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The effect of vitamin E and iron supplementation on free radical production in response to exercise

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THE EFFECT OF VITAMIN E AND IRON SUPPLEMENTATION ON FREE RADICAL PRODUCTION IN RESPONSE TO EXERCISE

by

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ABSTRACT

Twenty-five untrained female volunteers were exercised at 70% mVO2 to examine the effect of vitamin supplementation on free radical production during submaximal exercise. Subjects were randomly assigned to a vitamin E (400 IU d-alpha-tocopherol daily), vitamin E plus iron (400 IU d-alpha-tocopherol and 325 mg ferrous sulfate daily), or a control group (no supplement). Urinary malondialdehyde (MDA) levels were measured at rest before and after 3 months of supplementation and following 30 min of submaximal exercise after the 3-month period of daily vitamin supplementation. A 3 x 3 (Group x Time) repeated measures ANOVA revealed a significant three-way interaction in urinary MDA values ($p < .001$). Post-hoc 2 x 3 analysis determined this difference was evident between the control and supplement groups ($p < .001$), but no difference existed between the two supplementation groups ($p > .05$). Within groups, urinary MDA levels rose significantly after 30 min of exercise at 70% mVO2 in controls ($p < .05$), while these same values decreased significantly after exercise in both supplement groups ($p < .05$). A nonsignificant correlation existed between postexercise MDA and lactate levels ($r = -.294$), suggesting no substantial linear relationship exists between lactate and MDA production. These results indicate that vitamin E supplementation not only attenuates the rise in MDA associated with exercise but also may reverse this exercise effect. Furthermore, it seems that

supplementation with low doses of iron does not alter the influence of vitamin E upon free radical activity.

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A Thesis Presented to the Faculty of
the Division of Health, Physical
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Requirements for the Degree
Master of Science

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by
James C. Baldi
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Ithaca College

Division of Health, Physical Education, and Recreation

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CERTIFICATE OF APPROVAL

MASTER OF SCIENCE THESIS

This is to certify that the Master of Science Thesis of

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submitted in partial fulfillment of the requirements for the degree of Master of Science in the Division of Health, Physical Education, and Recreation at Ithaca College has been approved.

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DEDICATION

This thesis is dedicated to my family;

I have always drawn on your example

and support in pursuit of my goals.

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Chapter 1

INTRODUCTION

A free radical is any atom, group of atoms, or molecule with at least one unpaired electron in the outer orbital (Del Maestro, 1980). Free radicals of oxygen are normal products of aerobic respiration produced in very small concentrations during the reduction of oxygen in eukaryotic cells. It was recently determined that oxygen utilized at ambient concentrations may cause cellular damage in aerobic organisms by generating oxygen free radicals (Gutteridge, Rowley, Halliwell, Cooper, & Heeley, 1985).

Increases in free radicals can initiate the process of lipid peroxidation (LP) in which radicals attack the polyunsaturated portion of cell membranes. This attack initiates a chain reaction of membrane damage and often alters membrane permeability (Del Maestro, 1980). Free radical attacks can be very damaging to aerobic organisms; in fact, LP has repeatedly been associated with aging, cancer, diabetes, and many other disorders (Halliwell & Gutteridge, 1984; Jenkins, 1988).

Exercise has many positive attributes, but it may also lead to increased oxidative stress. Increased metabolism is repeatedly associated with increased levels of free radicals and LP indicators. In animals and humans, maximal (Davies, Quintanilha, Brooks, & Packer, 1982; Lovlin, Cottle, Pyke, Kavanagh, & Belcastro, 1987; Sumida, Tanaka, Kitao, & Nakadomo,

1989) and prolonged submaximal exercise (Balke, Snider, & Bull, 1984; Dillard, Litov, Savin, Dumelin, & Tappel, 1978) are known to elicit an increase in free radical and LP indicators. Exercise has also altered the concentration of enzymatic antioxidants that serve as free radical defense mechanisms in the body (Ohno, Sata, Yamashita, Doi, Arai, Kondo, & Taniguchi, 1986; Sumida et al., 1989), providing further evidence that certain types of exercise promote the production of free radicals.

Vitamin E (alpha-tocopherol) is one of many defense mechanisms protecting aerobic organisms from LP. Early studies indicated that vitamin E deficiency was associated with increased LP (Davies et al., 1982; McCay, Gibson, Fong, & Hornbrook, 1976; Noguchi, Cantor, & Scott, 1973; Whanger, Weswig, Schmitz, & Oldfield, 1977). However, later experiments confirmed that exercise-induced increases in free radical production were, in fact, attenuated by vitamin E supplementation (Brady, Brady, & Ullrey, 1979; Dillard et al., 1978; Sumida et al., 1989).

Iron is another factor that is important in free radical metabolism in that it promotes the liberation of reactive free radical species. Accordingly, researchers have speculated that iron overload may induce LP. Indeed, studies in humans with medical conditions related to gross iron overload demonstrated increased LP in erythrocytes (Rachmilewitz, Shohet, & Lubin, 1976) and the spleen (Heys & Dormandy, 1981). Another study found that pentane production (a LP indicator) increased dramatically over 7 to 8 weeks of intraperitoneally injected

iron in rats (Dillard, Downey, & Tappel, 1984).

The purposes of this study are first, to determine whether supplementation with either vitamin E or vitamin E and iron will affect urinary malondialdehyde (MDA) levels at rest and/or after 30 min of submaximal exercise and second, to see if there is any correlation between exercise-induced lactate (HLA) production and urinary MDA levels in response to exercise.

Scope of the Problem

This study was conducted to investigate whether supplementation with vitamin E and/or supplementation with a combination of vitamin E and iron has an effect on LP at rest and/or in response to 30 min of submaximal exercise. Thirty-three untrained female undergraduate students at Ithaca College volunteered to serve as subjects. They performed a graded exercise test to determine their peak oxygen consumption and then underwent 3 months of daily vitamin E supplementation, vitamin E and iron supplementation, or no supplementation. During this time, subjects were asked not to begin an exercise program. Following the 3-month treatment period, they participated in a 30-min submaximal exercise session. During the GXT and exercise session, three urine samples and two small blood samples were taken. The urine samples were analyzed to detect MDA levels across the different groups. The blood samples were analyzed to detect HLA levels and to compare these with urinary MDA production to see if a relationship existed between these metabolites.

Statement of the Problem

This study was conducted to determine whether 3 months of vitamin E supplementation decreases LP at rest or attenuates the increase in LP associated with 30 min of submaximal exercise at 70% of peak oxygen consumption (mVO_2) in women, and, if so, to determine whether the addition of iron supplementation has any effect on this.

Hypotheses

The hypotheses of the study were as follows:

H_1: Resting urinary MDA levels do not differ significantly among vitamin-E-supplemented, vitamin-E-and-iron-supplemented, and control subjects.

H_2: Urinary MDA levels do not differ significantly among vitamin-E-supplemented, vitamin-E-and-iron-supplemented, and control subjects in response to 30 min of submaximal exercise at 70% mVO_2.

H_3: No correlation exists between postexercise blood lactate and urinary MDA levels in response to 30 min of submaximal exercise.

Assumptions of Study

The following were assumptions of the study:

1. The subjects responded to the directions of the investigator and used maximum effort during the graded exercise testing session.

2. Free radical generation was responsible for LP that resulted in thiobarbituric acid reaction products (TBAR), a

measurable urinary marker, which is an indicator of MDA production.

3. Urinary MDA levels reflected blood MDA levels as they pertain to MDA produced in response to exercise.

4. The protocol used in this study was sufficient to elevate circulating vitamin E and iron levels in subjects after 3 months of supplementation.

Definition of Terms

The following terms are defined for the purpose of this study:

1. Malondialdehyde (MDA): A product of prostaglandin endoperoxide metabolism and nonspecific LP resulting from free radical oxidation of lipids (Marnett, Buck, Tuttle, Basu, & Bull, 1985).

2. Lipid peroxidation (LP): A series of oxygen free radical reactions resulting in a peroxide or hydroperoxide (H_2O_2) that will tend to spontaneously degenerate. The degeneration of peroxides and hydroperoxides produces additional radical centers and leads to structurally devastating consequences (Holman, 1954).

3. Thiobarbituric acid (TBA): A reagent solution used for a colorimetric assay of MDA (Marnett, Buck, Tuttle, Basu, & Bull, 1985).

4. Graded exercise test (GXT): A test to determine maximum oxygen consumption (mVO_2) on a treadmill. Maximal effort is required as the workload gradually increases until exhaustion.

5. Untrained subjects: Women who have not regularly trained aerobically for 3 months prior to the experiment.

Delimitations of Study

The delimitations of the study were as follows:

1. Thirty-three female undergraduate students from Ithaca College were recruited as subjects.

2. Only subjects who could be considered untrained participated in the study.

3. After a brief warm-up, 30 min of exercise at 70% mVO_2 was the only exercise studied.

4. Samples were taken from the exercise condition at only one time point (i.e., 30 min postexercise).

Limitations of Study

The limitations of the study were as follows:

1. The subjects were volunteers and might not be entirely representative of the total population of female undergraduates.

2. Results may only be generalizable to exercise at intensities around 70% mVO_2 for durations of about 30 min.

3. Results may only apply to urine samples taken about 30 min after exercise.

Chapter 2

REVIEW OF RELATED LITERATURE

In this chapter literature dealing with free radical chemistry, the importance of iron in free radical generation, the effects of exercise on oxygen free radical production and antioxidant defense mechanisms, and the effects of vitamin E supplementation is reviewed.

