Oxidative Stress and Antioxidant Defenses in Lymphocytes Following High Intensity Interval Training

by

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Abstract

A substantial body of evidence suggests strenuous exercise causes perturbations to the immune system and increases the formation of reactive oxygen species. Performing routine moderate exercise stimulates the immune system, while strenuous exercise has been shown to cause immunosuppression during recovery. Perturbations to these systems are known to occur from exercise, yet information regarding the interactions between antioxidants and the immune system is sparse. The purposes of this study were to: 1) examine the immune and oxidative stress responses following high intensity interval training (HIIT); 2) determine whether changes in antioxidant enzyme gene expression and enzyme activity occurs in lymphocytes following HIIT; and 3) determine whether changes in antioxidant expression and activity would improve lymphocyte cell viability following hydrogen peroxide (H_2O_2) exposure *in vitro.* Eight recreationally active males completed three identical high-intensity protocols. Each session was performed following an overnight fast. Blood samples were obtained pre-exercise, immediately post-exercise, 3hrs post-exercise, and 24hrs post-exercise. Total number of circulating leukocytes, lymphocytes, and neutrophils; as well as lymphocyte antioxidant enzyme activities, lymphocyte gene expression, lymphocyte cell viability, and plasma malondialdehye (MDA) levels were measured. Analytes were compared using a three (day) x four (time) ANOVA with repeated measures on both day and time. Significant differences were further explored using a Tukey post hoc analysis. The significance level for all analyses was set at $p < 0.05$. HIIT increased the number of lymphocytes and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). There were no significant increases in lymphocyte

SOD, CAT, or GPX gene expression. Circulating neutrophils significantly increased 3hrs post exercise. A significant increase in MDA was found following HIIT on Days 1 and 2. Lymphocyte cell viability following H_2O_2 exposure *in vitro* significantly increased on Days 2 and 3 compared to Day 1 of HIIT. Additionally, there was a significant decrease in cell viability at 3hrs compared to pre-exercise and 24hrs post-exercise. These findings indicate that lymphocytes respond to oxidative stress by increasing SOD, GPX, and CAT activity. Additionally, HIIT causes oxidative stress but did not induce a significant post-exercise lymphopenia. Analyses *in vitro* suggest lymphocytes may be fortified by increasing their antioxidant enzyme activities and become more resistance to subsequent episodes of oxidative stress. Furthermore, analysis *in vitro* confirms that lymphocytes are more vulnerable to cytotoxic molecules during recovery from exercise.

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CHAPTER I

INTRODUCTION

The term "oxidative stress" is an expression used to describe a range of deleterious states that result from an imbalance between the excessive formation of reactive oxygen species/free radicals (ROS/FR) and limitations in the antioxidant defense systems. An elevation in ROS/FR production that is unmatched by antioxidant defense mechanisms can be toxic to biomolecules, such as DNA, proteins, and lipids (58). This shift in the redox status of the cell from a "reducing" cellular environment to a more "pro-oxidant environment" has been implicated in the development of a number of pathologies, including atherosclerosis, hypertension, ischemia reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, immune dysfunction, aging, Parkinson's disease, and Alzheimer's disease (27, 53, 54, 118, 141). The process of redox regulation via ROS/FR and the antioxidant defenses within the cellular milieu represents a tightly controlled system that can have both deleterious and beneficial effects within the cellular environment.

Cellular sources of ROS/FR consist of the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, peroxisomes, and nitric oxide synthase (NOS) (33). The immune system is unique in that ROS/FR production by leukocytes that make up the immune system provides both humoral and cellular protection against infectious agents that initiate inflammatory responses (104). This acute immune response can occur following surgery, trauma, burns, infectious diseases, and muscle damage (82). The activation of the immune system triggers macrophages, neutrophils, lymphocytes, and other

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phagocytic cells to the sites of inflammation. During this process these phagocytic cells generate ROS and FR, which can be used for cellular signaling, killing of infectious organisms, induction of apoptosis, and stimulation of antioxidant repair processes (104).

Physical activity (both aerobic and anaerobic) is accompanied by an increased ROS/FR production (3, 5, 72, 90, 139). Additionally, during the post-exercise recovery period, the acute immune response resembles that of infection and inflammation (77, 93, 94, 114, 128, 130). The immune response during recovery has also been shown to be dependent on intensity and duration of exercise (91). The increase in oxidative stress seen during exercise and the subsequent elevation in the acute immune response during recovery from exercise provides a unique opportunity to study the relationship between stress and immunity.

High intensity interval training (HIIT) is a unique form of exercise that consists of both an aerobic and anaerobic component during exercise. Recent findings by a number of researchers have shown HIIT to yield rapid cardiovascular, metabolic, and performance adaptations in as few as six exercise sessions (13, 14, 15, 41, 42). Additionally, it has been suggested that HIIT provides a more powerful stimulus to modulate impaired fasting glucose/impaired glucose tolerance in pre-diabetic individuals than traditional exercise guidelines (40%-80% maximum aerobic capacity) (33). Based on these recent findings HIIT appears to be an efficient form of exercise that yields rapid improvements in health and fitness. The oxidative stress and immune response following HIIT has yet to be explored and may provide a unique opportunity to examine these paradoxical responses that are often present in pathological states.

Physical activity can consist of aerobic, anaerobic, or resistance types of activities. When assessing the oxidative stress and immune response following exercise it is often difficult to

compare results because of the vast differences between protocols (37). Performing routine exercise of any type is likely favorable compared to sedentary behavior, as trained athletes have been shown to have greater immunity against various toxins than untrained counterparts (86, 95). Therefore, the oxidative stress response during exercise may play a role in immune cell function when regular exercise is performed. The increased ROS/FR formed during exercise may up-regulate endogenous antioxidant enzyme expression in immune cells, perhaps enabling greater protection from subsequent bouts of oxidative stress. Thus, the purpose of this study was to examine the acute immune and oxidative stress response following HIIT and the subsequent changes in antioxidant enzymes (superoxide dismutase, glutathione peroxidase, and catalase) within isolated lymphocytes.

CHAPTER II

REVIEW OF LITERATURE

The benefits of routine exercise are well known to improve overall health, while physical inactivity is a primary or secondary risk factor for the development of chronic diseases, such as cardiovascular disease, hypertension, atherosclerosis, obesity, diabetes, osteoporosis, and cancer (1, 42, 51, 122). Routine physical activity enables the body to develop adaptive responses that lead to an improvement in overall health. While these short and long term benefits (such as increased HDL cholesterol and lower LDL cholesterol, increased lean muscle mass, reduced fat mass, lower resting heart rate, increased myocardial contractility, and improved immune function) occur when routine exercise is performed, the acute response from exercise is often paradoxical in nature, both enhancing and suppressing skeletal, cardiovascular, respiratory, and immune function (68, 122). It is well known that exercise leads to improved health and fitness. The exercise induced change at the whole body and cellular level likely follow the principle of "hormesis", which states that both stress and toxins at low doses may promote good health, but high doses appear to be detrimental or even lethal to the biological system (55, 102).

Exercise imposes both enhancing and suppressing effects on the immune system, that are directly related to the type, intensity, and duration of exercise (93). Exercise induced physiological changes to the immune system have been documented following short term high intensity (> 70% VO_{2max}), resistance, and long duration (> 1 $^1/_2$ hrs) types of exercise (74, 75, 80,

81, 112). The post-exercise response is similar to that seen with both infection and inflammation, which consists of an increase in circulating neutrophils (neutrophilia) and a decrease in circulating lymphocytes (lymphocytopenia) (77, 93, 94, 114, 128, 130). These interactions between exercise and the immune system provide a unique opportunity to link clinical and basic physiology, while evaluating stress and immune responses.

The immune response occurs following stressors, such as surgery, trauma, burns, infectious diseases, and muscle damage (93). The activation of the immune system triggers macrophages, neutrophils, lymphocytes, and other phagocytic cells to the site of inflammation. During this process these phagocytic cells generate reactive oxygen species (ROS) and free radicals (FR), which are involved in cellular signaling, killing of infectious organisms, induction of apoptosis, and stimulation of antioxidant repair processes (104). While ROS/FR are involved in a number of functional signaling processes within the cellular milieu, they are also believed to be involved in a number of pathological processes such as atherosclerosis, hypertension, diabetes, endothelial dysfunction, and neurodegenerative diseases (27, 53, 54, 118, 141). The immune response involves the recruitment of immune cells to the blood and other tissues, which utilize ROS/FR production to mediate the phagocytic response to the specific stressor.

Immunology research on exercise has focused on the suppressed immune system during the post-exercise recovery period, but it may be that the immune cells involved in these processes follow the principle of hormesis. Specifically, the increased oxidative stress response (partly induced by phagocytic cells) generated from increased ROS/FR production may actually up-regulate the antioxidant defenses within the immune cells themselves, perhaps rendering them less susceptible to the oxidative stress response during subsequent bouts of exercise. This

hypothesis appears likely, as trained athletes have been shown to have greater immunity against various toxins then untrained counterparts (86, 95). The immune cells provide one of the first lines of defense against infectious diseases and inflammation, perhaps the up-regulation of the endogenous antioxidant enzymes in immune cells follows the same principle as myocardial pre-conditioning seen in cardiac tissue following exercise (97). Nevertheless, the role of oxidative stress and the antioxidant defense systems role in immune cells remain to be determined.

The purpose of the following review is to 1) provide a detailed description of the biochemical and molecular properties of ROS/FR, 2) discuss the mechanisms and sites of ROS/FR production, 3) discuss the role of the cellular antioxidant defense systems, 4) describe oxidative stress and immune cell responses during various types of physical activity, 5) discuss current methods used to measure oxidative stress, and 6) describe the purpose of this study and methodology employed to examine these changes in immune function and antioxidant defenses following exercise.

Biochemistry and Molecular Properties of Free Radicals and ROS

Free radicals are highly reactive molecules or molecule fragments with one or more unpaired electrons in the valence shells (98, 117). ROS are oxygen-containing molecules that either may or may not have unpaired electrons but are highly reactive in tissues (141). An electron transfer that requires a high-energy input produces ROS/FR. Additionally, new radicals can be formed by previously formed radicals when reacting with other radicals or molecules (117).

Oxidative stress can be defined as an imbalance between ROS/FR production and the antioxidant defense mechanisms of a biological organism that results directly or indirectly in

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cellular damage (63). This imbalance can lead to the accumulation of oxidative damage in cell constituents, including DNA, proteins, and lipids. Generation of ROS/FR is considered a normal process in the life of aerobic organisms, and has a prominent role in maintaining cellular redox equilibrium. ROS/free radicals can be produced from a number of different mechanisms within the body; such as the mitochondrial electron transport chain, peroxisomes, NADPH oxidase, arachidonic acid metabolism, nitric oxide synthase, and xanthine oxidase (34).

Exercise is associated with a dramatic increase in oxygen uptake by the whole body and particularly by skeletal muscle (7). Most of the oxygen consumed by the body is utilized in the mitochondria for substrate metabolism and ATP production. An increased ATP demand accompanying exercise increases both aerobic and/or anaerobic metabolism, resulting in increased formation of ROS and FR that shift the cellular environment from a reduced state to a more oxidized state (27, 101). The potential sources of ROS/FR formation have been documented in the literature, but their exact roles remain to be determined (36).

The presence of low concentrations of ROS/FR are important for normal cellular redox status, tissue function, apoptosis, stimulation of antioxidant defense molecules, and intracellular signaling processes (91, 101). Their steady state concentrations are determined by the balance between their rates of production and their rates of removal by various antioxidants (116). The redox state of a cell is kept within a narrow range under normal conditions, similar to the manner in which a biological system regulates its pH. During high concentrations of ROS/FR formation, oxidative stress occurs, which is believed to be involved in muscle damage, aging, and a number of pathological processes such as cachexia, atherosclerosis, cancer, ischemia/reperfusion, inflammation, rheumatic arthritis, diabetes, immune dysfunction, endothelial dysfunction, and

neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (53, 54, 101, 142).

Therefore, only excessive ROS/FR production and/or decrease in detoxification mechanisms

lead to oxidative stress and the development of pathological conditions.

Mechanisms and sites of free radical and ROS production: ROS/FR production continually

occur from exogenous origins (exposure to radiation, air pollutants, intoxication by oxygen,

smoke, and alcohol), or from endogenous origins such as oxidative phosphorylation within the

mitochondria or extramitochondrial sources (peroxisomes, neutrophils, endothelial cells, plasma)

(22, 66, 132). Aerobic organisms require oxygen (O_2) in order to accept electrons during the

oxidation of energetic substrates. The generation of ROS/FR can occur via programmed

formation in the immune system or un-programmed formation during oxidative phosphorylation

(37). While ROS/FR production can occur via programmed or un-programmed formation, it is

widely believed that one of the forms and the majority of radical production during exercise are

due to a leak of electrons in the mitochondrial electron transport chain (111). Even during basal

metabolic states, radical formation can and does occur (111). Previous work and current

evidence on ROS/FR formation during resting and exercise conditions will be discussed

thoroughly in the following paragraphs.

Programmed free radical and ROS formation: In the immune system, neutrophils, lymphocytes,

and macrophages are critical components in the host defense response against antigens. These

immune cells produce superoxide ($O_2^{.-}$) by reacting with the reduced nicotinamide adenine

dinucleotide phosphate system (NADPH)-oxidase system (NOX). During this process two O_2

molecules interact with NOX, which consists of two membrane-bound elements (gp91^{PHOX} and

p22^{PHOX}), three cytosolic components (p67^{PHOX}, p47^{PHOX} and p40^{PHOX}), and a low-molecular-weight G protein (either rac 2 or rac 1), to generate O_2^{-} in the following reaction (6).

$$2\ O_2 + NADPH \Rightarrow 2\ O_2^{-} + NADP^+ + H^+$$
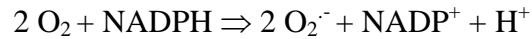
The function of NOX in phagocytic cells is to provide agents that kill organisms that are in contact with phagocytes (6). This is important in physiological processes that involve infection, inflammation, and any other antigen response that initiates an immune response. Thus, an important quantity of ROS/FR can be formed from immune cells and plays an essential homeostatic role (35). However, this process can become exaggerated in some situations, such as the initiation of hypertension and atherosclerosis, and may actually generate large amounts of O_2^{-} leading to oxidative stress (110). O_2^{-} rapidly reacts with nitric oxide (NO), forming the radical peroxynitrite (ONOO-) in the process (110). This is an important process that can lead to a cascade of various pathological states. NO is an important mediator of endothelium-derived relaxation and overall vascular function, therefore reducing the NO bioavailability can lead to endothelial dysfunction and development of hypertension and atherosclerosis.

