supplementation (220 IU Vitamin E/kg of diet) was shown to decrease MDA levels in rat myocardium following exhaustive exercise (Kumar et al., 1992). These results suggest that vitamin E supplementation may have prophylactic effects within the myocardium.

The effect of combined antioxidant supplementation on exercise induced oxidative stress has been studied in both human and animal subjects. A combination of vitamin C (200 mg) and vitamin E (400 IU) supplementation for four and a half weeks

was shown to reduce the creatine kinase response to a marathon race in humans (Rokitski et al., 1994). However, this same study showed no effect on plasma TBARS. Kanter et al. (1993) showed that six weeks of combined vitamin C, vitamin E, and beta-carotene supplementation decreased resting plasma MDA and pentane, but did not decrease MDA and pentane following 35 minutes of aerobic exercise in humans. Blood glutathione status was improved in male, human subjects after aerobic running following vitamin C (1000 mg/day), vitamin E (800 IU/day), and beta-carotene (10 mg/day) supplementation (Viguie et al., 1989). Vitamin C (1000 mg), vitamin E (600 mg), and beta-carotene (32 mg) supplementation for 32 days showed decreased plasma lipoperoxide concentration and the ratio of liperoxides to total antioxidant status in human subjects (Schroder et al., 2000).

Vitamin E (400 IU/day), beta-carotene (3 mg/day), and lutein (20 mg/day) supplementation for 30 days decreased DNA adducts and increased lag time of lipoprotein oxidation in dogs (Piercy et al., 2000). These results suggest that use of combined antioxidant supplementation may be prudent in protecting against exercise induced oxidative stress. However, the effects of combined supplementation have not been studied in the myocardium. Bloomer et al. (2006) reported that a combined antioxidant supplementation given for 2 weeks prior to a run of 30 minutes at 80% VO_2 max attenuated the increase in protein carbonyls and reduced the increase in glutathione status. You et al. (2005) reported that a combined antioxidant supplement to the diet of rats could reduce the amount of protein carbonyls accumulated within the muscle (both vastus lateralis and soleus) as a result of an exercise run.

Based on the current literature, it can be concluded that antioxidant supplementation may partially protect against exercise induced oxidative stress. The results are inconsistent, which could be attributed to differences in subject, exercise modes, supplementation dosage and form, and the markers of oxidative stress measured. Combination antioxidant supplementation with both vitamin C and vitamin E has not been investigated with a focus on oxidative stress in myocardium. The literature also shows that exercise induced oxidative stress may occur in the myocardium following exhaustive running. Eccentric exercise, particularly downhill running, has been shown to result in exercise induced oxidative stress in skeletal muscle and in blood. Therefore, it seems prudent to examine, in an animal model, if the effects of a combined antioxidant supplementation on exercise induced oxidative stress in the myocardium following aerobic downhill running would be beneficial.

In conclusion, eccentric exercise of sufficient intensity and duration has been reported to increase markers of oxidative stress in plasma and skeletal muscle. It is unclear if this would have the same effect in the myocardium. Additionally, antioxidant pretreatment has been shown to help protect skeletal muscle and blood from this stress. It is unclear if this same pretreatment of vitamin E and C would help protect the heart from this stress. Therefore the purpose of this study is to determine if a combined antioxidant treatment (vitamins C and E) can reduce the effects of oxidative stress in the myocardium as evidenced by the presence of protein carbonyls and MDA.

CHAPTER III

METHOD AND PROCEDURES

This chapter presents the methods and procedures that were used to investigate the changes in myocardial oxidative stress during the 48 hour period following 90-minutes of aerobic downhill running in rats with or without antioxidant supplementation in their diet. The organization of the chapter is as follows: animal selection, testing protocol, analysis of myocardial samples, and statistical analysis.

Animal Selection

Sixty-six healthy male Sprague-Dawley rats were used in this study. They were approximately six weeks old at onset. The rats were purchased from the Harlen-Teklad Company and housed in a temperature (21 ± 1 °C) and humidity ($35 \pm 5\%$) controlled room. This study was approved by the animal care and use committee using the procedures indicated.