Free Radical Chemistry

Free radicals are a minor, yet potentially harmful, by-product of aerobic respiration. Radicals, in particular OH, disrupt the structure of cell membranes by attacking the polyunsaturated fatty acid portion of the lipid bilayer. Researching free radical chemistry is complicated because of the difficulty in detecting short-lived radicals in human tissues. A solution to this problem is to measure metabolic products of free radical reactions. Past studies have measured pentane in expired air and thiobarbituric acid reaction products (TBAR), in particular MDA in blood, sweat, and urine.

Molecular oxygen alters the stability of many compounds, therefore it is essential that aerobic organisms reduce molecular oxygen to water. The reduction of O_2 to H_2O requires the addition of four electrons. According to Del Maestro (1980), in aerobic biological systems that reduction may occur in one step via the tetravalent pathway or one step at a time by the univalent pathway. The complete reduction of O_2 using the

univalent pathway results in three intermediates. Addition of one electron generates the superoxide anion radical (O_2-), addition of another electron at physiological pH is accompanied by a protonated H+ and generates hydrogen peroxide (H_2O_2), and addition of the third electron breaks the O-O bond and generates the hydroxyl radical ($OH\cdot$).

Tetravalent pathways reduce the majority of O_2 in aerobic organisms, thus avoiding the large scale production of free radical intermediates. In eukaryotic organisms, the cytochrome system in the inner membrane of the mitochondria is the primary means of tetravalent O_2 reduction. However, a small but significant percentage of O_2 is reduced univalently. In fact, in eukaryotic cells, there exist other enzyme systems whose sole purpose is to catalytically scavenge the free radical intermediates of univalent O_2 reduction. Superoxide dismutase (SOD) catalyzes the dismutation of O_2- to H_2O_2, catalase (CAT) is a hemoprotein that disproportionates H_2O_2 to H_2O and O_2, and glutathione peroxidase (GPx) reduces H_2O_2 by catalyzing its reaction with reduced glutathione (GSH) to form oxidized glutathione disulfide and H_2O. Unfortunately, $OH\cdot$, which is the most unstable and potentially dangerous of the free radical intermediates, has no cellular catalyzing enzyme and forms readily when free metals such as iron are present.

Free Radicals and Lipid Peroxidation

Though oxygen free radicals can attack any part of a cell, the most likely point of attack is the cell membrane, in

particular the phospholipid portion. Phospholipids contain both saturated and unsaturated fatty acids. The unsaturated fatty acids, which have fewer hydrogens available to the carbon chain, leave more potentially active carbons susceptible to attack by free radicals. In fact, Demopoulos (1973) found the content and degree of unsaturation are of major importance in determining the likelihood of free radical damage to cell membranes. Accordingly, he suggested that hydrogen bonding may protect against free radical attack because of the restrictive mobility it induces. Free radical attack of carbon atoms can actually cleave off the unsaturated fatty acid portion of the phospholipid and/or initiate a chain reaction of tissue damage called lipid peroxidation (Del Maestro, 1980).

According to Del Maestro (1980), LP is initiated when an oxygen radical species abstracts a hydrogen atom from the carbon chain of the polyunsaturated fatty acid portion of a cellular membrane. This leaves behind a carbon atom with an unpaired electron, or a carbon radical, which is very reactive. In order to stabilize, the carbon radical undergoes a molecular rearrangement to produce a conjugated diene structure, which quickly reacts with O_2, generating another oxygen radical. The new radical, in turn, cleaves another hydrogen from a neighboring fatty acid, and perpetuates the process of LP. This rearrangement of the fatty acid portion of the membrane is very damaging. Del Maestro (1980) noted that LP is repeatedly associated with cell injury and can affect many tissues in the

body. One study found a relationship between free radical generation and damage to cerebrospinal fluid, synovial fluid, and serum (Gutteridge, 1984). In another review, the author listed 11 major ailments, including aging, cancer, atherosclerosis, and diabetes, as harmful consequences of free radical mediated chemistry (Jenkins, 1988).

Free Radicals and Iron

As mentioned earlier, reduction of H_2O_2 may produce $OH\cdot$. However, glutathione peroxidase and catalase clear H_2O_2 without forming new radicals. Iron, which competes to react with H_2O_2, will reduce it and yield $OH\cdot$ via the Fenton reaction:

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

If O_2- is present in significant concentration, the oxidized iron that remains is quickly reduced:

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

Iron is thus recycled, allowing it to again react with H_2O_2. This allows a relatively small amount of iron to liberate a large amount of $OH\cdot$ if enough O_2- and H_2O_2 are present.

Halliwell and Gutteridge (1984) noted that because iron is so reactive, it exists in a free state only briefly *in vivo*. It quickly binds to different compounds depending on where it is in the body. The majority is bound to a variety of proteins, however, iron also binds to membranes, nucleic acids, and many low molecular weight chelating agents.

The binding of iron to a ligand potentially alters its reactivity with O_2- and H_2O_2 (Flitter, Rowley, & Halliwell, 1983;

Floyd, 1983). The ligand may allow more iron than normal to exist in solution or affect the redox potential of the $Fe_{2}+/Fe_{3}+$ half-cell by interfering with reduction by $O_{2}-$.

When the ligand is a protein, structural factors of the iron-protein complex often affect iron's reactivity with $H_{2}O_{2}$. Proteins bind very tightly to iron, and, in the case of larger proteins, often "wrap around" the iron molecule. It is thought that protein-iron complexes often block or hinder the binding of $H_{2}O_{2}$ to the iron molecule, inhibiting formation of OH·. In addition, the proteins bound to iron often scavenge any OH· that is liberated from the complex. It is generally thought that protein-bound or tightly bound iron does not promote the formation of OH· in systems containing $O_{2}-$ and $H_{2}O_{2}$ and is not likely to be a major factor in iron-dependent free radical production (Halliwell & Gutteridge, 1986). Cells of the body contain enzymatic defenses against free radicals (e.g., SOD, CAT, and the glutathione system), however, these enzymes are not prominent in extracellular fluid. Extracellular fluids are exposed to considerable amounts of $O_{2}-$ and $H_{2}O_{2}$. In fact, activated phagocytic cells are thought to release these compounds in response to foreign antigens (Holland, Alvarez, & Storey, 1982). It is thought that the body's defense against free radical attack in extracellular fluids lies in its ability to prevent metal catalysts from being active (Gutteridge, 1982; Gutteridge & Stocks, 1981). For instance, iron released into plasma will quickly bind to transferrin, which, at physiological

levels, does not promote LP or OH· formation (Halliwell & Gutteridge, 1986).

Iron also exists in a variety of loosely bound complexes. These include iron bound to membranes, ATP, and low molecular weight chelating agents. It is likely that these iron complexes may participate in free radical generating reactions (Halliwell & Gutteridge, 1984). Low molecular weight complexes also decompose lipid peroxides and form OH· radicals in systems containing O_2^- and H_2O_2 (Halliwell & Gutteridge, 1986). In particular, because the OH· radical reacts so close to its point of origin, iron bound to membranes may catalyze the reactions responsible for initiating LP and membrane damage (Schaich & Borg, 1988).

Because of the importance of iron in free radical chemistry, it is conceivable that excessive iron loading promotes tissue damage associated with free radicals. This idea is supported by symptoms of hereditary hemochromatosis, a condition in which nonheme serum iron is grossly elevated (Gutteridge, 1984; Gutteridge et al., 1985; Sagone, Greenwald, Kraut, Bianchine, & Singh, 1983). In one study, high occurrences of 10 serious disorders, including diabetes mellitus, cardiac abnormalities, and peripheral neuritis, are associated with hemochromatosis. It is thought that the increased radical-mediated tissue damage is caused by saturation of the antioxidative defenses of plasma. In the above-mentioned study, transferrin was frequently at or near 100% saturated in subjects tested (McLaren, Muir, & Kellermeyer, 1983).

Indeed, LP is repeatedly associated with iron overload. Rachmilewitz et al. (1976) found that erythrocytes from patients with iron overload underwent excessive LP associated with decreased vitamin E levels. Heys and Dormandy (1981) found that spleens from humans with iron overload formed TBAR, which are considered markers for LP. Again, TBAR values were inversely correlated with vitamin E levels. Dillard et al. (1984) injected rats with 100 mg of iron intraperitoneally and found that exhaled pentane levels (also a LP marker) increased dramatically over 7 to 8 weeks. These increased pentane levels were greater still when 200 mg of iron was injected, and all increases were attenuated by a variety of antioxidants studied, the greatest effect coming from vitamin E. Based on these data, severely elevated iron levels may elicit significant increases in oxygen free radical species and LP.

Iron supplementation is a method commonly used by women to combat or prevent anemia. Indeed, this treatment often prevents the effects of anemia under normal circumstances. No research has yet examined whether acceptable iron supplementation elicits an increase in free radicals or LP. It is thus unknown whether dosages commonly prescribed to treat or prevent anemia would be large enough to cause an effect similar to those seen with hemochromatosis (Gutteridge, 1984; Gutteridge et al., 1985; Sagone et al., 1983) or in experimental rats (Dillard et al., 1984), however, it is conceivable that some increase in free radical concentrations may exist in these patients.

Free Radicals and Exercise

Running, cycling, walking, and many other forms of aerobic exercise have gained popularity over the last decade. This is considered a positive trend, because the benefits of cardiovascular exercise are well documented. In fact, this trend may be occurring in response to the use of exercise for treatment and rehabilitation by medical practitioners. Cardiovascular exercise is a method often used to rehabilitate patients after myocardial infarction. In fact, it is often prescribed, in combination with diet and behavior modification, to increase cardiovascular fitness and help prevent cardiac events. There are obvious benefits to cardiovascular fitness, however, this exercise also leads to oxidative stress and increases in free radical production and LP (Balke et al., 1984; Davies et al., 1982; Dillard et al., 1978; Lovlin et al., 1987).