Un-Programmed free radical and ROS formation: During oxidative metabolism, oxygen receives two electrons and is reduced to water (H_2O), oxygen can also receive a single electron at a time and be converted into an O_2^{-} becoming highly reactive.

$$O_2 + e^{-} \Rightarrow O_2^{-}$$
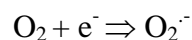
In this case, molecular oxygen typically reacts with species generated by single electron transfers, such as electron leaks between iron-sulfur clusters, Complex I and ubisemiquinone, and between ubisemiquinone and Complex III (11, 29). Sources of ROS/FR formation will vary

from organ to organ and depend on whether the mitochondria are actively respiring or highly

reduced (8). For example, Complex III appears to be responsible for most of the O_2^- production

in heart and lung mitochondria (135, 136), whereas Complex I appears to be the primary source

in the brain during normal conditions and is the primary source of ROS/FR in a variety of

pathological diseases ranging from aging to Parkinson's disease (8, 9).

During the Q cycle, ubiquinol (QH_2) binds to the Q_0 site of Complex III, while

ubiquinone (Q) binds to the Q_i site of Complex III. QH_2 transfers the first electron to the Rieske

iron-sulfur protein and the second electron is transferred to the b_L heme, resulting in the

production of a transient semiquinone (QH). The Rieske iron-sulfur protein becomes freed from

QH_2, and the first electron is transferred to an oxidized cytochrome C, which then continues

along the electron transport chain to molecular oxygen. The electron passed by QH_2 to the b_L

heme is used to reduce b_H heme, which then transfers the electron to Q at the Q_i site forming a

QH radical. A second Q cycle is necessary to transfer a second electron from cytochrome b,

reducing the QH to QH_2 (16, 134).

In the respiratory chain, the vast majority of the oxygen consumed is reduced to water by

a tetravalent reduction reaction catalyzed by coenzyme Q (CoQ), and utilizing energy from

electron transfer along the electron transport chain (35).

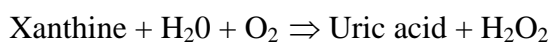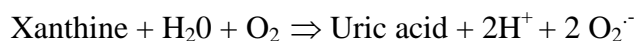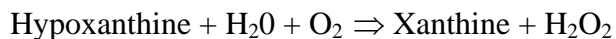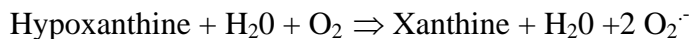$$O_2 + 4e\text{-} + 4H^+ \Rightarrow 2H_2O$$

However some O_2 forms O_2^- (65). It has been estimated that for every 25 oxygen molecules

reduced by normal respiration, one free radical is produced (21). This is an important concept, as

the rate of whole body O_2 consumption during exercise may increase 10-15 fold, and that O_2 flux

in active muscle may increase about 100 fold, leading to greater leak of electrons from the electron transport chain (119).

Under normal conditions QH is reduced to QH_2, whereas in conditions when electron transport is slowed by high membrane potential the O_2 dissolved in the membrane can react with QH and generate O_2^- (66). There is continuing debate on the exact sites of O_2^- production *in vivo*, but the best understood site is the Complex III contribution (66). O_2^- is released into the matrix at the Complex I site, whereas Complex III releases O_2^- to both sides of the inner membrane. Due to the proximal location of the Q_o site to the intermembrane space and the negative membrane potential within the matrix, the O_2^- should be expelled from the membrane with a higher probability to the cytosolic side (52). Nevertheless, half of the O_2^- produced at Complex III is released into the matrix (52).

It is generally accepted that exercise causes an increase in oxygen consumption by the mitochondria, which in turn yields an increase in ROS/FR formation within these organelles. However, some researchers feel the role of the mitochondria in the formation of ROS/FR in exercise may need to be recalculated (46). Early work by Chance et al. (1979) revealed that approximately 2% of oxygen used by the mitochondria is converted to ROS/FR only when these mitochondria are in the resting state (State 4 respiration) (21). However, when mitochondria are in State 3 respiration (ATP actively produced from ADP, with a high electron flow into oxygen), the proportion of oxygen converted to ROS/FR falls to a tenth of the resting state (46). Therefore, a number of researchers have begun to examine a number of extracellular sources of O_2^- associated with exercise, particularly xanthine oxidase (XO) (57, 92, 106).

Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid, generating either O_2^- or hydrogen peroxide (H_2O_2) in the following reactions:

$$Hypoxanthine + H_2O + O_2 \Rightarrow Xanthine + H_2O + 2\ O_2^-$$

$$Hypoxanthine + H_2O + O_2 \Rightarrow Xanthine + H_2O_2$$

$$Xanthine + H_2O + O_2 \Rightarrow Uric\ acid + 2H^+ + 2\ O_2^-$$

$$Xanthine + H_2O + O_2 \Rightarrow Uric\ acid + H_2O_2$$

XO has been found to generate free radicals during aerobic exercise, anaerobic exercise, and resistance training (46, 100, 140). The substrates for xanthine oxidase are hypoxanthine and xanthine which are derived from the degradation of ATP to AMP. Therefore, these substrates are likely only available during high intensity or exhaustive exercise. Gomez-Cabrera et al. 2003, reported that allopurinol (xanthine oxidase inhibitor) was able to protect against cell damage associated with exhaustive exercise in humans (12). The exact role of XO generation of ROS/FR during exercise remains largely unknown. ROS/FR's not only cause damage but also have a role in cellular signaling and may be vital in initiating transcription factors that are associated with cellular adaptation following exercise (45).

Properties of free radical and ROS's: The production of O_2^- warrants particular attention, although O_2^- is not highly toxic, it is the precursor to many other ROS/FR that can induce cellular damage. Therefore, it is essential for the cell to tightly regulate O_2^- production and removal. Once O_2^- is produced it can be converted to H_2O_2, catalyzed by the superoxide dismutase (SOD) enzyme (37).

$$2O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2$$
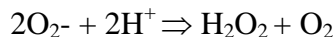
The mitochondrial matrix contains a specific form of SOD containing manganese in the active site (MnSOD), thus eliminating O_2^- formed in both the matrix and inner side of the inner membrane (68). The steady state concentration of O_2^- in the intermembrane is controlled by a different SOD isozyme containing copper and zinc instead of manganese (CuZnSOD) (50). Both isoforms work in conjunction to maintain optimal levels of $[O_2^-]$. It is important to note that in biological tissues, O_2^- can also be converted nonenzymatically into the nonradical species H_2O_2 and O_2 (125).

H_2O_2 is not considered a free radical because it has no unpaired electron, but is considered a ROS because of its toxicity and ability to form other ROS/FR (37). Once H_2O_2 is produced, it can easily penetrate through membranes because of its uncharged properties and low reactivity (66). Regulation of H_2O_2 is critical, as it is the main precursor to the hydroxyl radical (OH•) (37, 66, 134). This reaction proceeds via transition metal catalyzed reactions, most notably Fe^{2+} in the Fenton reaction.

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH\bullet + OH^-$$

The OH• is an extremely reactive oxidizing radical that reacts with most biomolecules at diffusion–controlled rates (66). The scavenging of this radical is an unlikely mechanism of action for any antioxidant *in vivo*. Once OH• is formed in the matrix, it likely does not cross the inner membrane, even though it is hydrophobic, because its average diffusion distance during its half-life is only about 2nm (66). These highly reactive radicals attack proteins, DNA, and induce non-enzymatic lipid peroxidation when present in the cytosol (37, 67). When produced in the matrix

side, they can also attack mitochondrial DNA (89). In order to minimize cell and tissue damage, the increased production of ROS must be matched by ROS detoxification mechanisms.

Role of the Cellular Antioxidant Systems

Under normal conditions there is a balance between ROS/FR formation and the antioxidant defense mechanisms. Subtle shifts in the redox balance are utilized in a vast array of cellular signaling pathways within the cell (69). In order for ROS/FR to induce tissue damage, there must be a sustained increase in ROS/FR that is not matched by the antioxidant defense systems (68). Exercise can produce an imbalance between ROS/FR and antioxidants. There is now strong evidence that strenuous physical exercise may be associated with an increased ROS/FR generation due to the dramatic increase in oxygen uptake (3, 29). The body is prepared to handle this increased ROS/FR production with an efficient antioxidant defense system consisting of antioxidant vitamins, thiols (glutathione (GSH) and sulfhydryls), and antioxidant enzymes (73). Antioxidant vitamins are involved in the direct trapping of free radicals and singlet oxygen; GSH plays an important role in the maintenance of cellular redox status, while the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)) catalyze the one-electron reduction of ROS (20, 79, 144). Enyzmatic antioxidants (SOD, CAT, GPX) and nonenzymatic antioxidants (Vitamin E, Vitamin C, GSH, β-Carotene, etc.) work in conjunction to detoxify ROS, preventing oxidative stress.

Antioxidant Enzymes: An antioxidant can be defined as a substance that helps to decrease the severity of oxidative stress either by forming a less active radical or by quenching the chain reaction of ROS and free radicals on proteins, lipids, carbohydrates, or DNA (31). As discussed above, SOD is the major defense against O_2^- and is the first line of defense against oxidative

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stress. The two other key antioxidant enzymes in the body are CAT and GPX (37). CAT and

GPX have similar primary functions, which is to decompose H_2O_2 to H_2O. GPX and CAT have

the same action on H_2O_2 but GPX is thought to be more efficient with high ROS concentration,

while CAT is more efficient with lower H_2O_2 concentration (4, 65). CAT can be found in every

cell (particularly cell structures that use oxygen in order to detoxify toxic substances), as well as

in mitochondria and other intracellular organelles (4). CAT converts H_2O_2 to water and oxygen

in the following reaction (37).

$$2\ H_2O_2 \Rightarrow 2\ H_2O + O_2$$
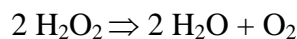
GPX is present in both the cell cytosol and mitochondria, and has the ability to transform H_2O_2

into water. This reaction uses reduced GSH and converts it into oxidized glutathione (GSSG)

using the enzyme GPX in the reaction (39).

$$2GSH + H_2O_2 \Rightarrow GSSG + 2\ H_2O$$
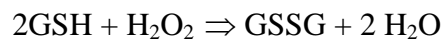
Adequate function of GPX requires the regeneration of GSH from its oxidative product GSSG

(68). This is accomplished by the glutathione reductase (GR) catalyzed reaction, utilizing

NADPH as the reducing agent.

$$GSSG + NADPH + H^+ \Rightarrow 2\ GSH + NADP^+$$

This reaction is important as it ensures that cells are kept in a reduced environment, which is

essential for the function for many enzymes and cofactors. GSH is a recyclable molecule,

making it an ideal antioxidant in the cell.

Thiols: Thiols are a class of molecules characterized by the presence of sulfhydryl residues (-SH) at their active site (120). They have numerous biological functions, such as protein synthesis, redox signaling, cell biogenesis and immunity, and play a major role in the antioxidant defense system. Already mentioned above, GSH is the major thiol present in an organism. GSH acts as a substrate for GPX in peroxidase ROS inhibition, can directly detoxify ROS, and enhances the functional antioxidant capacity of vitamins C and E (49, 75). GSH plays a multifunctional role in protecting tissues from oxidative damage. GSH reduces H_2O_2 and organic peroxides via GPX, acts as a scavenger of singlet oxygen and OH, and is used to reduce tocopherol radicals either by directly or indirectly reducing semidehydroascorbate radical (preventing lipid peroxidation) (68).

A loss of thiol proteins can appear during a period of oxidative stress, therefore changes in the GSH/GSSG ratio is a popular technique to assess oxidative stress (ROS and free radicals oxidize GSH into GSSG) (126, 131). When there is excessive ROS/FR production that exceeds the recycling capacity of GR, the GSSG levels rise, yielding a decreased GSH/GSSG ratio. In order to maintain this optimal ratio and to alleviate oxidative stress, cells are capable of exporting GSSG (79). Loss of thiols from repeated periods of oxidative stress are implicated in the etiology of neurodegenerative diseases, such as Parkinson's or Alzheimer's, and are observed in aging or following physical activity (125, 131).

In addition to enzymatic antioxidants and thiol compounds, there are non-enzymatic antioxidants that act on ROS/FR to limit oxidative stress. Vitamins E, C, A, β-carotene, uric acid, ferritin, albumin, and Coenzyme Q all help to prevent excessive oxidative stress (34, 58, 81, 90, 96).

Physical Activity and Oxidative Stress

Biological antioxidants play a vital role in protecting cells from exercise induced oxidative stress. There are an abundance of studies to correlate the impact of antioxidant status and exercise-induced oxidative damage (7, 25, 26, 136). An acute bout of exercise is known to increase activity of antioxidant enzymes (SOD, CAT, GPX) in skeletal muscle, heart, erythrocytes, lymphocytes and liver (17, 128). The threshold and magnitude of activation are different among enzymes and tissues (7). The exact mechanisms by which antioxidant enzymes are activated within a relatively short period of time during exercise are largely unknown (7).

Aerobic Exercise: In 1982, Davies et al. (29) were the first to show that exercise increases the production of ROS/FR in skeletal muscle. They used electron paramagnetic resonance spectroscopy to measure carbon-based radicals in rabbit muscle before and after exhaustive exercise. Jackson et al. (1985) subsequently extended this observation in humans, using the same technique to demonstrate increased ROS/FR in limb muscle following exercise (62). These findings showed that strenuous exercise increases biochemical indices of oxidative stress measured both systemically and in the working muscle (105).

Since this initial observation by Davies et al. (1982), there have been many studies investigating the effects of exercise on oxidative stress. Most of them have utilized methods incorporating aerobic exercise, involving running, cycling, and swimming (3, 5, 72, 90, 139). Performing aerobic exercise requires an increased VO_2, which may increase ROS/FR production. Therefore, many studies suggest that aerobic exercise increases ROS/FR production in animals and humans (3, 5, 72, 90, 139).