Test Protocol

Experimental Design

This experimental design was a 4 (sacrifice time) by 2 (treatment) trial design. Normal diet and antioxidant (vitamins C and E) diet were the two treatment conditions.

The four time points examined were; no exercise (NE), immediately post-exercise (0 PE), two hours post-exercise (2 PE), and 48 hours post-exercise (48 PE).

Animal Housing

All of the rats were used in compliance with the procedures approved by and filed with the UNCG Animal Review committee. The rats were housed two per cage at the UNCG Animal Facility in a temperature (21 ± 1 C°) and humidity ($35 \pm 5\%$) controlled environment. The rats had free access to food and water. Daily records were kept noting food intake and weight for each rat. The animals were housed for one week prior to any special treatment. This one-week period was also used to acclimate the rats to treadmill exercise through the use of one 15-minute running session on a rodent treadmill at a speed of 10-15 m/min and a 0° grade.

Antioxidant Supplementation

The rats were randomly divided into either the normal diet (N) or antioxidant diet (A) treatment group. The normal diet was standard rat chow purchased from the Harlan-Teklad Company. The antioxidant diet was supplemented with 2,000 mg of vitamin C and 1,000 IU of vitamin E/kg diet. These doses were determined to be safe and effective in reducing oxidative stress. This dose is similar to previous rat studies that used both antioxidants separately (Gohil et al., 1986; Reznick et al., 1992; Warren et al., 1992; McIntosh et al., 1993; Goldfarb et al., 1994). A period of two weeks was used as the diet supplementation period. It was computed that the amount of vitamin C taken per day was about 60 mg and 30IU for vitamin E based on 30 mg eaten per day for each rat.

Downhill Running

The rats were divided into a control rested group (C, N=18) and an exercised group (E, N=48). The exercised rats ran for 90 minutes on a rodent treadmill at a speed of 16 m/min and a grade of -16°. Armstrong et al. (1983) reported that this type of exercise resulted in muscle damage in rats. In a study using this same protocol, it was reported that this downhill running resulted in an increase in protein carbonyls in skeletal muscle (You et al., 2005). Eight rats were killed at each time point in both treatment groups.

Sample Collection

The rats were killed by decapitation and the hearts were rapidly removed and quickly frozen between pre-chilled clamps on dry ice (less than 2 minutes from sacrifice). The hearts were placed on dry ice and then stored in an -80° freezer for later analysis of protein carbonyls and MDA.

Biochemical Analysis

Myocardial Protein Carbonyl Concentration

Myocardial protein carbonyl concentration was measured using an ELISA kit from Zentech Company (P072). Ice cold 10 mM PBS, pH 7.4 was used to wash the muscle samples. They were then be blotted on absorbent paper and weighed. The samples were then homogenized at 0-4° C in 2 ml PBS, pH 7.4. The homogenates were centrifuged at 11,000 x g for 10 minutes at 4° C. The supernatants were then analyzed for protein concentration.

A volume of each sample containing 20 ug protein were put into 0.5 ml tubes and brought up to an equal volume with deionised water. Then, 0.8 volumes of ice cold 28%

(w/v) trichloroacetic acid (TCA) were added to each tube, mixed and incubated on ice for 10 minutes. The tubes were then centrifuged at 10,000 rpm for 3 minutes and the supernatant was removed, leaving a pellet of precipitate. 5 ul of EIA Buffer and 15 ul of a diluted dinitrophenylhydrazine (DNP) solution were added to each tube and vortexed thoroughly. Samples were then left to incubate at room temperature for 45 minutes. Then, 1 ml of EIA Buffer and 5 ul of each sampler were added to 1.5 ml tubes and mixed.

In the ELISA procedure, 200 ul of each sample was added to assigned ELISA-plate wells, covered and incubated at 4° C overnight. The plate was then washed with EIA Buffer (5 x approximately 300 ul per well). 200 ul of a diluted anti-DNP-biotin-antibody was added pr well and incubated for 1 hour at 37° C. The plate was then washed with EIA Buffer (5 x approximately 300 ul per well). After this, 200 ul of diluted streptavidin-horseradish-peroxidase (HRP) was added to each well and incubated for 1 hour at room temperature. The plate was washed with EIA Buffer (5 x approximately 300 ul per well). The chromatin reagent (200 ul) was then added to each well and color was allowed to develop for approximately 4-7 minutes. The reaction was stopped with 100 ul of stopping reagent per well. Absorbances were read at 450 nm directly after stopping the reaction in a plate reader. A standard curve was constructed by plotting the nmol/mg protein carbonyl concentration of the standards. Protein carbonyl content of samples (nmol/mg) was calculated from the standard curve by using the regression factors obtained from the standard curve. Protein carbonyl concentrations for each sample were determined in duplicate.