Most studies dealing with the relationship of exercise and free radicals have used animal models due to the difficulty in detecting free radicals in living systems. Davies et al. (1982) documented the effect of endurance exercise on free radicals in the liver and skeletal muscle of rats. In animal studies, by-products associated with LP, such as TBAR and pentane production, were detected. In other animal studies, the effect of endurance exercise on free radical markers in the liver (Suzuki, Katamine, & Tatsumi, 1983), skeletal muscle (Salminen & Vihko, 1983), brain, heart (Suzuki et al., 1983), and lungs (Quintanilha, 1984) were documented. Human studies have been limited to the

detection of LP by-products or to examination of the effect of exercise on scavenging enzymes or enzymes associated with cell damage (Brady et al., 1979; Ohno et al., 1986; Sumida et al., 1989). Pentane was detected in the breath (Balke et al., 1984; Dillard et al., 1978), and TBAR was found in blood (Kanter, Kaminsky, La Ham-Saeger, Lesmes, & Nequin, 1986; Kanter, Lesmes, Kaminsky, La Ham-Saeger, & Nequin, 1986; Lovlin et al., 1987; Ohno et al., 1986), sweat (Tompkins, 1989), and urine (Draper, Polensek, Hadley, & McGirr, 1984; Marnett et al., 1985).

Ullrey, Shelle, and Brady (1977) found elevated blood MDA in horses immediately after 10 min of exercise. This was one of the first suggestions that LP increased during exercise. Brady et al. (1979) found increased TBAR in liver and muscle tissue of rats subsequent to exhaustive swimming exercise. Though no liver or muscle enzymes were affected, a decreased activity of glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) in erythrocytes occurred immediately postexercise, suggesting these scavenging enzymes may be inhibited by exercise. In a similar study, Davies et al. (1982) found a two- to three-fold increase in free radical concentrations of the muscle and liver of rats following exhaustive exercise on a treadmill. They also noted a significant increase in the levels of LP products. These results coincided with decreased mitochondrial respiratory control and a general loss of integrity of sarcoplasmic and endoplasmic reticula. In another study, voluntary wheel exercise in rats fed diets high or low in rancid oil led to reduced MDA

accumulation in most tissues, but increased LP in the excised brains of these animals (Suzuki et al., 1983). The authors speculated that the causative agents of LP may have moved from muscle or liver tissue to the brain, and that MDA was not localized in specific areas. Marnett et al. (1985) supplied support for the idea that some tissue may metabolize LP products when they found that rats rapidly removed exogenously administered MDA in vivo and that MDA was found in all tissues studied.

Fewer studies have focused on humans. Dillard et al. (1978) found a 1.8-fold increase in pentane production in humans during exercise at 75% mVO_2 over resting values. Because expired pentane is used as an index of LP, this also suggested an exercise-induced increase in free radical production leading to LP. Balke et al. (1984) found a three-fold increase in n-pentane production in humans as a result of 20 min of cycling at 50% mVO_2, and suggested that significant LP occurs during moderate exercise in man. These results are similar to the findings of Dillard et al., who also found a significant increase in pentane production in response to cycling at 75% mVO_2.

However, the results of an experiment by Lovlin et al. (1987) contradict the findings of Balke et al. (1984) and Dillard et al. (1978). In this study, maximal exercise elicited a 26% increase in plasma MDA, but a 10.3% decrease in plasma MDA occurred at 40% mVO_2. At 70% mVO_2, plasma MDA levels were slightly, but not significantly, below resting levels. The

authors suggested that exercise to exhaustion significantly increases LP, but short bouts of submaximal exercise may actually inhibit LP. That study also indicated a correlation between lactate and MDA (r^2 = .51; p < .001) and a trend for increased MDA production as exercise intensity increased.

A possible explanation for the discrepancy between the findings of Lovlin et al. (1987) and other investigators may be a difference in exercise duration among the studies. In the study by Lovlin et al., subjects cycled for 5 min at 40% mVO_2, then rested for 5 min before cycling for 5 min at 70% mVO_2. In other studies (Balke et al., 1984; Dillard et al., 1978), the submaximal exercise bouts continued for at least 20 min. Davies et al. (1982), who also studied the effect of a submaximal workload, carried exercise to exhaustion in rats and found that damage induced by free radicals was gradual and cumulative, and depended mainly on the duration of work. This may provide a possible explanation for the findings of Lovlin et al (1987).

Sumida et al. (1989) also found a significant increase in serum levels of MDA immediately after exhaustive exercise on a cycle ergometer. In addition, they measured the activities of beta-glucuronidase and mitochondrial glutamic-oxaloacetic transaminase isozyme (m-GOT), indicators of cell damage known to increase after exhaustive exercise (Ohno et al., 1978; Salminen & Kihlstrom, 1985; Vihko, Rantamaki, & Salminen, 1978). They found a 19% increase for beta-glucuronidase and a 65% increase for m-GOT immediately after exercise. Based on these results, the

authors suggested an association between LP and cell damage.

Ohno et al. (1986) observed the effects of exercise on free radical scavenging enzyme systems of human erythrocytes. Of the enzymes studied, however, only total GR activity showed a significant increase immediately after 30 min of cycling at 75% mVO_2. This finding provides further evidence that exercise has an effect on the aerobic oxidative process through its effect on free radical scavenging enzyme systems. However, by elevating the levels of GR, which maintains reduced glutathione. the effect seems to be a protective rather than a harmful one.

It is generally assumed that the electron transport system of the mitochondria is responsible for the production of free radicals during exercise. Chance, Sies, and Boveris (1979) and Davies et al. (1982) found that enhanced mitochondrial respiration, in response to cardiovascular training, generated oxygen radicals as a by-product of oxidative metabolism. Further evidence of this can be found in a study by Salminen and Vihko (1983). They noted a significant difference in the rate of peroxidation between red and white skeletal muscle in rats. The red skeletal muscle contained more peroxidizable lipids than the white muscle. They suggested greater LP was due to the higher content of mitochondria, which are rich in phospholipids containing unsaturated fatty acids. However, it was also found that endurance training over a prolonged period increased the levels of reduced and total non-protein glutathione in red skeletal muscle leading to an increased resistance of skeletal

muscle to injuries caused by LP.

Another mechanism for free radical formation was postulated by Kellogg and Fridovitch (1975). They found that reperfusion of ischemic tissues could cause free radical formation via the xanthine oxidase system. Activation of this system would initiate a greater formation of uric acid. Sumida et al. (1989) found a great increase in uric acid concentration following exhaustive exercise that coincided with a significant increase in serum MDA levels. This led them to hypothesize that generation of free radicals after a bout of heavy exercise may occur by activation of the xanthine oxidase system as well as the mitochondrial respiratory chain.

In animal studies in which free radical concentrations in response to exercise were studied, researchers consistently found an increase in oxygen free radicals (Davies et al., 1982) or free radical markers (Brady et al., 1979; Quintanilha et al., 1984; Salminen & Vihko, 1983; Suzuki et al., 1983; Ullrey et al., 1977). In humans, both Dillard et al. (1978) and Balke et al. (1984) found increases in expired pentane after prolonged submaximal exercise, but Lovlin et al. (1987) found that plasma MDA levels actually decreased with short bouts of submaximal exercise at certain intensities. Lovlin et al. and Sumida et al. (1989), however, found that blood MDA levels increased significantly subsequent to maximal exercise. The discrepancy found in studies dealing with submaximal exercise may be explained by exercise duration or by the difference in markers

analyzed. From the data available, it would seem that maximal and prolonged submaximal exercise elicit an increase in LP indicators, but the effects of short bouts of submaximal exercise are not as well understood and may depend on exercise intensity.

Free Radicals and Vitamin E

The scavenging enzymes of the body are major factors in cellular control of free radical production. In addition, proteins, particularly transferrin, control production of free radicals extracellularly by inactivating iron. When these defenses are saturated and free radicals are allowed to initiate LP, other antioxidants are necessary. Selenium, ascorbate, and vitamin E have all shown an inhibitive effect on LP in response to increases in free radical concentrations (Demopoulos, 1973; Jenkins, 1988). Of these, vitamin E appears the most significant and consistent antioxidant.

Vitamin E, or alpha-tocopherol, exists in minute concentrations in the phospholipid portion of the plasma membrane of cells (Jenkins, 1988). As LP occurs in the membrane, a free radical species, usually OH·, pulls off a hydrogen from a polyunsaturated fatty acid (PUFA). This reaction gives rise to an organic radical that then reacts with a neighboring PUFA (Del Maestro, 1980). This process perpetuates in a chain reaction until an organic radical reacts with one of the alpha-tocopherol molecules in the membrane. The reaction of the organic radical with the alpha-tocopherol stabilizes the PUFA and forms an alpha-tocopherol radical. The alpha-tocopherol radical, however, is

stable and terminates the process, thereby discontinuing the disruption of the membrane and production of LP products (Tappel, 1968).

Early indications that vitamin E might be involved in terminating LP were found when it was learned that vitamin E deficiency led to an increase in LP indicators (McCay, Gibson, Fong, & Hornbrook, 1976; Noguchi et al., 1973; Whanger et al., 1977). Davies et al. (1982) examined the effects of vitamin E deficiency and exhaustive exercise in rats and discovered that nonexercised vitamin E deficient rats and exercise-exhausted control rats showed similar decreases in mitochondrial respiratory control, membrane integrity of the sarcoplasmic and endoplasmic reticula, and increases in LP indicators. In addition, when vitamin E deficient animals were exercised, the increase in free radicals in response to exercise was larger than that of control animals (Davies et al., 1982). Packer (1984), Quintanilha and Packer (1983), and Quintanilha, Packer, Davies, Racanelli, and Davies (1982) also found indications that vitamin E deficiency increased LP during exercise.