Lovlin et al. (1987) showed that antioxidant capacity is not overreached during exercise intensity less than 50% VO_{2max} (72). During exercise of this nature the antioxidant system is able to match the increased ROS/FR production, and radical-induced damage does not appear (72). Generally, the more intense the exercise bout, the greater the production of ROS/FR's and subsequent oxidative stress (72, 90). Ashton et al. (1998) (5) showed a correlation between oxidative stress and aerobic exercise intensity. However, other studies have shown no increase in oxidative stress following intense aerobic exercise (24, 30, 139). These studies consisted of 31km and 21km runs, and a 50k and 80k march. These contradictory results may be explained by antioxidant nutritional status, exercise intensity, training level, and methodology (37). Additionally, the mode of detection is also critical as the study showing an increase in oxidative stress used direct detection of ROS/FR (electronic spin resonance), while the studies showing no increase used indirect detection of oxidative stress (lipid and protein oxidation) (5, 24, 30, 138). In humans, a higher antioxidant enzyme activity was reported to correlate with VO_{2max}, and trained athletes were shown to have greater SOD and CAT activities in skeletal muscle (64).

SOD, CAT, GPX, and GR provide the first line of defense against ROS/FR generated during exercise (68). It has long been recognized that living organisms are capable of inducing the antioxidant defense system by relatively rapid mechanisms to cope with oxidative stress. Endurance exercise can modify both enzymatic and non-enzymatic antioxidant activity. Numerous studies have shown increased SOD, GPX, and CAT enzyme activity in both blood and tissue following aerobic exercise (25, 61, 67, 71). These adaptations may occur in as little as 5 minutes after ROS/FR production (96), however the increase of antioxidant enzyme activity does not appear to be proportional to exercise intensity (28).

Anaerobic Exercise: Anaerobic exercise is a form of exercise in which ATP is provided predominantly by the phosphocreatine or glycolytic energy systems, rather than oxidative phosphorylation. Information regarding the production of ROS as a result of anaerobic exercise is lacking compared with aerobic exercise (49). However, the studies that have been performed generally show an increase in oxidative stress following supramaximal exercise such as intermittent running, resistance exercise, and Wingate cycle tests (41, 44, 49, 76, 103).

The increase in ROS/FR during anaerobic exercise may be mediated similarly to aerobic exercise (via electron leakage along the ETC), or through other mechanisms, such as XO production, ischemia reperfusion, or phagocytic respiratory burst (49, 76, 113). Ischemic reperfusion in active skeletal muscle may be involved in oxidative stress during and after anaerobic exercise (113). During intense exercise, the purine nucleotide system is extremely active. The elimination of adenosine monophosphate (AMP) causes a buildup of hypoxanthine in skeletal muscle and plasma (56). Hypoxanthine is converted to uric acid via the xanthine dehydrogenase/xanthine oxidase system (XDH/XO). The XDH/XO catalyzed reaction reduces molecular oxygen to O_2^- and H_2O_2 (37). It has been hypothesized that during ischemia, oxygen concentrations are low while XO and hypoxanthine would be present in high concentrations in reperfused tissue. When oxygen is reintroduced, a burst of O_2^- and H_2O_2 can result, causing further tissue and cell damage (59).

Another potential source of ROS/FR production during anaerobic exercise arises from the inflammation and cellular damage following impact sports or eccentric exercises (76, 113, 115). Tissue hypoxia can lead to the freeing of transition metals (Fe and Cu) from their normal transporters, which can further catalyze free radical reactions (such as Fenton reaction) (17).

There is also a link between elevated lactate levels and the rise of oxidative stress markers. This would potentially reduce the concentration of NADH and NADPH, leading to an increased production of ROS (25).

Immune Response to Exercise: The benefits of regular moderate exercise on well-being and the immune system are well known (130). It has become clear that the immune response to exercise could be related to exercise intensity. Moderate exercise stimulates the immune system, while strenuous exercise induces immunosuppresion during the recovery period (93). The mechanisms associated with these exercise-associated immune changes include neuroendocrinological factors such as, epinephrine, norepinephrine, growth hormone, and cortisol, as well as oxidative stress (94).

Regular exercise causes perturbations, such as neutrophilia and lymphopenia, to the immune system (128). Neutrophilia has been documented following exhaustive exercise such as a marathon (114), while lymphopenia occurs following intense exercise (77). Lymphocyte concentration increases during exercise and falls below basal values after intense exercise of long duration (77).

Despite substantial evidence indicating that high intensity exercise induces oxidative stress and an acute immune response, there is little information assessing interaction between antioxidant defenses and the immune system during exercise (128). Antioxidant vitamins, such as vitamin E and vitamin C, have reportedly been found in large concentrations in neutrophils and lymphocytes, and play an important role in maintaining immune cell integrity and functionality (121). Lymphocytes and neutrophils express SOD, CAT, and GPX, and provide cellular defense against endogenous and exogenous sources of ROS/FR (127). It has also been

shown that lymphocytes increase their activity of SOD, CAT, and GPX in response to endogenous ROS (10).

The combination of duration and intensity of exercise yield differing results when assessing antioxidant enzyme activity in lymphocytes. Tauler et al. (2004) saw a decrease in lymphocyte antioxidant enzyme activity immediately following a maximal cycle ergometer protocol, but not following the submaximal protocol (128). In a follow up study, Tauler et al. (2006) found significant increases in GPX, SOD, and GR activity in lymphocytes following a mountain stage cycling protocol. (130). In addition to the two aforementioned studies, an additional study by the same group only found a significant increase in SOD enzyme activity and gene expression in lymphocytes following a cycling time trial performance (18). Morikawa et al. (2004) assessed levels of Mn-SOD mRNA in lymphocytes of high school boys subjected to 3 months of aerobic exercise training. They measured changes in Mn-SOD mRNA before and after a maximal exercise test. They found a significant increase in Mn-SOD mRNA following the max exercise test prior to the 3-month training protocol, but no difference pre and post max exercise test after the 3-month training cycle (83). From these results, the effects of exercise training on lymphocyte gene expression were able to improve Mn-SOD function for an acute bout of exercise (83). However, these results must be interpreted with caution, as the pre-exercise training baseline measures were made 48 hrs after the exercise session was completed and the post-exercise training samples were made 72 hours later. Hollander et al. (2001) confirmed increases in Mn-SOD expression in skeletal muscle of rats immediately after a single bout of exercise, one hour later and 2 hours later, but reported that they could not confirm significant changes after 48 hours (60).

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Hollander et al. (2001) hypothesized that Mn-SOD mRNA expression was increased in response to elevated oxidative stress resulting from the exercise (60). Work performed by Oh-shi et al. (1997) in rats, found elevated Mn-SOD mRNA expression in untrained rats, but not in the trained rats (88). The untrained group may have received greater damage from oxidative stress, whereas the trained group may have been equipped to withstand oxidative stress. Results appear to be equivocal as to whether exercise training improves antioxidant enzyme activity in lymphocytes. Some researchers have shown a decrease in antioxidant activity following an acute bout of exercise (128, 129), while others have demonstrated an increase in antioxidant activity (60, 88). Whether or not immune cells are able to adapt to the exercise conditioning remains to be determined.

High Intensity Interval Training

HIIT refers to repeated sessions of brief intermittent exercise, often performed at intensities > 90% of VO$_{2 \text{ Max}}$ (43). The duration of each interval depends on the training intensity; each effort may last from a few seconds up to several minutes in duration. Multiple efforts are separated by a few minutes of rest or low-intensity exercise that allow partial but often not a full recovery, depending on the effort exerted by the individual. Unlike strength training, which incorporates brief intense efforts performed against a heavy resistance to increase skeletal muscle mass, HIIT is normally associated with activities that require rhythmic muscle contractions such as cycling or running (42).

Recent HIIT Research

HIIT training can be incorporated into most exercise activities (swimming, cycling, running, rowing, skiing, etc.), but when taking place in a research environment it is convenient to

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utilize a cycle ergometer because of the ability to precisely control the work rate being performed. A common HIIT protocol used by a number of researchers (13, 14, 15, 43) utilizes the Wingate test, which involves 30 seconds of all-out maximal cycling against a high resistive force (0.075kp/kg body mass) on a specialized ergometer. The HIIT protocol consists of subjects performing the Wingate test four to six times, separated by 4 minutes of recovery. This protocol requires a total of 2 to 3 minutes of high intensity exercise per training session, and consists of three training sessions performed each week for 2 to 6 weeks (13, 14, 15, 43). The HIIT protocol performed by these researchers has yielded interesting physiological adaptations that are normally seen following ET lasting up to 6 hours per week rather than 1 hr of total HIIT per week.

Gibala et al. (2006) examined molecular and cellular adaptations in resting human skeletal muscle after six sessions of HIIT or ET performed over 2 weeks. By design, total training time commitment and exercise volume was lower in the HIIT group, yet skeletal muscle oxidative capacity and exercise performance were similar between groups (42). A recent follow up study by Burgomaster et al. (2008), using a similar protocol, found similar adaptations between selected markers of whole body and skeletal muscle carbohydrate and lipid metabolism. In addition, the total weekly exercise was 90% lower (225kJ versus 2250kJ per week) in the HIIT versus the ET group (14). Additionally, Rakobowchul et al. (2008) utilized this same model to compare peripheral arterial stiffness and flow-mediated dilation (104). They found similar improvements between the ET and HIIT group. Therefore, HIIT training is an appealing form of exercise training because it is a potent time-efficient strategy to induce similar metabolic and vascular adaptations that normally accompany traditional endurance exercise.

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HIIT is an interesting mode of exercise that consists of both an aerobic and anaerobic component during exercise. As mentioned above, the oxidative stress response during and following exercise often yields conflicting results. Some studies have shown no indication of oxidative stress following exercise of $< 50\%$ VO_{2max} (72) and long duration exercise (24, 30, 138), while others have shown an increase in oxidative stress following long duration exercise (5) and the response may be dependent on exercise intensity (72, 89). Additionally, anaerobic exercise has also been shown to increase oxidative stress (25, 44, 49, 76, 103). While anaerobic exercise, such as resistance training, may not consist of a significant increase in oxygen consumption; oxidative stress is still present (75). Based on the vast array of studies that have shown an increase in oxidative stress following aerobic and anaerobic exercise (3, 5, 25, 44, 49, 75, 102, 127, 128) it may be that the magnitude of oxidative stress is dependent on the energy balance within the cell. It is difficult to determine the specific sites of ROS/FR production during different duration and intensities of exercise. The mitochondria, XO, and NOX systems have all been implicated in the production of ROS/FR during exercise, but the exact contribution of each system during exercise remains to be determined. Based on these findings, using HIIT to examine the oxidative stress response during exercise and recovery from exercise allows the researcher to incorporate both an aerobic and anaerobic exercise component to maximize the oxidative stress response.

Determination of Oxidative Stress

Oxidative stress markers in humans have been measured in muscle, blood, and urine (25, 31, 49, 56, 61). The most commonly measured indirect markers are by-products of lipid

peroxidation, but changes in status of antioxidant compounds such as glutathione, protein and DNA oxidation, and antioxidant enzyme activities have been used (61). Electron spin resonance is a direct measure of free radicals and ROS, and has been used in many studies *in vitro* (5, 29, 61, 62).

Direct Detection of ROS: The electron spin resonance technique is a direct spectroscopic method that allows the direct measurement of ROS from their paramagnetic properties (5, 29). These measurements can be made *in vitro*, *in vivo*, or *ex vivo*, however, the most precise measurements made *in vivo* are not practical in humans because of the toxicity of the products used for such methods (26, 108). In addition, blood samples must be interpreted with caution because of the short-half life of the ROS, their strong ability to react with other molecules, and their weak concentration (5, 64).

Measurement of Protein Modification: Protein and DNA oxidation can be determined by changes in blood protein carbonyls. An increase of carbonyl is present in every phenomenon linked to oxidative stress (123). Thus, the measurement of carbonyl formation is the most usual method used to determine ROS damaged proteins (48). When ROS attack amino acids, carbonyl groups are produced and can be measured by HPLC or ELISA procedures (48, 80), however these methods have also been criticized as being non-specific and unreliable in human studies (61).

Measurements of Lipid Peroxidation: A basic approach to measuring oxidative stress is to measure the rate of peroxidation of lipids or fatty acids (37). When ROS are generated they can attack polyunsaturated fatty acids in the cell membrane leading to the chain of chemical reactions

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known as "lipid peroxidation". Determinants of lipid peroxidation include expired pentane, malondialdehydes (MDA), lipid hydroperoxides, isoprostanes, and conjugated dienes (137).

Lipid peroxidation can be measured by analyzing volatile hydrocarbon end products, such as pentane, hexane, and ethane in expired air. Pentane can be measured in expired air using a gas chromatographic technique; however this is a difficult technique, which explains its infrequent use (61, 80). This method is non-invasive but is not precise because these gases can be formed by pathways other than ROS production (108).

The most highly regarded method to determine lipid peroxidation is detection of F2-isoprostanes (84, 109, 143). F2-isoprostanes were discovered as lipid peroxidation products in 1990 by Morrow and colleagues; they are formed when arachidonic acid undergoes peroxidation *in vivo* (84). Because they are relatively stable molecules compared to lipoperoxides and aldehydes, they are considered as the most reliable markers of lipid peroxidation as recently evaluated by a multi-laboratory study of the National Institute of Environmental Health Sciences (70). Originally measured by gas chromatography/mass spectroscopy, there are now F2-isoprostane enzyme immunoassay kits that have been shown to be significantly correlated with the more sensitive gas chromatography-mass spectrometry method (99). F2-isoprostanes appear to be very useful to determine lipid peroxidation because they are stable and can be detected at very low levels (82). Increases in F2-isoprostane levels following exercise have consistently been shown by several investigators (74, 87, 112, 124).

The first well established determinant of lipid peroxidation is the measurement of Malondialdehyde (MDA). MDA is an aldehyde that has been frequently used as a marker of oxidative stress in response to exercise, and is measured by high pressure liquid chromatography

(HPLC), spectrophotometry, or spectrofluorescence (52). The most common method for MDA determinant is the thiobarituric acid (TBARS) assay, a low molecular weight lipid peroxidation product that mainly consists of MDA (61). The TBARS assay can determine lipid peroxidation in plasma, serum, urine, tissue homogenates, and cell lysates.