Myocardial Malondialdehyde Concentration

The myocardial malondialdehyde concentration was determined through the use of TBARS assay kit from Caymen Chemical (10009055). An ice cold solution of 10 mM PBS, pH 7.4 containing 4 mM BHT was used to wash the muscle samples. They were blotted dry on absorbent paper and weighed. Then, the samples were homogenized at 0-4° C in 2 ml PBS, pH 7.4, containing 4 mM BHT. The samples were then centrifuged at 11,000 x g for 10 minutes at 4° C. The resulting supernatants were used in the assay.

A volume of 200 ul of each sample was added to appropriately labeled 5 ml screw cap centrifuge tubes. Then, 100 ul of sodium dodecyl sulfate (SDS) solution was added to each vial and mixed. A volume of 4 ml of color reagent was then added forcefully down the side of each tube. Tubes were then capped and boiled in water for one hour. After an hour, the tubes were immediately removed and placed in an ice bath for 10 minutes to stop the reaction. The tubes were then centrifuged for 10 minutes at 1,600 x g at 4° C. After centrifugation, the tubes were placed back in the ice bath. 150 ul from each tube was then loaded into a plate and absorbance was read at 530-540 nm using a plate reader.

A standard curve was constructed using the absorbance values of each standard as a function of MDA concentration. MDA values for each sample were calculated from the standard curve. MDA concentrations for each sample were determined in duplicate.

Statistical Analysis

The statistical analysis for this study was done using SPSS statistical software. The data were analyzed using a multivariate ANOVA examining aerobic downhill running and antioxidant (vitamin C + E) supplementation. When a time main effect was

noted a post-hoc test (Bonferroni) was used to determine time differences if they existed. The run time in the exercised rats between the two treatment groups was compared using a student t-test. Significance was set at the 0.05 level.

CHAPTER IV

RESULTS

This chapter will present the results of this study. It will be organized into 3 sections; animal weight, run time, and biochemical analysis of myocardial samples (protein carbonyls and malondialdehyde).

Animal Weight

The animals were randomly assigned to each of the two treatment groups (Normal Diet or Antioxidant Diet) and then into exercise groups (Not Exercised {NE}, immediately post exercise {0 PE}, 2 hours post exercise {2 PE}, and 48 post exercise {48 PE}). Table 1 shows the initial animal weight (gm, mean \pm SE).

Table 1 Initial animal weight (gm)

Condition	NE	0 PE	2 PE	48 PE
Normal Diet (mean \pm SE)	170.88 \pm 4.62 (n = 9)	169.62 \pm 5.04 (n = 7)	161.59 \pm 5.23 (n = 9)	168.43 \pm 4.92 (n = 8)
Antioxidant Diet (mean \pm SE)	168.89 \pm 6.23 (n = 9)	174.13 \pm 3.94 (n = 7)	166.34 \pm 4.61 (n = 8)	170.11 \pm 5.42 (n = 9)

A statistical analysis was conducted using a 4 x 2 ANOVA. The results showed no significant differences in the initial body weight of the animals.

Table 2 Statistical data on initial body weights of animals

Source	Df	SS	F	Sig.
Time	3	551.9282	0.8411	0.4769
Treatment	1	81.8317	0.3741	0.5432
Time*Treatment	3	126.4520	0.1927	0.9010

Table 3 presents the final animal weight (gm, mean \pm SE) on the day that they were sacrificed. Each animal's weight increased in a normal manner throughout the entire study.