Because vitamin E inhibits radical-mediated tissue damage when other defenses have become saturated, vitamin E is particularly important to normal cell function during exercise. Dillard et al. (1978) found a significant increase in pentane production after exercise in humans and observed that 2 weeks of daily supplementation with high doses of d-alpha-tocopherol reduced pentane production. They concluded that exercise induced

LP and that LP was attenuated by vitamin E. Brady et al. (1979) also discovered the increase in TBAR subsequent to exercise was reduced by vitamin E in the liver but not in muscle tissue. Sumida et al. (1989) actually reported that blood levels of MDA decreased immediately, 1 hour, and 3 hours after exhaustive exercise in humans who took 300 mg of d-alpha-tocopherol acetate daily for 4 weeks, although MDA levels increased slightly for control subjects after exhaustive exercise. They also found that leakage of enzymes in control subjects was significantly increased after exercise, yet in those subjects who took vitamin E, enzyme leakage was significantly decreased. Brady et al. and Sumida et al. provided further evidence that exercise induced tissue damage associated with LP that may be attenuated by vitamin E.

The significance of vitamin E in the maintenance of free radical levels both at rest and in response to exercise seems well established. Evidence repeatedly supports that a deficiency of vitamin E results in increased LP markers (Davies et al., 1982; McCay et al., 1976; Noguchi et al., 1973; Packer, 1984; Quintanilha & Packer, 1983; Whanger, 1976) and a compromise of membrane integrity (Davies et al., 1982; Sumida et al., 1989). Evidence also indicates that vitamin E supplementation decreases these negative consequences of free radical chemistry (Brady et al., 1979; Dillard et al., 1978; Sumida et al., 1989).

Summary

Different defenses exist to control the levels of oxygen free radicals in aerobic organisms. In the cell, a variety of enzymes, such as SOD, CAT, and GPx, exist to reduce or change oxygen radicals into less harmful compounds (Del Maestro, 1980). Certain iron complexes (loosely bound iron) promote free radical formation, but other tightly bound complexes (e.g., protein bound) do not (Halliwell & Gutteridge, 1984). In fact, some (e.g., transferrin) provide defense mechanisms that exist in the extracellular fluids as effective inhibitors of free radical production by binding to and inactivating iron. Iron loading, however, may saturate these defenses, leading to increased indicators of LP (Dillard et al., 1984).

Research reveals that exercise at certain levels of intensity and duration promotes LP, as measured by increases in the concentrations of MDA in blood (Lovlin et al., 1987; Ohno et al., 1986), sweat (Tompkins, 1989), and urine (Draper et al., 1984; Marnett et al., 1985). This evidence can also be found in increased concentrations of expired pentane (Balke et al., 1984; Dillard et al., 1978). The observed increase in LP markers with exercise are thought to occur because of an increase in the activity of the mitochondria within cells caused by the oxidative stress of exercise and perhaps increased activity of the xanthine oxidase system (Kellogg & Fridovitch, 1975). An increased oxidative stress resultant from exercise is associated with vitamin E deficiency, and vitamin E supplementation apparently

attenuates that exercise effect (Dillard et al., 1978). Supplementing with antioxidants has also resulted in free radical levels of nonexercising subject populations that are lower than control group levels (Dillard et al., 1978).

Although iron loading and exercise may promote the production of free radicals and thus initiate LP, vitamin E supplementation seems to be an excellent defense against this process. In rats, vitamin E supplementation has effectively decreased elevated levels of LP indicators associated with iron loading (Dillard et al., 1984; Draper et al., 1984), however, no similar human studies have been conducted.

Chapter 3

METHODS AND PROCEDURES

In this chapter the methods and procedures used in this study are outlined. Specifically, the chapter deals with (a) selection of subjects, (b) testing procedures and instrumentation, and (c) treatment of data.

Selection of Subjects

Data collection for this study was conducted from December 1989 to March 1990. Subjects were recruited by a verbal announcement (Appendix A) to classes in the Division of Health, Physical Education, and Recreation and the School of Humanities and Sciences at Ithaca College. Thirty-three females, ranging in age from 18 to 23 years, volunteered and were cleared to participate. Before testing, each subject filled out a medical history questionnaire (Appendix B) and read and signed an informed consent form describing the experimental procedures (Appendix C). In addition, a 24-hour history was filled out before each day of testing (Appendix D). If a candidate had no contraindications for exercise testing as stipulated by the American College of Sports Medicine (1986) and had not trained aerobically for 3 months prior to testing, she was allowed to participate. Of the original 33 subjects who began the study, only 25 completed it. Eight subjects were forced to discontinue the study due to illness or nonadherence to the test protocol.

Testing Procedures and Instrumentation

All subjects completed two data collection sessions. The first was a graded exercise test (GXT), and the second was a submaximal exercise test that occurred after the supplementation period, approximately 3 months subsequent to the GXT. Following the GXT, aerobic capacities were matched and subjects were assigned to one of three groups. One group took 400 IU of d-alpha-tocopherol (Henkel Corporation, #5 Oval Softgel) daily, another group took the same dosage of vitamin E and 325 mg ferrous sulfate (Carls Drugs Fe-Tabs with 65 mg elemental iron) daily, and a control group took no supplements during the 3-month period. Standard directions were given for each test, with an opportunity for further explanation if requested. Both of the data collection sessions are described in more detail in the following sections.

Graded Exercise Test

Aerobic capacity (mVO_2) was assessed by running to exhaustion on a treadmill. Before entering the lab, each subject refrained from drinking alcohol for 12 hours and refrained from eating for at least 3 hours. Between 60 and 120 min before the test, subjects drank 500 cc of water to standardize hydration. Upon entering the lab, subjects produced a small urine sample that was immediately frozen at -80° C and stored for later analysis. Height, weight, resting blood pressure, and resting heart rate were taken while the researcher acquainted the subject with the testing procedures. The subject was fitted with a

Daniels breathing valve, supporting headgear, and a noseclip to prevent air leakage. Respiratory gases were collected, and metabolic data were derived from Applied Electrochemistry O_2 and Beckman CO_2 gas analyzers and a Rayfield gas meter, providing input to an Apple IIe computer that determined the following printed data: ventilatory volume (l/min), VO_2 STPD (ml/kg/min), VCO_2 (l/min), and respiratory quotient.

The exercise protocol began at a workload of 4 mph at 0% grade and increased 1 mph every minute until the subject felt she could not run faster comfortably. Thereafter, workload increased 2% in grade for each 2-min stage until voluntary exhaustion. During the GXT, heart rate was monitored each minute and at the point of maximum effort, using a Uniqcic Pro Trainer heart rate monitor. Following the GXT, subjects actively cooled down for 3 to 5 min and then rested in the lab until heart rate returned to near resting levels, at which time they were permitted to leave.

Submaximal Exercise Session

The same instructions were followed by subjects before entering the lab as were described for the GXT. After giving a urine sample upon arrival, subjects were then asked to drink 500 cc of water before exercising. While they drank this, a small blood sample (50 ml) was collected in a capillary tube for analysis of preexercise lactate concentrations on a Yellow Springs Instruments Model 27 analyzer. These samples were compared to 5.0+/-0.1 and 15.0+/-0.3 mmol/l lactate standards provided by the manufacturer. Standards were analyzed before

each sample analysis. Subjects then exercised on the treadmill at 70% mVO_2 as determined during the GXT and as monitored by heart rates recorded each minute during the submaximal exercise. Immediately upon completion of 30 min of exercise, subjects were seated, and another 50-ml blood sample was taken and analyzed for postexercise lactate concentrations. Subjects then actively cooled down by walking for 5-10 min. Between 30-36 min postexercise, subjects produced a final urine sample. All urine samples were immediately frozen at -80°C and analyzed subsequently for MDA.

Thiobarbituric Acid Assay

Urine samples were thawed for 4 hours, following which a 1-ml aliquot was combined with 1 ml of 1% TBA and 1 ml of 20% acetate buffer (pH = 3.5) in a test tube. Samples were then incubated at 90°C for 20 min to allow the TBA reaction to occur. After being cooled at room temperature, samples were passed through a Waters C18 Sep-Pak and eluted in 2 ml methanol. The eluate was collected in a cuvette and analyzed on a Perkin-Elmer LS5 spectroflourometer that measured the flourescence of the solutions with excitation at 515 nm and emission at 553 nm. This procedure was repeated in triplicate for each urine sample, and average readings were used in subsequent statistical analysis. To convert spectroflourometric unit readings to molar values, results were compared to a 2.2 µmol MDA standard (24.1 spectroflourometric units), which was analyzed before the samples.

Subject Supplementation

Subjects were supplemented for 90 days between the GXT and submaximal exercise test. Two supplements were used in this study. The vitamin E group took 400 IU d-alpha-tocopherol. The second group, vitamin E and iron, took the same dosage of vitamin E and also ingested 325 mg ferrous sulfate (65 mg elemental iron) over the same period. The dosages used in this study were chosen to mimic dosages used commonly as a daily supplement.

Treatment of Data

Statistical significance was tested at the .05 level. A 3 x 3 (Group x Trial) repeated measures ANOVA was used to determine whether any differences existed in MDA levels among groups and across the three trials. If differences were noted using this analysis, subsequent 2 x 3 repeated measures ANOVA analysis further pinpointed between which groups MDA differences existed. Simple ANOVAs revealed differences among groups at individual time points, and post-hoc Tukey tests examined differences in MDA within the groups to determine at which time points any differences occurred. A Pearson correlation was used to see if any relationship existed between postexercise MDA and lactate values for all groups.