Conclusion and Purpose of the Study

In conclusion, based on recent findings, HIIT provides a stimulus that changes whole body and cellular function quite rapidly. HIIT provides both an aerobic and anaerobic component during exercise allowing us to assess the oxidative stress and immune response following exercise. While there is a large anaerobic component during this mode of exercise, there is also a significant oxygen requirement involved during HIIT. HIIT provides a maximum stimulus that may facilitate an increase in mitochondrial ROS/FR production, as well as an increase in the XO/NOX production of ROS/FR, leading to an increase in oxidative stress. This increased oxidative stress may appear detrimental to cellular function, but this non-lethal bout of oxidative stress may follow the principle of hormesis (which states that both stress and toxins at low doses may promote good health, but high doses appear to be detrimental or even lethal to the biological system) and actually up-regulate antioxidant defenses to protect the cell from future oxidative stress exposure.

As previously mentioned, the presence of low concentrations of ROS/FR are important for normal cellular redox status, tissue function, initiating apoptosis, stimulation of antioxidant and repair processes, and intracellular signaling processes (91, 101), while under high concentrations they are believed to be involved in muscle damage, aging, and the development of pathological processes such as cachexia, atherosclerosis, cancer, ischemia/reperfusion,

inflammation, rheumatic arthritis, and neurodegenerative diseases such as Alzheimer and Parkinson diseases (53, 54, 101, 142).

With these principles in mind, HIIT is a form of intense exercise that yields rapid changes in oxidative and glycolytic enzyme activity, improves exercise performance, and improves endothelial function (12-15, 42, 43, 104). These changes occur quite rapidly at the cellular level, therefore the immune cell response may respond to this stimulus in a similar fashion. There may be periods of post-exercise oxidative stress in which the immune cells are vulnerable to both necrosis and apoptosis, but these cells may be able up-regulate endogenous antioxidant enzyme defenses enabling them to combat future insults of oxidative stress.

Therefore, the aim of this study is to examine the acute immune response and oxidative stress response in recreationally active individuals following HIIT. This will be accomplished by measuring oxidative stress (TBARS), and mRNA and enzyme activity of the following endogenous antioxidants (SOD, CAT, and GPX) in isolated lymphocytes. In addition, isolated lymphocytes will be exposed to various concentrations of hydrogen peroxide to determine whether HIIT is able to protect the lymphocytes against a subsequent oxidative stress via up-regulation of endogenous antioxidant enzymes.

III. JOURNAL MANUSCRIPT

OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE RESPONSES IN LYMPHOCYTES FOLLOWING HIGH INTENSITY INTERVAL TRAINING

ABSTRACT

The purposes of this study were to: 1) examine the immune and oxidative stress responses following high intensity interval training (HIIT); 2) determine whether changes in antioxidant enzyme gene expression and enzyme activity occurs in lymphocytes following HIIT; and 3) determine whether changes in antioxidant expression and activity would improve lymphocyte cell viability following hydrogen peroxide (H_2O_2) exposure *in vitro.* Eight recreationally active males completed three identical high-intensity protocols. Each session was performed following an overnight fast. Blood samples were obtained at pre-exercise, post-exercise, 3hrs post-exercise, and 24hrs post-exercise. Total number of circulating leukocytes, lymphocytes, and neutrophils; as well as lymphocyte antioxidant enzyme activities, gene expression, cell viability, and plasma malondialdehye (MDA) levels were determined. Analytes were compared using a three (day) x four (time) ANOVA with repeated measures on both day and time. A Tukeys post hoc analysis was utilized when significant differences were found. The significance level for all analyses was set at $p < 0.05$. HIIT increased the number of lymphocytes and the lymphocyte activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). There were no significant increases in lymphocyte SOD, CAT, or GPX gene

expression. Circulating neutrophils significantly increased 3hrs post exercise. A significant increase in MDA was found following HIIT on Days 1 and 2. Lymphocyte cell viability following H_2O_2 exposure *in vitro* significantly increased on Days 2 and 3 compared to Day 1 of HIIT. Additionally, there was a significant decrease in cell viability at 3hrs compared to pre-exercise and 24hrs post-exercise. These findings indicate that lymphocytes respond to oxidative stress by increasing antioxidant enzyme activity. Additionally, HIIT causes oxidative stress but did not induce a significant post-exercise lymphopenia. Analyses *in vitro* suggest that lymphocytes may be fortified by increasing their antioxidant enzyme activities and become more resistance to subsequent episodes of oxidative stress. Furthermore, the analysis *in vitro* confirms that lymphocytes are more vulnerable to cytotoxic molecules during recovery from exercise.

INTRODUCTION

Oxidative stress is a physiological condition that occurs when there is a significant imbalance between reactive oxygen species/free radicals (ROS/FR) and the body's antioxidant defenses. An elevation in ROS/FR production that is unmatched by antioxidant defense mechanisms can be toxic to bio-molecules, such as DNA, proteins, and lipids (17). This shift from a "reducing" cellular environment to a more "pro-oxidant" environment has been implicated in the development of a number of pathologies, including atherosclerosis, hypertension, ischemia reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, immune dysfunction, aging, Parkinson's disease, and Alzheimer's disease (3, 4, 7, 14, 15, 43, 53). The process of redox regulation via ROS/FR and the antioxidant defenses within the cellular milieu represents a tightly controlled system that can have both deleterious and beneficial effects within the cellular environment.

Physical activity is characterized by an increased ROS/FR production and post-exercise immunosuppression (2, 3, 22, 32, 52). It is well known that physical activity stimulates adaptations that lead to improvements in overall health. While these short and long term adaptations (such as lower cholesterol, increased lean muscle mass, reduced fat mass, lower resting heart rate, reduction of blood pressure, increased myocardial contractility, improved immune function) occur when routine exercise is performed, the acute response from exercise is often paradoxical in nature, both enhancing and suppressing skeletal, cardiovascular, respiratory, and immune function (21, 45). It is well known that exercise leads to improved health and exercise performance. The exercise-induced changes at the whole body and cellular level likely follow the principle of "hormesis", which states that both stress and toxins at low doses may

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promote good health, but high doses appear to be detrimental or even lethal to the biological

system (16, 37). Additionally, a low grade oxidative stress from chronic exercise may be

necessary to up-regulate the antioxidant defense system, thus providing protection from ROS/FR

formed during subsequent acute bouts of exercise or non-exercise related conditions (10).

The benefits of routine, moderate intensity exercise on well-being and the immune

system are well known (50). It has become clear that the immune response to exercise could be

influenced by exercise intensity. Moderate exercise stimulates the immune system, while

strenuous exercise has been shown to provoke immunosuppression during the recovery period

(33). The mechanisms associated with these exercise-associated immune changes include

oxidative stress, as well as neuroendocrinological factors such as epinephrine, norepinephrine,

growth hormone, and cortisol (34). Additionally, the oxidative stress associated with exhaustive

exercise may play a role in immune system impairment (28). Exercise causes perturbations to the

immune system, such as neutrophilia and lymphopenia, during the post-exercise recovery period

(47). Neutrophilia and lymphopenia have been documented following both exhaustive long

duration exercise and strenuous high intensity exercise of short duration (24). Lymphocyte

concentration increases during the exercise session and falls below basal values after intense

exercise of long duration (24). Despite substantial evidence indicating that strenuous exercise

induces oxidative stress and an acute immune response, there is little information assessing

interaction between antioxidant defenses and the immune system during exercise (47).

Antioxidant vitamins, such as vitamin E and vitamin C, reportedly found in large concentrations

in neutrophils and lymphocytes play an important role in maintaining immune cell integrity and

functionality (44). Lymphocytes and neutrophils express superoxide dismutase (SOD), catalase

(CAT), and glutathione peroxidase (GPX), which provide cellular defense against endogenous

and exogenous sources of ROS/FR (46). It has also been shown that lymphocytes increase their activity of SOD, CAT, and GPX in response to endogenous ROS/FR, while neutrophils decrease their antioxidant enzyme activities (5). Tauler et al. (2006) examined the antioxidant adaptations in lymphocytes during a cycling mountain stage, and found an increase in SOD, GPX, and glutathione reductase (GR), but this increase in enzyme activity did not prevent the oxidative damage in lymphocytes (48).

The increase in oxidative stress seen during exercise and the subsequent elevation in the acute immune response during recovery from exercise provides a unique opportunity to study the relationship between stress and immunity. Cellular sources of ROS/FR consist of the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, peroxisomes, and nitric oxide synthase (NOS) (9). The immune system is unique in that ROS/FR production by leukocytes provides both humoral and cellular protection against infectious agents and inflammatory responses (38). This acute immune response can occur following surgery, trauma, burns, infectious diseases, and muscle damage (26). Immunology research on exercise has focused on the suppressed immune system during the post-exercise recovery period, but it may be that the immune cells involved in these processes follow the principle of hormesis. Specifically, the increased oxidative stress response (partly induced by phagocytic cells) generated from increased ROS/FR production may actually up-regulate the antioxidant defenses within the immune cells themselves, perhaps enabling greater protection from oxidative stress during the subsequent bout of exercise or non-exercise related oxidative stress. This hypothesis is supported by evidence that athletes have shown to have greater immunity against various toxins than their untrained counterparts (29, 35).Therefore, examination of mononuclear cells during the post-exercise recovery period may provide a better

understanding of the link between ROS/FR and the host defense response, and the up-regulation of antioxidant genes involved in maintaining redox balance.

The aims of this study were to examine the acute immune and oxidative stress responses following one week of high intensity interval training (HIIT). Circulating neutrophil and lymphocyte numbers were assessed before and after three HIIT sessions. Lymphocyte antioxidant gene expression, enzyme activity, and cell viability were assessed to determine whether this mode of exercise was able to fortify the antioxidant defense enzymes within these cells. Responses for all variables were assessed within treatment days and between treatment days.

METHODS AND PROCEDURES

Participants

Male volunteers aged 19 to 35 years meeting the following inclusion criteria were invited to enroll in the study: 1) Vo_{2peak} between 35 ml/kg/min and 55 ml/kg/min; (2) healthy, as determined by Medical Par Q Screening (Appendix A); 3) currently engaging in no more than three days per week of moderate strength and/or endurance training (self-reported, Appendix B); and 4) currently not taking any antioxidant supplementation or non-steroid anti-inflammatory drugs (NSAIDS).

Preliminary Procedures and Assessments

Qualifying volunteers were scheduled for an initial visit to the Auburn University Thermal Lab for further screening. Upon arrival, volunteers were provided with and asked to sign an institutionally-approved informed consent document prior to any screening processes (Appendix C). Volunteers meeting all of the inclusion criteria then continued with

anthropometric measurements and preliminary physiological assessments. Participants were also asked to keep a one-week food diary during the week of preliminary screening in order to obtain a nutrient profile for each individual.

Physiological Assessment

Anthropometric measurements including height and weight were obtained. Height was determined to the nearest 0.25 inch with a stadiometer and weight was measured to the nearest 0.25 kg using a calibrated scale (Michelli Scales, Harahan, LA). Three-site skinfold assessment was obtained using skinfold calipers (Lange Skinfold Caliper, Beta Technology Inc., Cambridge, MD) and body density was calculated. Participants performed a 30-second Wingate maximal anaerobic power test on a cycle ergometer with resistance determined by bodyweight (0.075kp/kg body weight) during the first visit, and then performed an incremental peak VO_2 test on an electronically-braked cycle ergometer (Quinton Excalibur, Quinton Instrument Company, Bothell, WA) during the second visit (separated by at least 48 hours from the first visit). Each participant pedaled at 100 watts for five minutes, 150 watts three minutes, 200 watts for three minutes, and then wattage increases of 25 watts per minute until volitional exhaustion. Oxygen uptake was measured with an automated metabolic testing system (True Max 2400 Metabolic Testing System, Parvo Medics, Salt Lake City, UT). The highest observed oxygen uptake averaged for 45 seconds was considered the peak oxygen consumption (VO_{2peak}). VO_2 values were plotted against work-rate for the first three workloads, and a linear regression line was used to calculate % VO_{2peak} at a given work-rate (Table 3).

Experimental Procedures

Participants performed a high intensity interval training (HIIT) protocol, which consisted of three exercise sessions separated by 48hrs each over the course of one week. Participants were asked to refrain from other physical activity during the course of the study and to continue similar dietary practices (using the preliminary diet recall log, which was provided to each participant). Participants performed each exercise session at the same time of day, separated by 48hrs of recovery.

Upon arrival to the lab, participants provided a urine sample for determination of hydration status. Samples were analyzed for specific gravity using a refractometer (American Optical Corp., Keene, NH), and the participant was cleared to exercise if urine specific gravity was less than 1.020 g/ml. Venous blood samples were taken from the antecubital vein prior to exercise, immediately post-exercise, 3hr, and 24 hrs after each exercise session.

Exercise Conditions:

Participants performed a HIIT protocol on an electrically-braked cycle ergometer (Quinton Excalibur, Quinton Instrument Company, Bothell, WA). Participants performed a 20-minute protocol, consisting of four minutes of cycling at 15% of maximum anaerobic power (Max-AP) followed by 30 seconds at 90% of Max-AP. These workloads were based upon pre-trial Wingate tests. This cycle was repeated four times within each protocol, ending with two minutes at 15% of Max-AP. It should be noted that the cycle ergometer did not transition wattages in a square-wave fashion. Wattage increased/decreased at each power transition at a rate of 150 watts per second.

Blood Sampling Procedures

Participants reported to the lab on testing days after an overnight fast. A venous butterfly needle (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, 23G x ¾) was inserted into a superficial vein in the antecubital region of the arm and then capped with an intermittent injection port (Kawasumi Laboratories, Inc., Tampa, FL). Venous blood samples were collected with suitable vacutainers with EDTA as the antiocoagulant. Immediately following blood draw, fresh whole blood was used to quantify lymphocytes, neutrophils, hemoglobin and hematocrit using an automated analyzer system (Coulter LH750, Hematology Analyzer). Hemoglobin and hematocrit concentrations from whole blood samples were used to estimate plasma volume shifts resulting from the exercise sessions via the method of Dill and Costill (8).

Lymphocyte isolation

Blood collected in EDTA tubes was mixed with an equal volume of phosphate buffered saline (8ml of blood/8ml PBS) and carefully layered onto a lymphocyte separation solution (8ml) and centrifuged at 400 x g at 22°C for 30 min. The lymphocyte layer was removed and the slurry was washed twice with phosphate buffered saline and centrifuged for 10 min at 260 x g at 22°C. The isolated lymphocytes were: 1) used for gene expression studies; 2) used for antioxidant enzyme activity assays or 3) cultured in RPMI 1640 medium/10% fetal bovine serum and 2% phytohemagglutinin (PHA–GIBCO) for cell viability studies.