Table 3 Final animal weight (gm)

Condition	NE	0 PE	2 PE	48 PE
Normal Diet (mean \pm SE)	296.22 \pm 6.18 (n = 9)	292.59 \pm 5.84 (n = 7)	286.33 \pm 6.68 (n = 9)	294.80 \pm 5.74 (n = 8)
Antioxidant Diet (mean \pm SE)	297.19 \pm 7.18 (n = 9)	304.79 \pm 5.87 (n = 7)	299.64 \pm 5.94 (n = 8)	295.08 \pm 6.01 (n = 9)

A statistical analysis was conducted using a 4 x 2 ANOVA. The results (Table 4) indicated that there was no significant effect on final animal weight by time, treatment, or time*treatment interaction.

Table 4 Statistical data on final body weights of animals

Source	Df	SS	F	Sig.
Time	3	278.1073	0.2834	0.8372
Treatment	1	731.4063	2.2358	0.1403
Time*Treatment	3	610.9904	0.6226	0.6033

Run Time

In all, 48 rats ran on the treadmill. Of the 48 exercised animals, 38 of them finished the full 90 minutes of downhill running. Six animals finished with a run time between 45 and 90 minutes. Four animals finished with a run time of less than 45 minutes.

Within the normal diet group, 18 animals ran for 90 minutes, 4 ran between 45-90 minutes (these were 83.18 min, 77.63 min, 69.53 min, and 47.05 min respectively), and 2 ran less than 45 minutes. Within the antioxidant diet group, 20 animals ran for 90 minutes, 2 ran between 45-90 minutes (78.45 min and 67.97 min), and 2 ran for less than 45 minutes. None of the animals that ran for less than 90 minutes were included in the chemical data analysis.

Two animals from each group were injured on the tail or foot during the run and were not able to finish at least half of the downhill run. These animals were not included in run time and biochemical analysis since the injuries might have an influence on treadmill running and the extent of exercise-induced oxidative stress.

Table 5 presents the run time of the exercised animals (min, mean \pm SE).

Table 5 Animal run time using only non injured animals (min)

Condition	Running Time
Normal Diet (mean \pm SE)	86.25 \pm 2.16 (n = 22)
Antioxidant Diet (mean \pm SE)	88.47 \pm 1.11 (n = 22)

A statistical analysis was conducted using a student t-test. The results indicated that there was no significant difference in run time between treatment groups.

Table 6 T-test for run time comparing the groups

Source	Df	Difference	t	Sig.
Running Time	42	2.2286	0.9184	0.3637

Protein Carbonyl Concentration

Protein carbonyl (PC) concentration (nmol/mg protein, mean \pm SE) is presented in Table 7 for each group at each time point. A statistical analysis was performed using a multivariate ANOVA (SPSS). Only animals that ran the prescribed 90 minutes were included in the analysis to avoid difference effects of exercise duration on this variable. The results indicate that there was no significant treatment or time*treatment interaction

effect on PC concentration. However, there was a significant time main effect independent of treatment. Significance was reached at a 0.020 level. The post hoc test indicated no significant individual differences although the 2 hour post exercise time approached significance ($p=0.064$).

Table 7 Protein Carbonyl concentration (nmol/mg protein)

Condition	NE	0 PE	2 PE	48 PE
Normal Diet (mean \pm SE)	0.083 \pm 0.032 (n = 8)	0.273 \pm 0.158 (n = 4)	0.216 \pm 0.088 (n = 7)	0.285 \pm 0.108 (n = 8)
Antioxidant Diet (mean \pm SE)	0.112 \pm 0.039 (n = 9)	0.466 \pm 0.190 (n = 7)	0.913 \pm 0.457 (n = 5)	0.425 \pm 0.161 (n = 8)