Summary

Twenty-five untrained college-aged women were matched for mVO_2 and assigned into either a vitamin E, vitamin E plus iron, or control group to examine the effects of vitamin E and iron supplementation on exercise-induced urinary MDA production. Data

collected before and after a submaximal exercise session were analyzed using a repeated measures ANOVA design to determine differences among groups and across time. Subsequent post-hoc Tukey tests were used to identify differences across time within each group. The possibility of a relationship between blood lactate levels and urinary MDA was also examined using a Pearson correlation.

Chapter 4

ANALYSIS OF DATA

This study was conducted to investigate the effect of vitamin E and vitamin E plus iron supplementation on free radical production at rest and in response to submaximal exercise. In addition, lactate was measured before and after exercise to investigate whether there was any correlation between lactate levels and TBAR production in response to exercise. Statistical analyses of these data are described in this chapter.

Description of Subjects

The physical characteristics of the subjects in this study are listed in Table 1. Subjects were untrained prior to and throughout the duration of the study. Ages ranged from 18 to 22 yr, and weight ranged from 48.6 to 76.0 kg. Aerobic capacities (mVO_2) were between 31.7 ml/kg/min and 53.4 ml/kg/min, as determined by a graded exercise test (GXT). Subjects' submaximal workloads (70% mVO_2) were determined from the GXT, and exercise intensity was maintained over a 30-min period by monitoring heart rates, which averaged from 144 bpm to 179 bpm. These data indicate a wide range in weight and exercise capacities among the subjects used in this study.

Repeated Measures ANOVA

The dependent variable studied in this experiment was TBAR, which is a chemical marker for MDA. This measurement was made over 3 trials to determine if a statistically significant

Table 1

Physical Characteristics of Subjects

Subject	Age (yr)	Weight (kg)	mVO2 (ml/kg/min)	GXT Peak Workload/HR (mph/grade/bpm)	70% Peak Workload/HR
Group 1 (Control)					
1	18	72.7	38.3	6.0/6.0/207	5.5/0/166
2	21	55.9	41.3	7.0/3.5/192	4.5/2/167
3	18	56.0	38.1	9.0/2.0/192	5.3/0/159
4	21	59.1	46.6	8.0/4.0/196	6.5/0/150
5	19	59.0	50.4	7.0/6.0/207	6.3/0/166
6	20	59.1	40.4	8.0/2.0/198	5.3/0/171
7	21	59.0	33.0	7.0/2.0/212	3.8/2/144
8	22	67.3	37.1	8.0/2.0/174	4.3/0/162
9	20	68.0	53.4	9.0/2.0/206	6.0/0/176
Group 2 (Vitamin E)					
1	21	56.8	42.0	7.0/4.0/187	6.4/0/167
2	21	74.1	46.4	7.0/4.0/203	5.5/0/179
3	21	67.0	39.2	8.0/2.0/194	5.8/0/165
4	19	76.0	38.0	7.0/2.0/230	3.8/2/168
5	21	75.0	33.8	6.0/2.0/193	4.0/2/164
6	21	50.1	50.4	9.0/4.0/204	7.1/0/175
7	20	65.9	38.3	6.0/4.0/206	4.5/2/181
8	19	73.0	37.8	7.5/1.0/183	5.8/0/169

(Table continues)

Subject	Age (yr)	Weight (kg)	mVO2 (mL/kg/min)	GXT Peak workload/HR (mph/grade/bpm)	70% Peak workload/HR
Group 3 (Vitamin E and Iron)					
1	20	64.0	41.0	9.0/1.0/194	5.3/0/165
2	18	60.0	36.9	8.0/2.0/196	4.5/3/158
3	22	56.8	31.7	7.5/1.0/189	4.2/2/161
4	20	59.5	36.6	7.0/4.0/213	5.0/0/176
5	22	48.6	50.7	8.0/4.0/198	6.7/0/176
6	20	62.7	40.6	8.0/2.0/210	4.2/3/162
7	22	51.4	42.9	8.0/4.0/197	5.8/0/169
8	18	61.8	37.6	6.0/6.0/194	5.8/0/168

difference in TBAR existed among the 3 groups as a result of vitamin supplementation over time. As reported in Table 2, a 3 x 3 (Group x Time) analysis indicated a significant group by trial interaction ($F[4,44] = 10.67$, $p < .001$). Figure 1 illustrates that control values remain the same between Trials 1 and 2, and then increase following exercise (Trial 3), but the supplement group had a slight decline across all three Trials. A 2 x 3 analysis of the two supplement groups across time indicated that iron did not alter the effect of vitamin E ($F[1,14] = 0.15$, $p > .05$), thus the two supplement groups were not significantly different and were pooled into one group ($n = 16$) for further analysis (Table 3). A subsequent 2 x 3 ANOVA (Table 4) showed a significant difference between control and supplement groups across the three trials ($F[1,23] = 9.48$, $p < .001$). Figure 2 illustrates the nature of the between-groups difference. A test of simple main effects using ANOVA determined supplementation decreased the rise in postexercise MDA found in the controls ($F[1,23] = 41.92$, $p < .001$). The preexercise trial (Trial 2) showed a considerable difference from Trial 1 in the supplement group, although this was not significant at the .05 level.

Within the control group, a significant difference ($F[2,16] = 15.081$, $p < .001$) existed across the 3 trials. Individual Tukey tests showed no significant difference in urinary MDA levels between Trials 1 and 2 ($p > .05$). However, postexercise MDA levels were significantly higher ($p < .05$) than the levels for both Trial 2 and Trial 1.

Table 2

ANOVA Summary Table for Differences in MDA Among Control and Supplement Groups Across Time

Source of Variation	SS	df	MS	F	p
Group	48.75	2	24.37	4.62	.021
Subjects within Group	116.01	22	5.27		
Trials	3.79	2	1.89	2.35	.107
Group x Trials	34.34	4	8.59	10.67	.000
Subjects within Group x Trials	35.41	44	0.81		

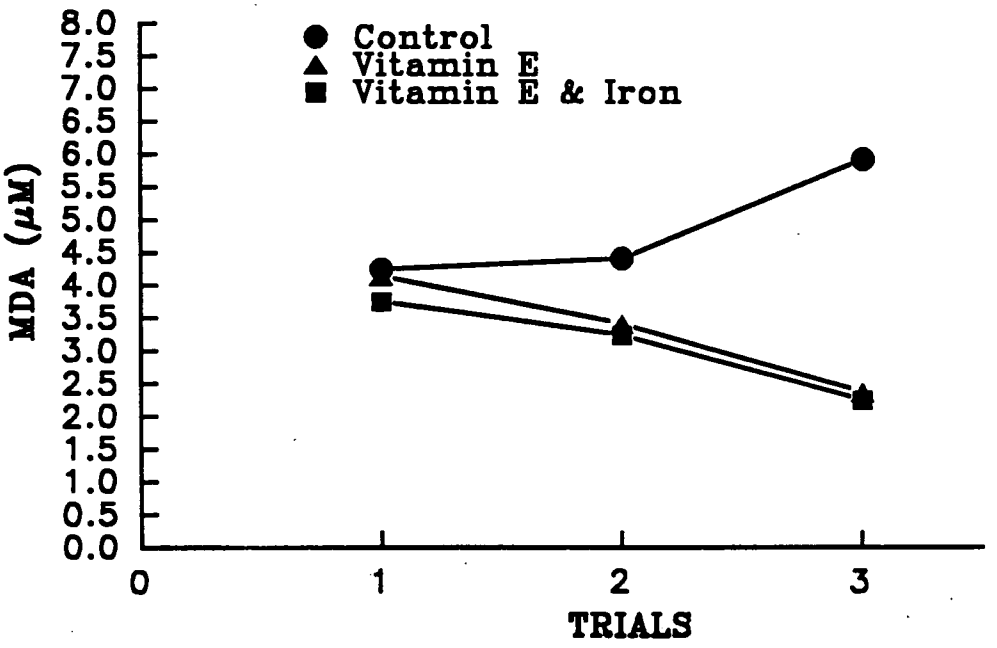

Figure 1. A significant group-by-trial interaction existed among the three groups across the three trials. (Trial 1 is preexercise, presupplementation; Trial 2 is preexercise, postsupplementation; and Trial 3 is postexercise, postsupplementation.)