RNA Isolation from Lymphocytes

Lymphocyte RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA). 4ml of RLT buffer was mixed with 40ul of mercaptoethanol. The lymphocyte pellet was mixed with

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600ul of the RLT/mercaptoethanol buffer and placed into a QIA shredder column. Samples were centrifuged at 10,000 x g for 2 minutes at room temperature and the flow through was discarded. The lysate was added to a gDNA eliminator spin column and centrifuged at 10,000 x g for 30 seconds; the column was discarded and the flow through was saved. The sample was mixed with 600ul of 70% ethanol, vortexed, transferred to a spin column and placed into a 2ml collection tube. Samples were centrifuged at 800 x g for for 15 seconds and the flow through was discarded. Samples were washed with 700ul of RWL buffer, centrifuged at 800 x g for 15 seconds and the flow through was discarded. To prevent further contamination, the spin column was transferred to a new 2ml collection tube, washed twice with 500ul of RPE buffer and centrifuged at 10,000 x g for 2 minutes. The collection tube was discarded and replaced with a new 1.5ml tube. Nuclease free water (30ul) was added to the column and incubated at room temperature for 5 minutes. The tube was centrifuged for 2 minutes at 10,000 x g and the RNA in the flow through was immediately stored at -80°C until analyzed for gene expression.

RNA Purity and Concentration Analysis

Sample RNA was analyzed spectrophotometrically for purity and concentration. The optical density of RNA samples was determined by UV spectroscopy using a SmartSpec plus spectrophotometer (Bio-Rad, Hercules, CA). Sample RNA was diluted 1:10 in 1XTE buffer (10 mM/L Tris-HCl, pH 7.5, 1mM/L EDTA), transferred into a clean cuvette and analyzed spectrophotometrically using 1X TE buffer as a blank. Purity was assessed using the ratio of absorbance from 260 nanometers and 280 nanometers ($A_{260/280}$).

First Strand cDNA synthesis

First strand cDNA was synthesized from total RNA using the iScript first strand synthesis kit (BioRad). Each reaction contained one microgram total RNA, reverse transcriptase buffer, one microliter of reverse transcriptase in a final volume of 20 microliters. The reaction was carried out on a BioRad MyIQ thermocycler under the following conditions: 25°C for five minutes followed by 42°C for thirty minutes then 85°C for five minutes with a final hold at 4°C.

Real –Time PCR Primer Design

Gene specific primers for superoxide dismutase 1 Cu/Zn-SOD (Accession number AY450286), superoxide dismutase 2 Mn-SOD (Accession number BC035422.1), glutathione peroxidase (NM_002084), and catalase (NM_001752), as well as the two housekeeping genes, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (NM_002046) and acidic ribosomal phosphoprotein Po (Arbp) (X03205). (Table 1)

<u>Table 1.</u> Gene Specific Primers for PCR

Cu/Zn SOD (human)	Forward 5' -AGGCCTCGCACTTCTCGAA
Accession # AY450286	Reverse 5' -CTACAGGTACTTTAAAGCAACTCT
Mn-SOD (human)	Forward 5' -GGCCTGATTATCTAAAAGCTATTTGG
Accession # BC035422.1	Reverse 5' -CGATCGTGGTTTACTTTTTGCA
GPX (human)	Forward 5' -TTCCCGTGCAACCAGTTTG
Accession # NM_002084	Reverse 5' -TTCACCTCGCACTTCTCGAA
CAT (human)	Forward 5' -TTTTCCCAGGAAGATCCTGAC
Accession # NM_001752	Reverse 5' -ACCTTGGTGAGATCGAATGG
GAPDH (human)	Forward 5' -GCAAATTCCATGGCACCGT
Accession # NM_002046	Reverse 5' -GCCCCACTTGATTTTGGAGG
Arbp (human)	Forward 5' –CGGCTACCACATCCAAGGAA
Accession # X03205	Reverse 5' -GCTGGAATTACCGCGGCTGC

Real-Time PCR

Real-time PCR analysis of CuZn-SOD, Mn-SOD, GPX, and CAT were performed in lymphocyte RNA samples. Expression of CuZn-SOD, Mn-SOD, GPX, CAT, as well as the two housekeeping genes GAPDH and Arbp were measured in duplicate on the myIQ iCycler real-time PCR detection system. No change was detected in housekeeping gene expression. The reaction mixture consisted of 12.5 ul SYBR green supermix, 10.5 ul nuclease–free H_2O, 1ul of primer mix (forward and reverse suspended in 1X TE buffer), and 1 ul of cDNA. The RT-PCR cycle was as follows: an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for

15 seconds, 60°C for 20 seconds, and 68°C for 1 minute. Each sample was run in duplicate, and a cDNA-free sample was run with each primer to ensure no DNA contamination. Melt curve analysis was used to determine the presence of 1 amplicon, and no primer dimerization. Data were normalized to the housekeeping genes GAPDH and Arbp. Data were analyzed using a modification of the delta delta Ct method as described by Vandesompele *et. al* (51) and gene expression was expressed as a fraction of that occurring pre-exercise.

Antioxidant Enzyme Activity

The activities of superoxide dismutase, catalase, and glutathione peroxidase were measured in lymphocytes. All activities were determined with a SmartSpec plus spectrophotometer (Bio-Rad, Hercules, CA). Cell numbers were calculated for each sample. Cell samples were ruptured via the addition of 500ul of deionized H_2O per 2×10^6 cells. All assays were performed in duplicate.

Superoxide Dismutase Activity Assay

Superoxide dismutase activity was measured by an adaptation of the method of McCord and Fridovich (25). This assay utilizes the xanthine/xanthine oxidase method to generate O_2^-. Cytochrome c in the solution is easily reduced in the presence of O_2^- and will increase the absorbance at 550nm. SOD is capable of inhibiting this reaction; therefore, 1 unit of SOD activity is defined as the amount of SOD required to inhibit this reaction by 50%. The number of SOD units in the sample assay was calculated using the formula:

$$\text{units} = \% \text{ inhibition}/(100\text{-}\% \text{ inhibition})$$

Catalase Activity Assay

Catalase (CAT) activity was measured by the spectrophotometric method of Aebie (1) based on the decomposition of hydrogen peroxide (H_2O_2). This reaction depends on the concentration of H^+ donor and the steady-state concentration or rate of production of H_2O_2 in the solution. The active catalase- H_2O_2 Complex I is formed first. The decomposition of H_2O_2, in which a second molecule of H_2O_2 serves as the H^+ donor for complex I, proceeds rapidly. The decomposition of H_2O_2 can be followed directly by absorbance at 240nm. The assay was performed for one minute at 25°C using the following parameters: three second initial delay, followed by absorbance reading every 5 seconds for one minute. One unit was defined as the amount of catalase that will decompose 1uM of H_2O_2 to H_2O and O_2 per minute at pH 7.0 at 25°C at substrate concentration of 10mM H_2O_2.

Glutathione Peroxidase Activity Assay

Glutathione peroxidase activity was measured using the spectrophotometric method of Flohe and Gunzler (11). This assay utilizes a coupled reaction of glutathione peroxidase with glutathione reductase. Glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) to oxidized glutathione, (GSSG) while glutathione reductase utilizes NADPH as the substrate to reduce GSSG back to GSH. Based on these principles, the rate of change in absorbance of NADPH per minute is followed at 340nm. One unit of glutathione peroxidase activity is defined as the amount required to oxidize 1uM of GSH/min corresponding to 0.5uM of NADPH oxidized per minute.

TBARS Assay

TBARS assay was performed in plasma as a marker of plasma oxidative stress levels. TBARS assays were determined using TBARS Kits purchased from Cayman (Cayman Chemical, Ann Arbor, MI, Catalog No. 1009055). Plasma was obtained following centrifugation of whole blood (400 x g 30 minutes). Equal volumes of plasma and sodium dodecyl sulfate solution were added to a 5 ml tube (100ul of each solution). A color reagent, consisting of thiobarbituric acid (TBA), acetic acid, and sodium hydroxide, was added to the 5ml tube (4 ml of color reagent). Samples were boiled in a water bath for one hour. After one hour the tubes were immediately placed into a water bath to stop the reaction between MDA and TBA. Tubes were centrifuged for 10 minutes at 1600 x g at 4°C. Samples were loaded into 96 well plates and absorbance was measured spectrophotometrically at 540nm. A standard curve was used to determine MDA concentration for each sample. Interassay coefficient of variation was 8.3%, intrassay coefficient of variation was 7.6%.

Lymphocyte Cell Viability Studies

Lymphocyte samples from pre-exercise, 3hrs-post exercise, and 24hrs-post exercise for each exercise session were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 2% phytohemagglutinin. Lymphocytes (1×10^6 cells/ml) were cultured and exposed to each of the following conditions: no hydrogen peroxide (H_2O_2), 200uM H_2O_2, 500umM H_2O_2, or 1mM H_2O_2 for four hours. This was used to determine whether the HIIT protocol protected the lymphocytes against a subsequent oxidative stress *in vitro*. Cell viability was determined by the Trypan blue (TB-0.4% SIGMA-ALDRICH) exclusion assay. Following the 4hr incubation period, the culture medium was removed from the culture plate and placed into 6ml centrifuge

tubes. Cells were centrifuged at 500 x g for 10 min at 22°C. The supernatant was aspirated and the cell pellet was resuspended in 1ml PBS. 10ul of cell suspension was mixed with 10ul of Trypan blue dye, this mixture was loaded into a hemocytometer. The numbers of both live (cells do not take up the trypan blue) and dead cells (cells take up the trypan blue) were counted in the 4 outer squares of the hemocytometer. The cell viability was calculated using the following equation:

Viable Cell % = total number of viable cells per ml of aliquot / total number of cells per ml of aliquot *100

Statistical Analysis

This experiment was a within subjects design with each participant serving as his own control. Group characteristic were reported as means ± SD. A three (day) x four (time) analysis of variance (ANOVAs) with repeated measures on both day and time was used to compare superoxide dismutase, catalase, glutathione peroxidase (enzyme activity), TBARS, total leukocyte number, neutrophil number, lymphocyte number, and cell viability for each day. Gene expression of antioxidant enzymes were compared to Day 1 pre-exercise expression. Gene expression was also assessed within days using pre-exercise expression as control.

The independent variables for this experiment were day (Day1, Day2, and Day 3) and time (pre-exercise, post-exercise, 3hrs post-exercise, and 24hrs post-exercise). The dependent variables for this study were SOD, CAT, GPX (gene expression and enzyme activity), TBARS, total leukocyte number, neutrophil number, lymphocyte number, and cell viability. Significant differences were further explored utilizing a Tukey post hoc analysis. The significance level for

this study was set at $p < 0.05$. Data were analyzed with the Statistical Package for the Social Sciences (SPSS, version 16.0, SPSS, Inc., Chicago, IL.)

RESULTS

Participants

In total, nine male volunteers met the study inclusion criteria and agreed to participate in the study. One participant withdrew from the study because of time commitment; therefore eight participants began and completed the entire study protocol. Participant baseline physiological characteristics are presented in Table 2.

Table 2. Baseline physiological characteristics

Variable	Mean ± SD	Minimum	Maximum
Age (yrs)	22 ± 2	21	28
Height (cm)	181 ± 4.8	177	187
Weight (kg)	83.0 ± 13.6	68	112
Body fat %	12 ± 6	6	24
VO_{2peak} (mL/kg/min)	44.6 ± 8.2	32.0	55.0
Wingate (Watts)	871 ± 134	670	1066

All values are presented as mean ± SD and minimum and maximum values.

Exercise Intervention

All participants were able to complete each of the three exercise conditions. Each of the exercise sessions lasted exactly 20 minutes, and was identical in power settings and cycle ergometer settings for each participant. Participants completed the exercise protocol following an overnight fast.

Exercise was performed during the same time of day and was separated by 48 hours. Each of the three exercise protocols was identical within each of the eight participants. Percentage of VO_{2peak} was based upon each participant's power output at VO_{2peak}. Exercise intervention data are presented in Table 3.

Table 3. Exercise Session Data

Variable	15% MAP	90% Max-AP
Average power (Watts)	130.1 ± 19.9	784.0 ± 121.2
% VO_{2peak}	54.9 ± 10.8	274.5 ± 58.6
Total time (min)	18.0	2.0*

All values are presented as mean ± sd. Max-AP = maximum anaerobic power as measured by Wingate; % VO_{2peak} = percentage of peak oxygen uptake at each intensity based on power output at VO_{2peak}; %. Total time = total exercise time at each intensity for each exercise session. * = Wattage increased/decreased to set value at 150 watts per second at each power transition.

Effect of Exercise on Leukocyte, Neutrophil, and Lymphocyte Counts:

There were no significant differences between days, therefore data were pooled for all three days. Changes in leukocyte, neutrophil, and lymphocyte number pre-exercise, post-exercise, 3hrs post-exercise, and 24hrs post-exercise are reported in Table 4.

Leukocytes

There was a significant increase in leukocyte number post-exercise and 3hrs post-exercise as compared to pre-exercise and 24hrs post-exercise ($p < 0.05$). There were no significant differences in leukocyte number between exercise days.

Neutrophils

There was a significant increase in neutrophil number 3hrs post-exercise compared to pre-exercise, post-exercise, and 24hrs post-exercise ($p < 0.05$). There were no significant differences in neutrophil number between exercise days.

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Lymphocytes

Post-exercise lymphocyte number was significantly increased compared to pre-exercise, 3hrs post-exercise, and 24hrs post-exercise p < 0.05. However, there were no significant differences in lymphocyte number between exercise days.