Table 8 Statistical data on protein carbonyl concentration

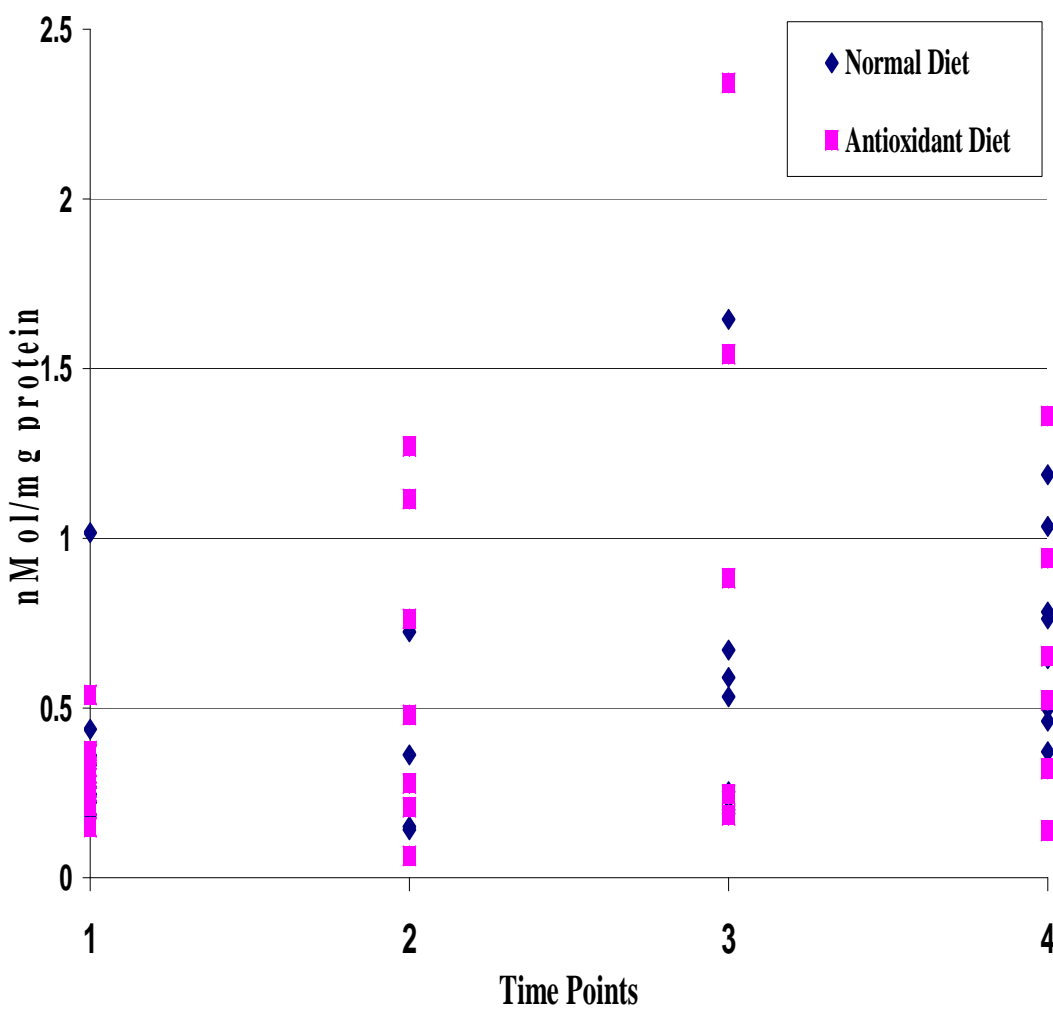
Source	Df	SS	F	Sig.
Time	3	1.596	3.584	0.020*
Treatment	1	0.419	2.820	0.100
Time*Treatment	3	1.072	2.408	0.079

*significant difference independent of treatment, $p \leq 0.05$

Figure 1 presents a scatter graph for myocardial PC concentration in response to exercise and diet. Sacrifice time points are represented on the x axis: 1- Rest, 2- 0PE, 3- 2 hr PE, 4- 48 hr PE. This shows the increased variation occurred in both groups with PC at the different times after exercise.

Figure 1.

Myocardial PC in Response to Exercise and Diet



Malondialdehyde Concentration

Malondialdehyde (MDA) concentration (nmol/mg protein, mean \pm SE) is presented in Table 9 for each group at each time point. A statistical analysis was conducted using a multivariate ANOVA. The results indicated that there was no significant time, treatment, or time*treatment interaction effect on MDA concentration.