Table 3

ANOVA Summary Table for Differences in MDA Between Control and Combined Supplement Groups Across Time

Source of Variation	SS	df	MS	F	p
Group	0.67	1	0.67	0.15	.704
Subjects within Group	62.09	14	4.44		
Trials	21.96	2	10.98	11.20	.000
Group x Trials	0.16	2	0.08	0.08	.920
Subjects within Group x Trials	27.45	28	0.98		

Table 4

ANOVA Summary Table for Differences in MDA Across Time Within Groups

Source of Variation	SS	df	MS	F	p
Group	48.08	1	48.08	9.48	.005
Subject within Group	116.68	23	5.07		
Trials	.83	2	0.42	0.54	.587
Group x Trials	34.18	2	17.09	22.09	.000
Group @ Trial 3	74.85	1	74.85	41.92	.000
Error	41.06	23	1.79		
Trials @ Control	15.00	2	7.50	15.08	.000
Error	7.96	16	0.49		
Trials @ Treatment	17.80	2	8.90	8.52	.001
Error	31.35	30	1.05		
Subjects within Group x Trials	35.58	46	0.78		

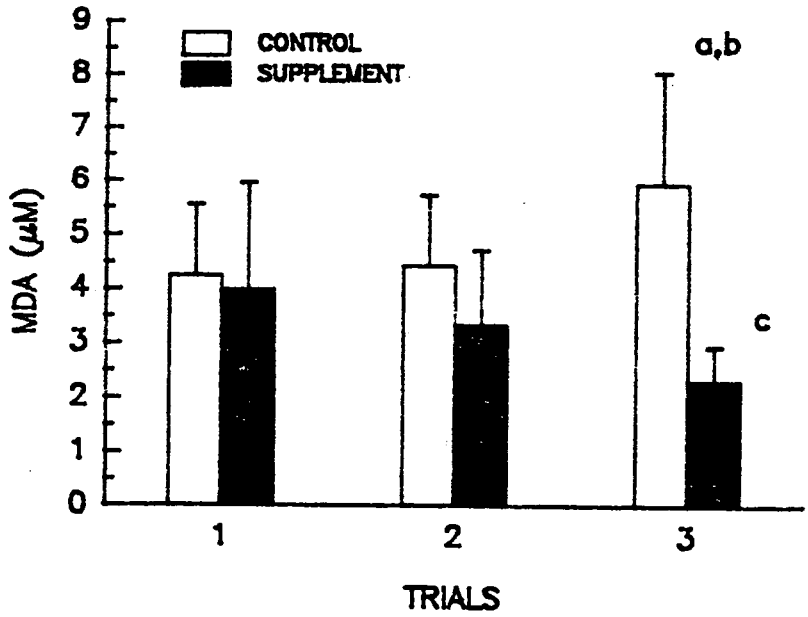

Figure 2. Control and supplemented MDA values for initial presupplementation resting (1), postsupplementation resting (2), and postsupplementation postexercise (3) trials. ([a]Post-exercise control MDA was significantly greater than postexercise supplemented MDA [$p < .001$]. [b]Postexercise control MDA levels were significantly greater than resting MDA levels [$p < .05$]. [c]Postexercise supplemented MDA levels were significantly less than resting MDA levels [$p < .05$].)

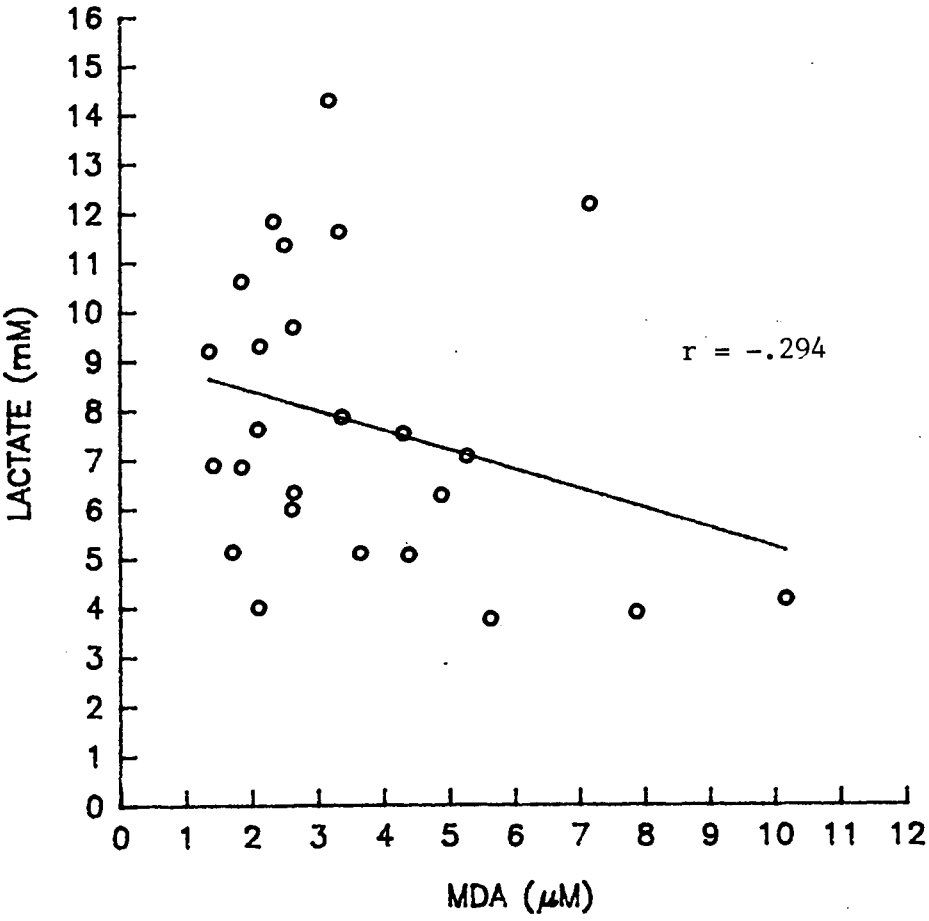

Figure 3. No significant linear correlation existed between postexercise MDA and lactate.

ANOVA was also run within the supplement group, and again a significant difference ($F[2,30] = 8.521$, $p < .001$) existed across the 3 trials. These data are reported in Table 4. Tukey tests showed that postexercise MDA levels were significantly lower than the two resting levels ($p < .05$).

MDA/Lactate Correlation

The purpose of this correlation was to investigate whether a relationship existed between TBAR and lactate production in response to the submaximal exercise bout. Postexercise MDA levels were matched with postexercise lactate concentrations and a correlation of $r = -.294$ was observed. These data, shown in Figure 3, indicate that no linear correlation exists between TBAR and lactate after submaximal exercise.

Chapter 5

DISCUSSION OF RESULTS

The results of this study showed that urinary levels of thiobarbituric acid reaction products (TBAR), a biochemical marker for malondialdehyde (MDA), increase in response to moderate intensity submaximal exercise and that vitamin E supplementation may actually reverse this event. Furthermore, these results showed that combining iron supplementation with vitamin E did not influence the effect of vitamin E on MDA production ($p > .05$). Lastly, a correlation between postexercise MDA and lactate levels showed no substantial relationship existed between the two metabolites. A discussion of these results is found in this chapter in the following sections: (a) TBAR in urine, (b) TBAR response to exercise, (c) effects of vitamin E on resting and exercise LP indicators, (d) the effect of iron and vitamin E on LP, and (e) the relationship of lactate and LP.

TBAR in Urine

The present study found that MDA was detectable in urine before and after 30 min of submaximal exercise. These urinary MDA values were used to assess the effect of submaximal exercise on oxygen free radical production. MDA levels recorded in this study are of the same order of magnitude as those found by Draper, Polensek, Hadley, and McGirr (1984) who reported that small amounts of MDA are excreted in an acid-hydrolyzable form in

urine and that excreted MDA was an effective index of LP. Present findings verify the use of urinary MDA as an index of free radical production. Urinary MDA analysis was used in this study because it is an easy and convenient method of assessing free radical production during exercise and may provide some information about MDA clearance after exercise.

TBAR Response to Exercise

Many investigators have shown increases in LP indicators in response to maximal (Davies et al., 1982; Lovlin et al., 1987; Sumida et al., 1989) and prolonged submaximal exercise (Balke et al., 1984; Dillard et al., 1978). In support of these findings, control subjects in this study exercised at 70% mVO_2 for 30 min and showed a significant increase ($p < .05$) in urinary MDA levels 30 min postexercise.

Marnett et al. (1985) found MDA values higher in the bladder than in all other tissues studied 30 min after MDA injection in rats. These values dropped greatly by 2 hours postinjection. In the present study, urinary MDA samples were collected 30 min postexercise in an effort to obtain samples at a time associated with the greatest urinary clearance of MDA. Results of the present study showed increases in urinary MDA 30 min postexercise, although previous studies monitoring TBAR in blood and tissue homogenates found the greatest increases in MDA immediately after exercise (Davies et al., 1982; Sumida et al., 1989). In fact, Sumida et al. found an immediate postexercise increase in serum MDA that subsided 1 hour later. Although a

time course analysis of urinary MDA was not completed in the present study, it is believed that the 30-min-postexercise urine sample represents a mean peak urinary MDA value. The logic for this assumption is explained in the following review of the renal response to exercise.

In a review of the renal response to exercise, Zambraski (1990) stated that moderate to intense exercise causes significant decreases in renal blood flow and urine volume. Heavy exercise also causes a decrease in urinary electrolyte concentration (Poortmans, 1984). Glomerular filtration rate (GFR) appears to be inversely related to exercise intensity (Kachadorian & Johnson, 1970) and widely affected by exercise duration (Poortmans & Labilloy, 1988). Normal renal function appears to resume within 1 hour after exercise (Zambraski, 1990). The present study did not quantify these effects by use of a marker for renal function, such as creatinine or inulin excretion. A future study should be conducted to compare urinary MDA values after exercise with such a marker.

If it can be assumed that renal activity resumes normal function within 1 hour after exercise (Zambraski, 1990) and that MDA collects in the kidney 30 min after blood levels are elevated (Marnett et al., 1985), then urinary MDA excretion should increase within 1 hour of the termination of exercise. Therefore, it is likely that urinary MDA values measured 30 min postexercise reflect blood MDA levels immediately postexercise. Perhaps the significant increases in urinary MDA found 30 min

postexercise in control subjects reflect a protective effect of excretion by reducing circulating levels of MDA, which is itself a mutagen (Basu & Marnett, 1984; Yau, 1979).