Table 4. Leukocyte, Neutrophil, and Lymphocyte Changes Following Exercise

Cells 10^3/ul blood	Pre-Ex	Post-Ex	3hr-Ex	24hr-Ex
Leukocytes	6.5 ± 0.3	$9.35 \pm 0.38^*$	$8.55 \pm 0.51^*$	6.13 ± 0.27
Neutrophils	3.12 ± 0.15	3.63 ± 0.2	$5.61 \pm 0.42^{\#}$	3.18 ± 0.16
Lymphocytes	2.65 ± 0.15	$4.56 \pm 0.21^{@}$	2.24 ± 0.13	2.33 ± 0.14

All values are reported as mean ± SEM. * significant increase in leukocyte number compared to Pre and 24hr values. # Significant increase in neutrophil number compared to Pre, Post, and 24hrs. @ Significant increase in lymphocyte number compared to Pre, 3hr, and 24hr. p < 0.05

Plasma Oxidative Stress Determinant Following Exercise:

Oxidative stress was measured indirectly using a TBARS assay kit. The MDA/TBA adduct formed was measured spectrophotometrically at 540nm. MDA levels were determined pre-exercise, post-exercise, and 3hrs post-exercise for each day of exercise. MDA levels were significantly elevated immediate-post-exercise as compared to pre-exercise on Day 1 and Day 2, and 3hrs post exercise on Day 2 (p < 0.05). There were no significant differences in MDA levels on Day 3 of exercise (p < 0.05). These data show that oxidative stress occurred following HIIT on both Day 1 and Day 2, but did not significantly increase on Day 3 of HIIT. MDA concentration for each day and time period is presented in Figure 3.
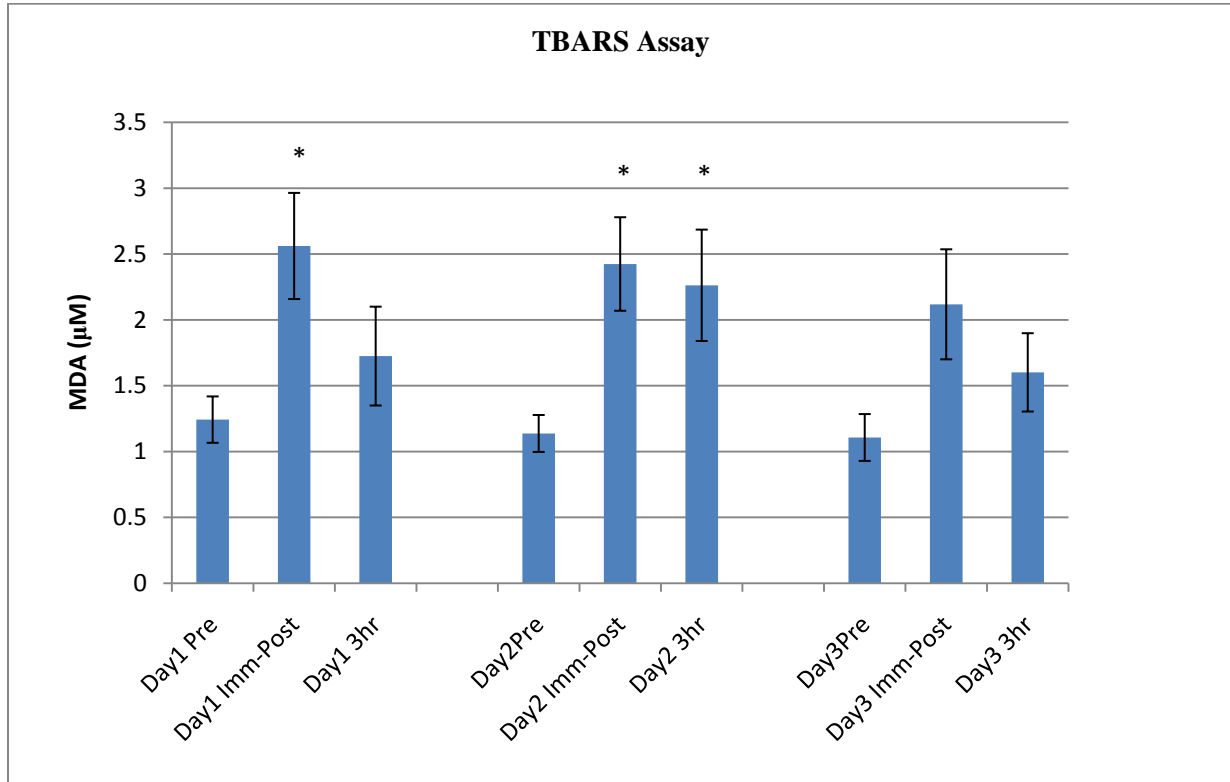
TBARS Assay

Figure 1.

Values are reported as mean ± SEM. * Significant increases from pre-exercise levels ($p < 0.05$).

Lymphocyte Antioxidant Gene Expression Following Exercise:

Antioxidant gene expression for CuZn-SOD, Mn-SOD, CAT, and GPX were assessed in lymphocytes following HIIT. Gene expression was assessed between days and within days. All changes in gene expression between days were compared to pre-exercise on Day 1 (control value). In order to determine gene expression changes within days, all 3hr post-exercise values were compared to pre-exercise expression. No significant differences in antioxidant gene expression were observed at any of the sampling times. Antioxidant gene expression is presented in Figure 4, 5, 6, and 7.
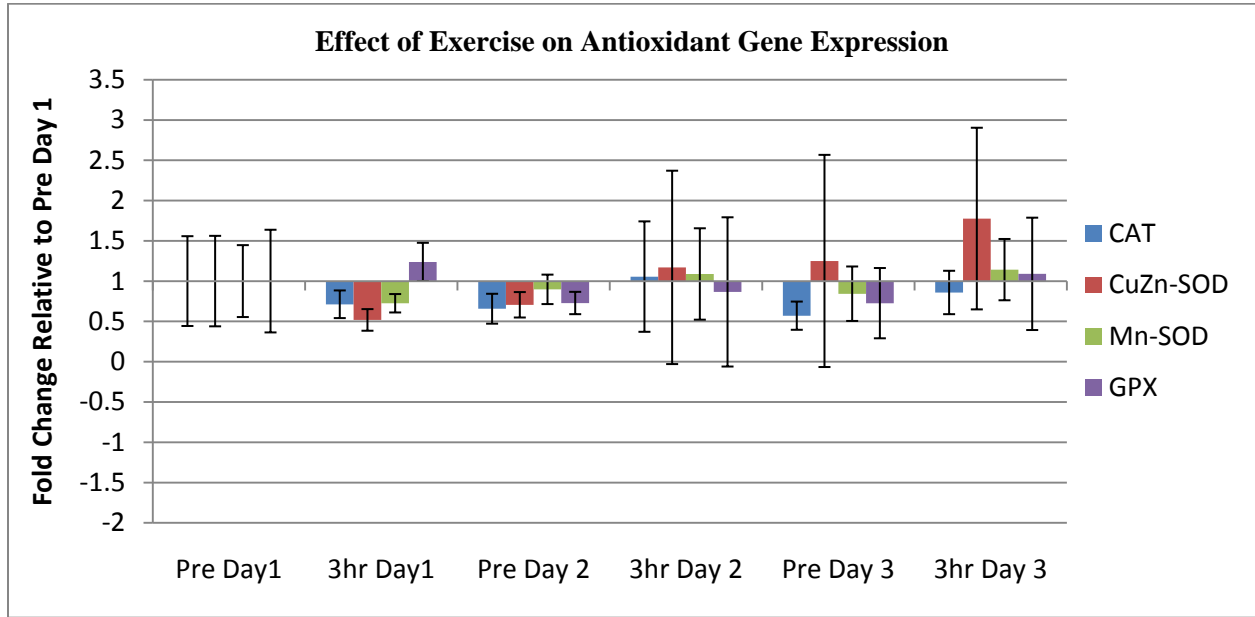
Figure 2. Effect of Exercise on Antioxidant Gene Expression in Lymphocytes

Values are reported as mean ± SD. Gene expression was measured using RT-PCR. Pre-Day1 exercise was arbitrarily referred to as 1. All changes in gene expression were compared to Pre-Day1. No significant increases in gene expression were observed.

Figure 3. Day 1 Antioxidant Gene Expression

Values are reported as ± SD. Pre-exercise arbitrarily referred to as 1. Post-exercise changes in expression were compared to Pre-exercise. No significant differences were found between pre-exercise and 3hrs post-exercise for antioxidant enzyme gene expression ($p < 0.05$).

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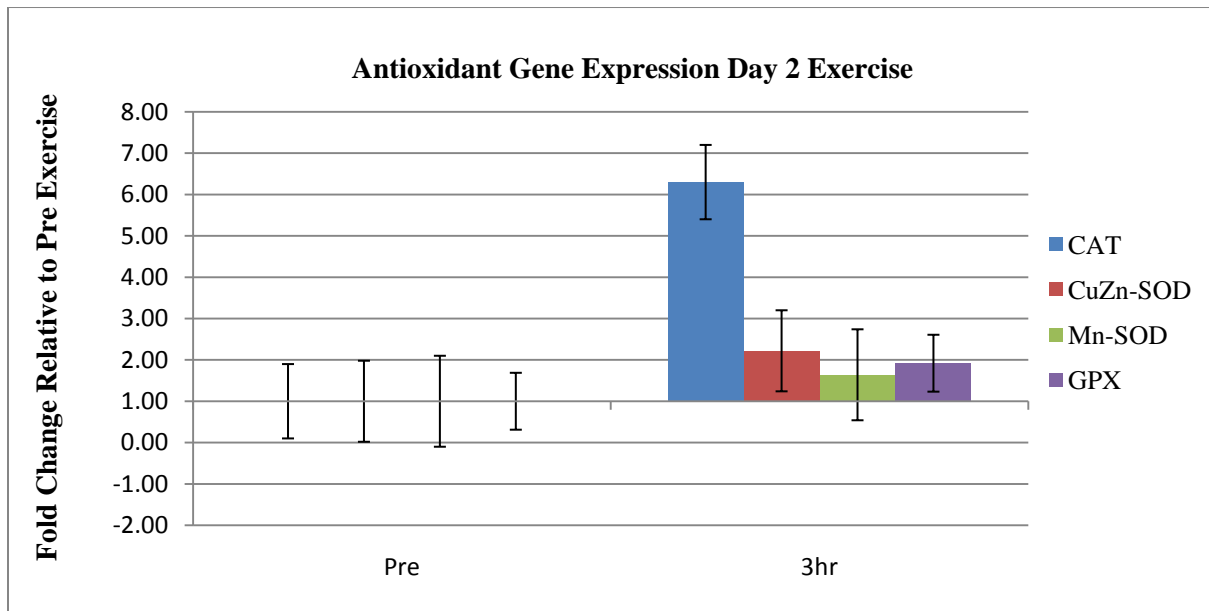
Figure 4. Day 2 Antioxidant Gene Expression

Values are reported as ± SD. Pre-exercise arbitrarily referred to as 1. Post-exercise changes in expression were compared to Pre-exercise. No significant differences were found between pre-exercise and 3hrs post exercise for antioxidant enzyme gene expression ($p < 0.05$).
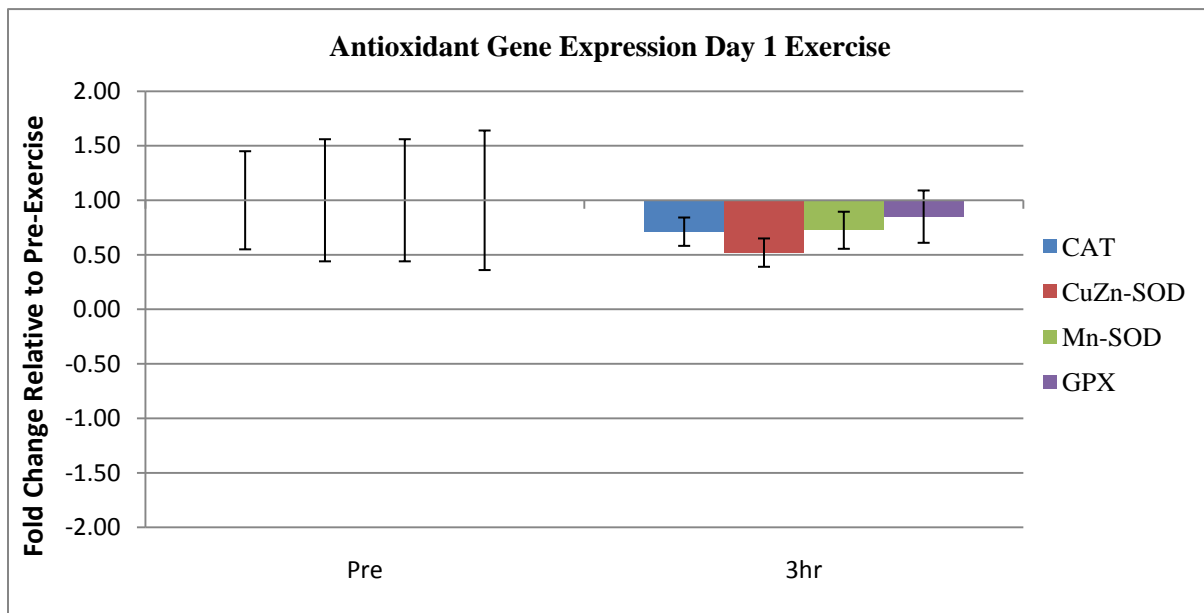

Figure 5. Day 3 Antioxidant Gene Expression

Values are reported as ± SD. Pre-exercise arbitrarily referred to as 1. Post-exercise changes in expression were compared to Pre-exercise. No significant differences were found between pre-exercise and 3hrs post exercise for antioxidant enzyme gene expression ($p < 0.05$).
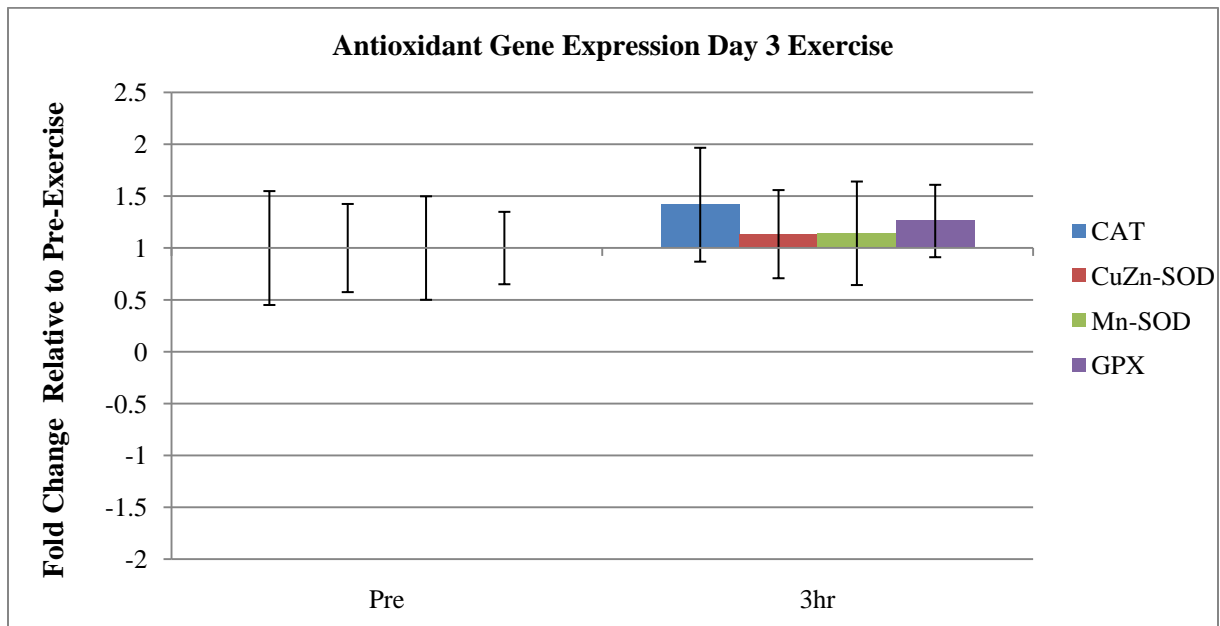
Changes in Lymphocyte Antioxidant Enzyme Activities Following Exercise:

        SOD, GPX, and CAT enzyme activities were determined in lymphocytes following three

HIIT sessions. Enzyme activities were determined pre-exercise, post-exercise, 3hrs post-

exercise, and 24hr post exercise. Changes in lymphocyte antioxidant enzyme activities following

one week of HIIT training are presented in Table 5.