Table 9 Malondialdehyde concentration (umol/gm)

Condition	NE	0 PE	2 PE	48 PE
Normal Diet (mean \pm SE)	0.445 \pm 0.023 (n = 8)	0.447 \pm 0.017 (n = 7)	0.447 \pm 0.023 (n = 9)	0.414 \pm 0.009 (n = 8)
Antioxidant Diet (mean \pm SE)	0.455 \pm 0.021 (n = 9)	0.445 \pm 0.012 (n = 7)	0.430 \pm 0.015 (n = 8)	0.419 \pm 0.012 (n = 9)

Table 10 Statistical data on malondialdehyde concentration

Source	Df	SS	F	Sig.
Time	3	0.011	1.389	0.255
Treatment	1	0.000	0.009	0.925
Time*Treatment	3	0.002	0.228	0.876

CHAPTER V

DISCUSSION AND CONCLUSIONS

The present study was designed to determine whether antioxidant supplementation influences oxidative stress in the myocardium of male rats in response to a 90-minute aerobic run. This chapter will discuss the results of the study and suggest conclusions and future directions for research. The chapter will be organized as follows: running and oxidative stress markers and antioxidant supplementation and oxidative stress markers.

Prolonged Running and Oxidative Stress Markers

In the present study, myocardial muscle tissue was analyzed. Based on the results from both the MDA and PC in the present study, the hypothesis that 90-minutes of aerobic downhill running would induce oxidative stress in the myocardium was partly accepted since a significant time effect was found in PC but not in MDA.

MDA levels in the myocardium did not change significantly at any of the time points. This finding is in contrast to results from Kumar et al. (1992) and Goldfarb et al. (1996). Kumar et al. (1992) reported that myocardial MDA levels increased after exhaustive swimming exercise in rats. Goldfarb et al. (1996) reported lipid peroxidation, as measured by TBARS, in the myocardium of rats after an acute run to exhaustion. Both of these studies exercised their rats to exhaustion. Goldfarb et al. (1996) exercised the rats with 1 hour of running on a motorized rodent treadmill at 21 m/min up a 12% grade. The

present study used 90 minutes of downhill running at 16 m/min on a -16% grade. The intensity of exercise used in the present study was much less and most likely was not high enough to elicit significant oxidative stress in the myocardium. It is possible that higher intensity downhill running could increase myocardial MDA during the post exercise time points. The reason for the downhill run was that this downhill run resulted in muscle damage within skeletal muscle as reported by You et al. (2005). Future research may be needed to determine if higher intensity exercise can increase myocardial MDA levels. It would also be of importance to note if exercise that does not result in exhaustion can result in an increase in myocardial markers of oxidative stress.

PC levels in the myocardium did reach significance for a time main effect ($p = 0.02$) independent of treatment. The PC values immediately after and 2 hours after the run were highest in the antioxidant diet group, although they were not significantly different than those of the normal diet group. It is possible that with a larger sample size, the levels of PC immediately after and 2 hours after running might reach significance. In a recent study, You et al. (2005) reported a significant increase 2 hours after exercise in certain skeletal muscles in these animals with this exercise protocol. It also should be pointed out that these authors did not report any significant changes in MDA within the blood or in the muscles with this exercise.

Antioxidant Supplementation and Oxidative Stress Markers

Although two studies have reported vitamin E supplementation can attenuate myocardial oxidative stress in rats (Kumar et al., 1992 and Goldfarb et al., 1996), this is the first study to investigate the effects of a combined (vitamin E + vitamin C)

antioxidant supplementation following eccentric exercise. Based on the results of MDA and PC, the hypothesis that antioxidant supplementation would reduce the extent of oxidative stress in the myocardium was rejected.

Antioxidant supplementation did not demonstrate an effect on MDA in myocardium either at rest or following a 90-minute downhill run. Therefore, the antioxidant supplementation for this length of time in these animals did not alter the extent of lipid peroxidation in the myocardium. It is possible that a more intense level of exercise may have increased the MDA level and the effect of antioxidant supplementation could have been beneficial. Two previous studies employing exhaustive exercise have shown increases in myocardial MDA in response to exercise and an attenuation of this lipid peroxidation marker in response to vitamin E supplementation (Kumar et al., 1992 and Goldfarb et al., 1996). The difference between the present study and the other two studies are type of exercise and antioxidant treatment. It is possible that the combined vitamin C and vitamin E treatment acted as pro-oxidants. Vitamin C at high levels has been shown to act as a prooxidant (Paolini et al. 1999).