Lovlin et al. (1987) found an increase in plasma MDA in response to maximal exercise, as would be expected. However, their results following submaximal exercise differed from previous studies in that they found a significant decrease in plasma MDA levels during exercise at 40% mVO_2 and no change in MDA at 70% mVO_2. The difference between the Lovlin et al. results and the results of the present study may be related to exercise duration. In this study, subjects were exercised at 70% mVO_2 on a treadmill for 30 min. Previous studies that document a rise in LP indicators in response to submaximal exercise used durations of 20 min (Balke et al., 1984) or 1 hour (Dillard et al., 1978). In addition, Davies et al. (1982), who found a twofold to threefold increase in muscle and liver free radical concentrations when submaximal exercise was taken to exhaustion, suggested that free radical-induced damage is cumulative and depends mainly on the duration of work. Lovlin et al. exercised subjects at submaximal intensities for only 5 min, and that may not be long enough for free radical levels to increase. In keeping with most previous research findings, data from the present study provide evidence that prolonged submaximal exercise elicits an increase in LP indicators in untrained humans.

Effects of Vitamin E on Resting and Exercise LP Indicators

In previous investigations it has been found that vitamin E

supplementation lowers resting MDA levels (Dillard et al., 1978; Kanter, Nolte, & Holloszy, 1990). In the present study, 3 months of vitamin E supplementation showed a trend towards this effect, however, this result was not statistically significant at the .05 level. This discrepancy may simply be the effect of the small number of subjects who received supplementation (n = 16) in this study. The discrepancy may also be due to different dosages of vitamin E used in these studies. Dillard et al. and Kanter et al. used 1200 IU and 800 IU d-alpha-tocopherol daily, respectively, but subjects in this study consumed only 400 IU each day. Unfortunately, no pre- or postsupplementation measurements of vitamin E concentrations were made in this study. information that might have further helped to explain the discrepancy between this study and previous investigations. Assuming that lower resting levels of MDA were a result of higher vitamin E levels in previous studies, appropriate supplementation of vitamin E may provide an important antioxidative defense against the production of free radicals. This resting benefit may not be evident at the lower supplement dosages used in the present study. Considering the potentially harmful effects of LP (Jenkins, 1988), the use of vitamin E supplementation deserves further investigation.

Although the results of the present study show that urinary MDA levels increased significantly ($p < .05$) after 30 min of exercise at 70% mVO_2 in control subjects, urinary MDA levels actually decreased significantly ($p < .05$) in response to the

same exercise bout in subjects who had taken 400 IU d-alpha-tocopherol for the previous 90 days. Based on these results, it appears that vitamin E supplementation not only attenuates, but may actually decrease free radical production seen with exercise.

Dillard et al. (1978) and Kanter et al. (1990) both noted attenuation of exercise-induced increases in LP indicators after vitamin E supplementation, but it is difficult to explain the present decline in urinary MDA seen after exercise in subjects taking vitamin E. The same form of vitamin E (d-alpha-tocopherol) was used as in these previous studies, but the present study showed a more extreme vitamin E effect using a lower daily dosage (400 IU) than Dillard et al. (1200 IU) or Kanter et al. (800 IU). In all these studies, the supplementation duration (90 days) and timing of the last dosage were similar, making these unlikely causes for difference. Similarities in exercise intensities and durations between the studies also negate the likelihood that these are reasons for the difference.

One explanation for the different effect of 400 IU daily vitamin E supplementation on exercise MDA may involve the use of urinary MDA as an indicator of LP, rather than using expired pentane or blood MDA measures. Because no marker of renal function (creatinine or inulin) was measured in this study, it is impossible to determine whether the effects of exercise on renal function may have influenced these measurements. Zambraski (1990) pointed out that renal function resumes normal levels

within 1 hour postexercise. At this time, it can only be assumed that urinary MDA concentrations from samples collected 30 min postexercise are an appropriate measure for the occurrence of LP. Finally, though a strict bladder clearance and hydration protocol was used prior to exercise, it is impossible to be sure that collected urine samples were actually produced during or after exercise. It is conceivable, though unlikely, that these samples consisted of filtrate collected before the onset of the hydration protocol. However, these explanations seem unlikely because control subjects underwent an identical protocol to supplemented subjects and showed results similar to previous studies in nonsupplemented subjects (Dillard et al., 1978; Balke et al., 1984).

The results of this study showed a reversal of the increase in LP indicators in response to exercise due to vitamin E supplementation, which is similar to the work of Sumida et al. (1989) and generally supports the concept that vitamin E inhibits the increased production of MDA associated with exercise-induced LP. Vitamin E, therefore, helps avoid the potentially harmful side effects of increased free radical production. In addition, it appears that urinary clearance of MDA occurs quickly after exercise, and may itself be a defense mechanism.

The Effect of Iron and Vitamin E on LP

In the present study, urinary MDA levels from subjects who took a combination of vitamin E and 325 mg ferrous sulfate did not differ significantly across all measurements from subjects

who took only vitamin E ($p > .05$). However, when the two groups were combined into one supplementation group, postexercise MDA levels were significantly lower than levels for the control group ($p < .05$). Dillard et al. (1984) found that intraperitoneal iron injection in rats led to a significant increase in LP indicators, but these increases were significantly attenuated by a variety of antioxidants, including vitamin E. The results of the present study suggest that iron supplementation similar to the doses used here, in combination with vitamin E supplementation, do not lessen the protective effect of vitamin E alone in preventing MDA production via free radical-mediated reactions. Because extremely elevated iron levels are associated with increases in LP (Dillard et al., 1984; Rachmilewitz et al., 1976), further study should be focused on higher supplementation doses of iron.

The Relationship of Lactic Acid and LP

The results of the present study show no linear relationship between lactate (HLA) and urinary MDA levels in response to submaximal exercise ($r = -.294$). Lovlin et al. (1987) found a significant correlation ($r^2 = .51$; $p < .001$) between HLA levels and plasma MDA levels. The results of Lovlin et al. (1987) reflect greater HLA and MDA levels after exercise at 70% mVO_2 than after exercise at 40% mVO_2. It is well documented that HLA accumulation rises with increased exercise intensity. It is also not surprising that MDA levels would be higher after exercise at 70% mVO_2, because another study has shown increases in LP indicators with a similar exercise intensity (Dillard et al.,

1978). However, because no documentation exists to show that MDA production increases in response to exercise at 40% mVO_2, it is unknown whether the lack of an increase in MDA at this intensity occurred because of insufficient lactate accumulation or lack of a significant oxidative stress caused by exercise. Because of the lack of LP research at such mild exercise intensities (i.e., 40% mVO_2), it seems premature to suggest that a relationship between HLA and MDA exists. Moreover, no acceptable rationale to suggest a causal relationship between HLA and MDA can be presented at this time, nor was one presented by Lovlin et al. In the present study, HLA and MDA production were measured across subjects at one exercise intensity, and no linear relationship existed between the postexercise levels of the two metabolites. It seems that no biochemical relationship exists between MDA and HLA accumulation, however, a spurious relationship is likely to exist between the two, with exercise intensity being the moderating variable.

Summary

It is known that maximal exercise elicits an increase in LP indicators (Davies et al., 1982; Lovlin et al., 1987). The results of the present study show this increase is also reflected in urinary MDA levels following submaximal exercise, bolstering a growing pool of evidence suggesting submaximal exercise promotes LP (Balke et al., 1984; Dillard et al., 1978). The present study also showed that vitamin E supplementation reduced the production of urinary MDA after exercise. These results are similar to

those of Sumida et al. (1989) and indicate that vitamin E somehow inhibits LP associated with the increased oxidative stress of exercise.

Iron overload is associated with increased LP (Dillard et al., 1984; Rachmilewitz et al., 1976). Data from the present study indicate that combining a typical daily dosage of ferrous iron with vitamin E supplementation did not influence the protective effect of vitamin E supplementation ($p > .05$). In this experiment, no linear relationship was found between postexercise circulating HLA and urinary MDA levels. It would seem that no direct biochemical relationship exists between HLA and MDA production.

Chapter 6

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This study was designed to determine the effects of vitamin E and vitamin E plus iron supplementation on malondialdehyde (MDA) production at rest and in response to a submaximal bout of exercise. In addition, this study was designed to determine if a relationship existed between lactic acid and MDA production. The subjects were 25 undergraduate female students from Ithaca College.

Resting urinary MDA levels were measured. This was followed by a 3-month supplementation period. Urinary MDA measures were then analyzed before and after 30 min of treadmill exercise at 70% mVO_2. Repeated measures ANOVA was used to determine if any significant differences existed in MDA levels across sampling times or between groups. Results indicated that controls showed a significant rise in postexercise MDA levels over preexercise levels ($p < .05$), but the combined supplementation group showed significantly lower postexercise MDA levels than control levels ($p < .05$). These results are in agreement with previous findings that exercise causes increased LP (Davies et al., 1982; Dillard et al., 1978), however, they also indicate that vitamin E supplementation may reduce exercise-induced increases in LP. Iron supplementation does not significantly alter the protective effect of vitamin E supplementation, however, data from this

study do not conclusively dispel the idea that iron may have some effect on vitamin E.

Blood samples were taken before and 30 min after the submaximal exercise session and analyzed for lactate (HLA) concentrations. No significant linear correlation existed between postexercise HLA and MDA levels.

Conclusions

The results of this study led to the following conclusions regarding the effect of submaximal exercise and vitamin E supplementation on MDA production:

1. Urinary MDA levels increase significantly in response to 30 min of exercise at 70% mVO_2 in control subjects.

2. Supplementation with vitamin E not only attenuates the increased MDA production in response to exercise (as found in control subjects), but actually elicited a significant decrease in urinary MDA levels in response to exercise. This effect was not significantly altered when an iron supplement was combined with vitamin E supplementation.

3. No linear relationship exists between lactate and MDA production.

Recommendations

The following recommendations for further study were made after completion of this investigation:

1. Further study should be directed toward determining if larger doses of iron (i.e., dosages used by patients with anemia) or ferric iron supplementation affect the action of vitamin E in

inhibiting oxygen free radical formation. In such a study, pre- and postsupplement blood levels of vitamin E and iron should be taken to determine the effect of the supplementation protocol.