Table 5. Changes in lymphocyte antioxidant enzyme activities following HIIT training.

Enzyme	Pre-Ex	Post-Ex	3hr-Ex	24hr-Ex
Catalase (Units/10^9 Cells)				
Day 1	6.1 ± 0.7	11.9 ± 1.5*	7.5 ± 2.2	11.5 ± 3.1
Day 2	7.7 ± 0.9	12.9 ± 1.9*	11.4 ± 2.3	10.4 ± 2.3
Day 3	8.9 ± 0.8	11.1 ± 2.6	9.3 ± 1.4	8.6 ± 1.7
Superoxide (Units/10^9 Cells) **Dismutase**				
Day 1	39.4 ± 5.4	57.4 ± 6.7*	56.9 ± 6.0*	41 ± 8.2
Day 2	44.8 ± 3.4	63.2 ± 6.1*	59.8 ± 6.3*	42.4 ± 6.6
Day 3	41.1 ± 5.1	61.6 ± 6.9*	55.9 ± 6.3*	41.7 ± 5.9
Glutathione (Units/10^9 Cells) **Peroxidase**				
Day 1	36.6 ± 5.3	61 ± 6.3*#	41.4 ± 4.8	40.3 ± 6.3
Day 2	45.2 ± 5.4	52 ± 3.6#	39.4 ± 5.3	36.6 ± 4.5
Day 3	41.8 ± 6.3	57 ± 4.8*#	39 ± 4.5	38.3 ± 2.7

Values are reported as mean ± SEM. * Significantly greater than pre-exercise activity ($p < 0.05$). # Significantly greater activity compared to 3hrs and 24hrs post exercise ($p < 0.05$).

Superoxide Dismutase:

There were no significant differences in activity between days 1, 2, or 3 at any sample time for SOD, therefore data were pooled into one data set and statistically analyzed at each sample time for this enzyme. SOD activity was significantly elevated post-exercise (45.6%) and 3hrs-post exercise (37.1%) compared to both pre and 24hr-post exercise levels ($p < 0.05$). SOD activity returned to pre-exercise levels by 24hrs. SOD activity is presented in Figure 8.
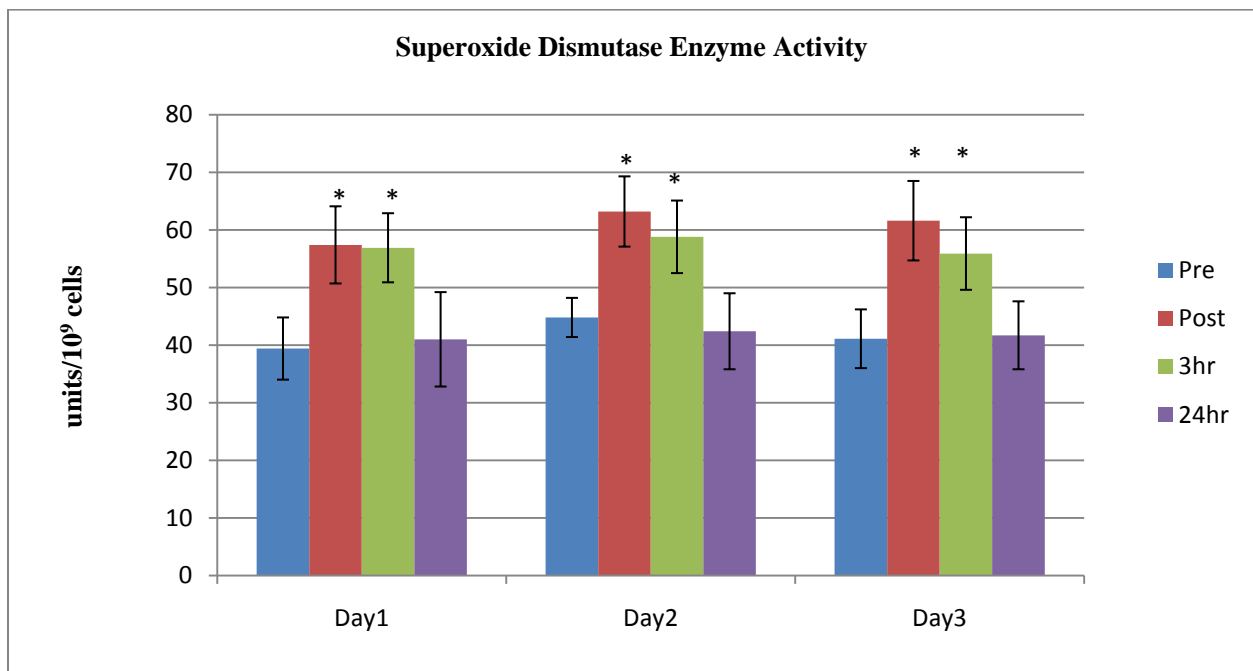

Figure 6. Superoxide Dismutase Activity

Values are reported as mean ± SEM. * Significant increase in activity compared to pre and 24hrs post exercise $p < 0.05$.

Glutathione Peroxidase:

GPX activity was significantly elevated from pre to post exercise on Day 1 (67%) and Day 3 (36%) ($p < 0.05$). No significant differences were found pre to post exercise on Day 2.

Additionally, post exercise GPX activity was significantly elevated from 3hr and 24hrs post exercise on Day 1, Day2, and Day 3 ($p < 0.05$). GPX activity returned to pre-exercise levels by 3hrs-post exercise. GPX activity is presented in Figure 9.

Figure 7. Gluathione Peroxidase Activity

Values are reported as mean ± SEM. * Significant increase in activity compared to pre-exercise $p < 0.05$. # Significant increase in activity compared to 3hr and 24hrs-post exercise $p < 0.05$.

Catalase:

CAT activity was significantly elevated from pre to post exercise on Day 1 (95.4%) and Day 2 (66%) ($p < 0.05$). No significant differences in CAT activity were found at any other sampling times. CAT activity is presented in Figure 10.
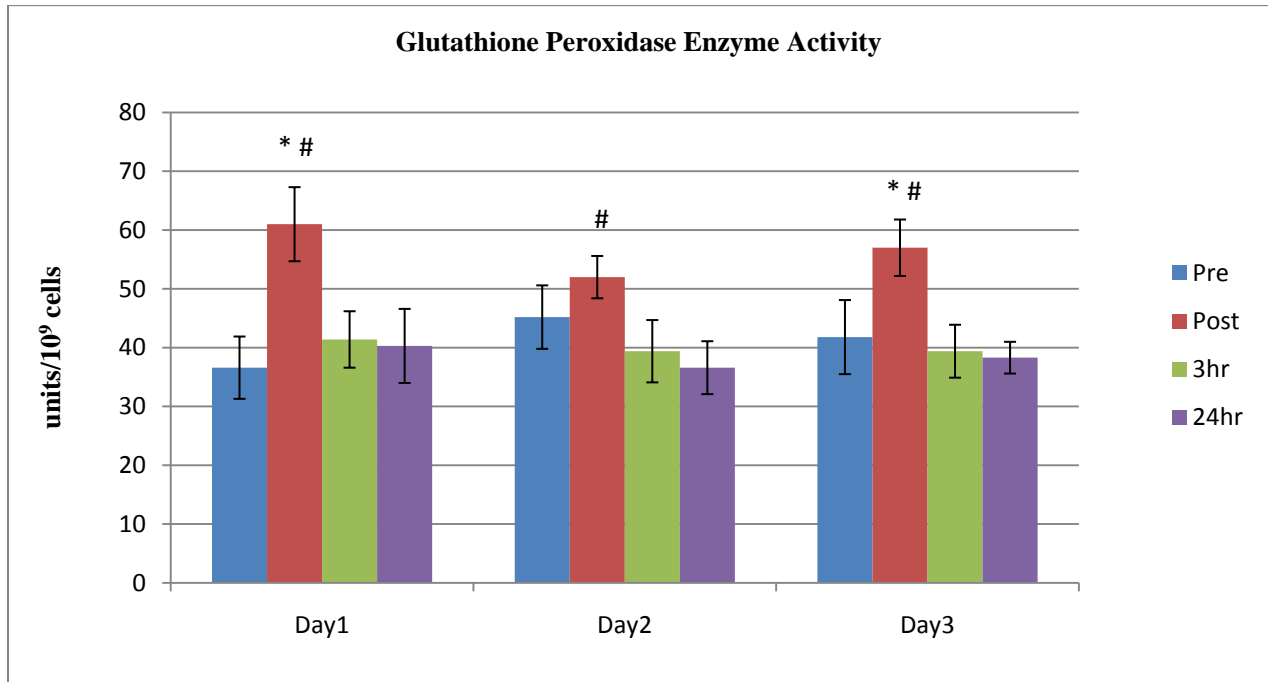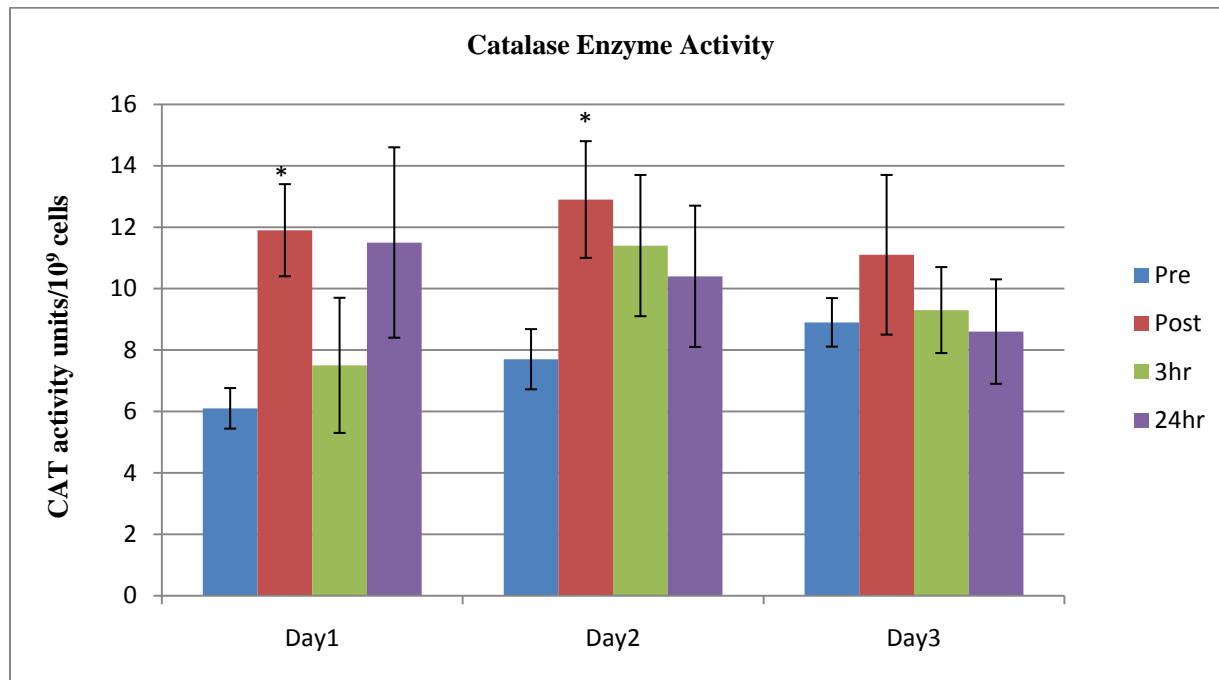
Figure 8. Catalase Activity

Values are reported as mean ± SEM. * Significant increase in activity compared to pre-exercise $p < 0.05$.

Effect of exercise on Lymphocyte Viability Following H_2O_2 Treatment *in vitro*:

There were significant differences between control, 200uM, 500uM, and 1mM H_2O_2 treatment ($p = 0.01$); thus a dose response with the four different treatments was observed. Each concentration of H_2O_2 treatment was analyzed independently of one another in order to determine if cell viability improved between Days 1, 2, and 3 following treatment. A significant increase in cell viability was observed at Day 2 and Day 3 compared to Day 1 during the 3hr post exercise period for the 1mM H_2O_2 challenge ($p < 0.05$). Cell viability data 3hrs post exercise is presented in Figure 9. There were no significant differences observed between the other three concentrations.