In the present study, antioxidant supplementation did not show a significant effect on PC in myocardium either in the non-exercised heart condition or following 90 minutes of running. Although PC appeared to increase immediately after and 2 hours after exercise in the antioxidant group, this did not reach statistical significance. The change of PC to about 7.5 fold higher at the 2 hour post exercise time ($p= 0.064$) compared to the rested animals is greater than the increases reported by You et al. (2005) in the plasma and vastus lateralis at this time point. There was a large variability in the PC values

which contributed to this lack of significance. Thus, the small sample size used in the study and the variability between the animals probably helped contribute to the lack of significance. It is possible that a larger sample size could have demonstrated an antioxidant supplementation effect on protein oxidation to this downhill running.

The data also suggests that the antioxidant treatment in these animals may have exacerbated the effect of the exercise on heart oxidative stress. The hearts taken after the exercise tended to have a greater PC but this did not reach significance ($p=0.064$). Independent of the time the values were similar from immediately after, 2 hr and 48 hrs after exercise. In contrast, the antioxidant treated animal's PC values appeared to be higher at each time point but again due to a small sample size and large variation, did not reach significance. This suggests that the combined treatment with vitamin E and vitamin C may actually have promoted oxidative stress at the heart level. This is in contrast to the response observed by You et al. (2005) in the blood and skeletal muscle which reported an attenuated oxidative response with this treatment. It is also in contrast to the report that vitamin E alone could help attenuate exercise induced lipid peroxidation (Kumar et al. 1992, Goldfarb et al. 1996).

It is unclear why the heart responded to the antioxidant treatment differently than the skeletal muscles examined by You et al. (2005) in these same animals. This may be related to the vitamin C in the antioxidant supplement diet as vitamin C has been reported to be a pro-oxidant in certain conditions (Paolini et al. 1999). In fact, Neiman et al. (2002) reported that high dose vitamin C had no effect on oxidative stress markers and inflammatory markers in response to an ultramarathon in humans. However, this same

group reported that this same high dose vitamin C could attenuate cortisol levels in response to an ultramarathon (Peters et al., 2001). Paolini et al. (1999) reported that high dose vitamin C (500mg/kg b.w. for 4 days) could generate large amounts of the superoxide radical in rat hearts. It has also been reported that high dose vitamin C in humans can increase oxidative stress given after eccentric exercise (Childs et al., 2001). It is possible that the present dose of vitamin C (~ 60 mg day/rat) coupled with the vitamin C naturally produced by rats, was sufficient to promote oxidative stress within the myocardium. This may explain why the present study reported a tendency to increase PC at the 2 hour post exercise time. Clearly greater numbers of animals are needed to increase the power to substantiate these preliminary findings.

Summary

The present study demonstrated that 90 minutes of downhill running did not induce significant increases in oxidative stress in the myocardium of male rats as measured by MDA. PC levels did reach the significant level ($p = 0.02$) over time, independent of treatment. Additionally, two weeks of vitamin E (1,000 IU vitamin E/kg diet) and vitamin C (2,000 mg/kg diet) pretreatment did not significantly influence MDA levels at rest, nor following the running. The PC data suggests that immediately after and 2 hours after running the combined antioxidant supplementation was not beneficial and may have actually promoted oxidative stress but this needs to be confirmed with a larger sample size.

Recommendations

There are some recommendations for future researchers to have a better understanding of the effect of combined antioxidant supplementation on myocardial oxidative stress in response to eccentric exercise. The recommendations are as follows.

- 1) Determine if eccentric exercise at a higher intensity can result in oxidative stress in the myocardium.
- 2) Determine if antioxidant supplementation can reduce oxidative stress in myocardium of trained individuals. Trained individuals typically upregulate their defense system in skeletal muscle. It is unclear if this pattern of oxidative stress in response to exercise is upregulated in the heart.
- 3) Examine other markers of oxidative stress such as glutathione ratio. Glutathione may be a more sensitive and therefore more accurate marker of oxidative stress than MDA.
- 4) Examine if a larger sample size might show significance between the control group and the antioxidant diet group as measured by PC.
- 5) Examine other combinations of antioxidants to determine if they are beneficial in preventing exercise induced oxidative stress within the heart.

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