2. Investigation of the exact mechanism by which vitamin E suppresses LP is necessary. Further study should be focused on the interaction of vitamin E supplementation with oxygen free radicals and/or antioxidative enzyme systems in cellular membranes.

3. A training study should be conducted to determine whether aerobic and anaerobic training influence MDA levels.

4. A similar study combining a submaximal test with a maximal test should be conducted to examine whether exercise intensity influenced the protective effect of vitamin E.

5. A controlled time course study should be conducted using blood and urine samples to clarify the role of urinary MDA in removing MDA produced during exercise.

6. A study of varying exercise durations should be used to determine the importance of exercise duration to the rate of LP.

Appendix A

RECRUITING CONVERSATION

My name is Chris Baldi. I am a graduate student working on my Master's Degree in Physical Education at Ithaca College. I am conducting a study related to the body's use of oxygen during exercise. Harmful by-products are formed when the body consumes oxygen. Certain chemicals stimulate or inhibit the formation of these harmful by-products. Iron, for example, seems to stimulate the process, but vitamin E inhibits it. My project is designed to determine whether exercise, which increases the body's oxygen consumption, increases production of these harmful by-products, and if iron and vitamin E have any effect on this.

I am looking for 30 female volunteers who have not actively engaged in a regular exercise program or a sport program within the last 6 months to participate in this study. Your time involvement will be 3-4 hours, during which you will be required to allow me to record your oxygen consumption ability while you exercise on a treadmill. In addition, urine samples and small blood samples from your finger would be needed.

If you are interested in participating in this study or would like any further information, please write your name and phone number down, and I will contact you.

MEDICAL HISTORY QUESTIONNAIRE

NAME _____ AGE _____ SEX _____ DATE _____

CHECK IF YES

PAST HISTORY
(In the past have you ever had?)

- Rheumatic Fever ( )
- Heart Murmur ( )
- High Blood Pressure ( )
- Heart Trouble:
  - Rhythm Abnormalities ( )
- Disease of Arteries ( )
- Varicose Veins ( )
- Lung Disease ( )
- Injuries to back, knees, ankles ( )
- Epilepsy ( )
- Diabetes ( )
- Stroke/Heart Attack ( )
  - How long ago? _____
- Operations ( )
  - What Kind? _____
- Other ( )

FAMILY HISTORY
Have any blood relatives had? (parents, sister, etc.)

- Heart Attacks ( )
- Heart Operations ( )
- High Blood Pressure ( )
- High Cholesterol ( )
- Diabetes ( )
- Congenital Heart Disease ( )
- Other ( )

PRESENT SYMPTOMS
(Have you recently had?)

- Chest Pain ( )
- Shortness of Breath ( )
- Heart Palpitations ( )
- Lightheadedness ( )
- Cough on Exertion ( )
- Coughing up Blood ( )
- Back Pain ( )
- Arthritis ( )
- Swollen Legs ( )
- Use more than one pillow for sleep ( )
- Awaken short of breath ( )
- Loss of Consciousness ( )

If other is checked explain here: _____

RISK FACTORS

1. Smoking—Do you smoke? Yes ( ) No ( )
   - Cigarettes ( )
   - Cigars ( )
   - Pipe ( )
   - How long have you been smoking? _____
   - How many do you smoke a day? _____
   - Quit ( )
   - If quit, how many did you used to smoke a day? _____
   - For how many years did you smoke? _____
2. Have you gained ( ) or lost ( ) weight in the last two months? Yes___ No___ How much? ___
3. Do you presently engage in physical activity? For example: (Bowling, Tennis, Gardening) Yes _____ No _____
   - What Kind? _____
   - How Often? _____
4. How far do you walk each day? Approximately—1–2 miles _____ (Mild Activity)
   3–4 miles _____ (Moderate Activity)
   More _____ (Heavy Activity)
5. Is your occupation—Sendentary ( ), Inactive ( ), Active ( ), Heavy Work ( )
   Explain your occupation: _____
6. Do you have discomfort, shortness of breath, or pain with exercise? Yes___ No ___
   If yes, what type of exercise: _____
7. Are you taking any medications: Yes___ No___ If yes, name the medications: _____
8. When was your last physical exam? _____
   Were you instructed not to exercise at that time? _____

Appendix C

INFORMED CONSENT FORM

1. a) Purpose of the study. To determine whether exercise elevates the levels of malonaldehyde in the urine of humans and to determine whether iron or Vitamin E influences this event. Malonaldehyde is a by-product of a series of chemical reactions in the body involving oxygen, and it has detrimental effects if it exists at elevated levels.

b) Benefits. You will be aiding in research aimed towards understanding the body's use of oxygen during exercise. You will also receive information on your aerobic capacity and gain knowledge of oxygen use during exercise.

2. Method. The first testing day will require between 60 and 90 minutes of your time. After volunteering, you will be asked to give a urine sample. You will also perform a graded exercise test to exhaustion on a treadmill. You will then be asked to participate in one of three groups. One group will be asked to take a Vitamin E supplement for 3 months prior to beginning the exercise portion of the study. Another group will be asked to take Vitamin E and iron supplements for the same period. The final group will be asked to abstain from taking any supplements for an equal period of time prior to the exercise test. You will then be asked to participate in one more testing day, which will also require between 60 and 90 minutes of your time. The testing days during the exercise portion will be arranged at mutually

convenient times on weekdays from 7:00 to 12:00 a.m. or Saturdays and Sundays from 7:00 a.m. to 2:00 p.m. Testing sites will be on campus at the Exercise Physiology lab in Hill Center. Testing procedures will be as follows:

Day 1

You will be tested for maximum effort oxygen consumption ability (peak VO_2) which requires strenuous exercise on a treadmill for approximately 5-15 minutes. This will require your breathing into an air valve during your time on the treadmill and wearing a heart rate monitor. You will begin by simply walking slowly, then increasing speed and grade in stages of 1 or 2 minutes respectively. At the time you feel fatigued and want to stop, you will be slowed down to a walk while you continue breathing into the air tube for approximately 2 or 3 minutes. You will also be asked to provide a urine sample. Approximately 1 hour of your time is needed for explanation and testing.

Day 2

Two hours prior to exercising, you will void your bladder (no sample will be collected). You will then consume 500 cc of water (about 17 oz) within the next hour. Upon your entering the lab, a urine sample will be collected, and you will then consume another 500 cc of water. In addition, a small blood sample will be taken from your finger. You will then exercise on the treadmill for 30 minutes. The workload will be 70% of the peak VO_2 established during Day 1. You will again be wearing a heart rate monitor, however, you will not be breathing into an air

valve. Immediately after exercise, you will sit down while another blood sample is taken from your finger. You will then actively cool down by walking for 5-10 minutes. Thirty minutes after the completion of your exercise, you will supply another urine sample.

3. Will this hurt? Exercise at heavy and moderate workloads is required. There is not an extreme risk of physical harm, however, there is evidence that certain bodily changes may occur during exercise. In addition to normal response, these changes may include abnormal blood pressure, fainting, abnormal heart beat, and possibly heart attack or stroke. These adverse effects are very rare and occur in one of every 10,000 tests. These events become even more unlikely because we are dealing with a young healthy group of subjects and are monitoring you during the test. In addition, you will experience the discomfort associated with having blood drawn from your finger. In summary, the most likely negative effects of participation in this study are some transient and minor discomfort or temporary muscle soreness.

4. Need more information? Additional information can be obtained from either Chris Baldi (273-5648) or Dr. Gary Sforzo (274-3359). All questions are welcomed and will be answered.

5. Withdrawal from the study. Participation is voluntary. You are free to withdraw your consent and discontinue at any time. In addition, if you are unable to tolerate the exercise sessions or the blood samplings, you may withdraw from the study.

6. Will the data be maintained in confidence? In all written

records, you will be identified throughout the testing procedure by subject number (e.g., Subject 10) to ensure confidentiality of information collected. Subsequently data will only be reported in an anonymous fashion.

7. I have read the above, I understand its contents, and I agree to participate in the study. I further agree to accurately complete a medical history questionnaire prior to participation. I am 18 years of age or older and do not know of any physical restrictions that should preclude my participation in this during project.

_____ _____

Signature Date

24-HOUR HISTORY

NAME: _____ DATE: _____

TIME: _____

HOW MUCH SLEEP DID YOU GET LAST NIGHT? (Please circle one)

1 2 3 4 5 6 7 8 9 10 (hours)

HOW MUCH SLEEP DO YOU NORMALLY GET? (Please circle one)

1 2 3 4 5 6 7 8 9 10 (hours)

HOW LONG HAS IT BEEN SINCE YOUR LAST MEAL OR SNACK? (Please circle one)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 (hours)

LIST THE ITEMS EATEN BELOW:

_____

WHEN DID YOU LAST:

Have a cup of coffee or tea _____

Smoke a cigarette, cigar, or pipe _____

Take drugs (including aspirin) _____

Drink alcohol _____

Give blood _____

Have an illness _____

Suffer from respiratory problems _____

WHAT SORT OF PHYSICAL EXERCISE DID YOU PERFORM YESTERDAY?

WHAT SORT OF PHYSICAL EXERCISE DID YOU PERFORM TODAY?

DESCRIBE YOUR GENERAL FEELINGS BY CHECKING ONE OF THE FOLLOWING:

_____ Excellent _____ Bad

_____ Very, Very Good _____ Very Bad

_____ Very Good _____ Very, Very Bad

_____ Neither Bad nor Good _____ Terrible

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