3hr Post-Exercise Cell Viability
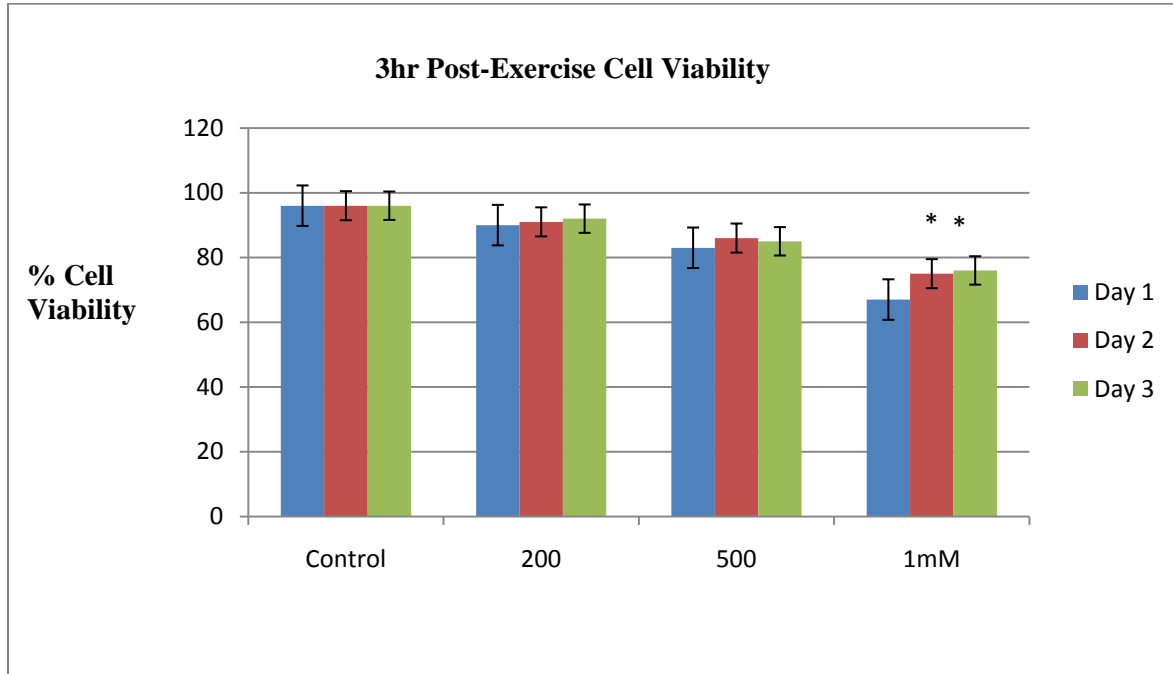
Figure 9. Lymphocyte cell viability between days

Values are reported as mean ± SEM. * Significant differences from Day 1 1mM at p < 0.05. Both Day2 and Day 3 showed significant increases in cell viability compared to Day 1 following the 1mM peroxide exposure.

Cell viability was compared between pre-exercise, 3hrs post-exercise, and 24hrs post-exercise conditions. Cell viability was significantly decreased at 3hrs post-exercise compared to pre-exercise and 24hrs post-exercise (p < 0.05) Cell viability at each time period is presented in Figure 10.
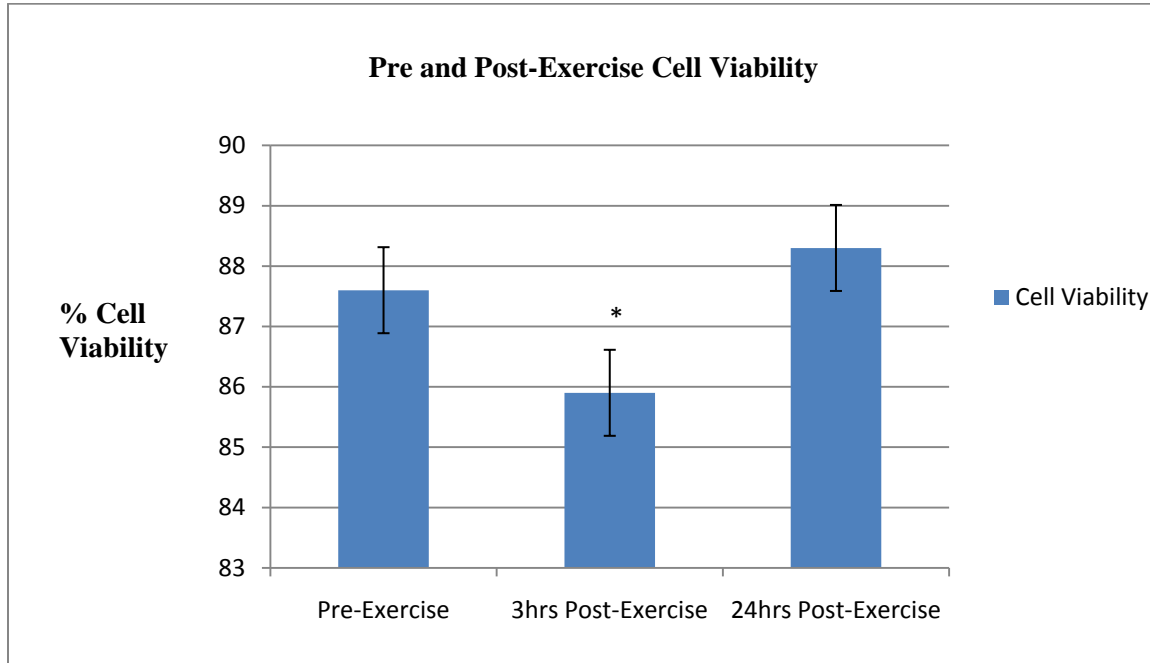
Figure 10. Lymphocyte cell viability pre-exercise, 3hrs post-exercise, and 24hrs post-exercise.

Values are reported as ± SEM. *Significant decrease in cell viability compared to pre-exercise and 24hrs-post exercise $p < 0.05$.

DISCUSSION

The purposes of this study were to: 1) assess the immune and oxidative stress response following one week of HIIT; 2) determine whether changes in antioxidant enzyme gene expression and enzyme activity occurs in lymphocytes following HIIT; and 3) determine whether changes in antioxidant expression and activity would improve lymphocyte cell viability following hydrogen peroxide exposure *in vitro*. The main findings of this investigation were that: 1) HIIT elicits both an acute immune and oxidative stress response; 2) post-exercise lymphopenia did not occur following HIIT; 3) lymphocytes appear to respond to oxidative stress by increasing SOD, GPX, and CAT activity; not by increasing gene expression; and 4) analysis

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of cell viability *in vitro* suggests that exercise may fortify lymphocytes by increasing their antioxidant enzyme activities.

Exercise increases the production of circulating leukoyctes, lymphocytes, neutrophils, and increases the formation of ROS/FR's (12, 21, 24, 28, 29, 33, 34). Strenuous exercise is known to cause perturbations to the immune system (21, 22, 24, 35) and increase the formation of reactive oxygen species exceeding the capacity of the antioxidant defense system (10, 19, 20). The ability to stimulate the immune system by performing routine moderate exercise has been well documented (28, 29, 33, 34), while strenuous exercise has been shown to cause immunosuppression during the recovery period (33). Perturbations to each of these systems are known to occur from exercise, yet information regarding the interactions between antioxidants and the immune system are sparse.

There are several consistent patterns found in the blood following acute exercise (24, 33, 46, 47). There is an increase in the number of circulating neutrophils during exercise, which continues to increase well into the recovery period (24, 33, 46, 47). The lymphocyte concentration increases during exercise, yet often falls below baseline values during the recovery period (24, 33, 46, 47). The increased lymphocyte concentration is due to the recruitment of all lymphocyte subpopulations into the blood (33, 34). Following exercise of a strenuous nature, lymphocyte concentration often decreases and function can be suppressed (33, 46). In this study HIIT elicited an acute immune response as observed by elevated leuukocyte counts. Total leukocyte number significantly increased immediately following exercise and remained significantly elevated at 3hrs post exercise. Neutrophil number began to increase immediately following exercise and was significantly elevated at 3hrs post exercise. Lymphocyte number was

significantly elevated post exercise and returned to near pre exercise levels at 3hrs, thus post exercise lymphopenia did not occur following the HIIT protocol. Additionally, the acute immune responses were similar across all days of exercise (Table 4).

The oxidative stress response following exercise is less clear than the acute immune response following exercise. Fisher-Wellman and Bloomer (2009) reviewed a vast number of prior studies assessing oxidative stress following both aerobic and anaerobic exercise. They critically showed the conflicting results of both the occurrence and nonoccurrence of oxidative stress following exercise throughout the literature (10). In the present study, significant increases in plasma MDA levels were found immediately post exercise on Days 1 and 2. Additionally, there was a significant increase 3hrs post exercise on Day 2 (Figure 3). There were no significant increases in MDA levels on Day 3 of exercise. These data indicate that there may be variability in the oxidative stress response when assessed at several times during multiple exercise sessions. This is one of the first studies in humans to assess the transient changes of these systems following multiple sessions of HIIT exercise. The majority of human studies assessing antioxidant enzyme activity and oxidative stress biomarkers use a single exercise session to compare pre-exercise to post-exercise levels, or assess pre-training and post-training changes (13, 23, 30, 41, 49, 54).

The immune and oxidative stress responses are thought to be dependent on intensity, and perform a critical role during inflammation and cellular damage (23, 40, 42). Neutrophils and macrophages are phagocytic cells that generate ROS/FR when there is excessive tissue damage or inflammation (40, 42). While we did not directly measure neutrophil ROS/FR production, we did see a significant increase in circulating neutrophils during the post-exercise recovery period

(Table 4). It has been suggested that the increase in circulating neutrophils seen following strenuous exercise may actually contribute to oxidative stress (21). With these principles in mind, it appears that performing exercise at higher intensities impairs the immune system during recovery. While this may be the case, this short term stress that occurs following exercise may follow the same principle as cardiac pre-conditioning, in which exercise associated stress up-regulates heat shock proteins and antioxidant enzymes that elicit greater long term cardioprotection (36). Introducing the immune system to exercise associated oxidative stress may be deleterious during recovery, but may be advantageous long term. This hypothesis seems likely as the enzymatic antioxidant defenses have shown the ability to adapt to oxidative stress by increasing their activities.

Lymphocytes are key components of the adaptive immune system. Ascorbate and vitamin E are present in large concentrations in both neutrophils and lymphocytes, and play a critical role in maintaining immune cell integrity and function (40). However, the lymphocyte endogenous antioxidant enzyme adaptations following exercise are less well known. Prior investigations assessing lymphocyte antioxidant enzyme activity have yielded equivocal results; some studies have shown an increase in SOD (6, 50) and GPX (50), while others have shown no change in SOD (47) and GPX (6, 47) activity. Our study showed a significant increase in SOD activity post exercise and 3hrs post exercise following each day of HIIT (Figure 7). GPX activity significantly increased post exercise following Days 1 and 3 (Figure 8). While the aforementioned studies did not find any significant increases in CAT activity, we found a significant increase in CAT activity post exercise on Days 1 and 2 (Figure 9). These data suggest that antioxidant enzymatic defenses increase activity in order to adapt to oxidative stress.

Lymphocyte oxidative stress levels were not directly measured in this study, thus we can only speculate that lymphocyte oxidative stress levels were similar to plasma oxidative stress levels. Tauler et al. (2006) measured plasma MDA and lymphocyte protein carbonyl levels following a mountain stage cycling protocol and found significant increases in both indices of oxidative stress (50), while a follow up study measured MDA and protein carbonyl levels in lymphocytes following a cycling stage road race and found no significant increases in either marker (6). The conflicting results may be due to the more stringent nature of exercise in the mountain stage compared to road cycling. These observations again illustrate the difficulty of interpreting mechanisms associated with oxidative stress and immunity, as one would expect an increase in oxidative stress following a 165km all out cycling time trial.

The present investigation showed that lymphocyte SOD, GPX, and CAT respond to oxidative stress by increasing their activity. SOD appears to contribute more than GPX and CAT, as GPX and CAT responses were more transient in nature than SOD, which is in agreement with previous studies (48, 50). In order to determine if some of these adaptations occur at the pre-transcriptional level, gene expression was determined for CuZn-SOD, Mn-SOD, CAT, and GPX. There were no statistically significant increases in gene expression 3hr post exercise for any day of exercise, indicating that lymphocyte antioxidant adaptation occurs primarily at the post-transcriptional level (at least over the time frame investigated in this study). These data differ from previously reported results, in which significant increases in CuZn-SOD and Mn-SOD gene expression were observed post exercise (6) Additionally, Morikawa et al. (2004) assessed changes in Mn-SOD expression in lymphocyte in high school boys subjected to 3 months of aerobic exercise training. They found a significant increase in Mn-SOD mRNA following the max exercise test prior to the 3-month training protocol, but no significant difference in Mn-SOD

expression following the 3-month training cycle (27). The vast differences in exercise mode, nutritional status, and initial fitness make it difficult to compare results between protocols. Based on our finding and previous findings (6, 27), the changes in antioxidant enzyme gene expression are not clear. Our exercise protocol consisted of high-intensity cycling for a brief time period; this may not have stressed the immune system to the same degree as the prolonged cycling protocol seen in prior studies. While the up-regulation of lymphocyte antioxidant genes is unclear, the up-regulation of Mn-SOD mRNA in response to exercise in skeletal muscle has been well documented in the literature (12, 18, 31).

It has been suggested that the increased number of circulating lymphocytes seen during exercise are recruited from other tissue pools, such as the spleen, lymph nodes, and the gastrointestinal tract (33). Additionally, animal models have shown that there is redistribution from the circulation back into tissue pools following exercise (39). While the post exercise lymphopenia represents suppressed immunity, the ability of lymphocytes to adapt to oxidative stress challenges *in vitro* has yet to be explored. Thus, the current investigation isolated lymphocytes from pre-exercise, 3hrs post exercise, and 24hrs post exercise blood samples each day. Lymphocytes were cultured and exposed to 1mM H_2O_2 for 4hrs, our results showed a significant increase in lymphocyte cell viability at 3hrs post exercise during Days 2 and 3 compared to Day 1 (Figure 1). Additionally, it appears that the cells were most vulnerable to the H_2O_2 treatment during the 3hr post exercise time period. There was a significant decrease in lymphocyte cell viability during the 3hr time period compared to both pre exercise and 24hrs post exercise (Figure 2). While it appears that lymphocytes may be fortified following exercise and show greater resistance to subsequent episodes of oxidative stress, we did not find any significant correlation between cellular viability, and antioxidant gene expression or activity. The

cell culture data support the postulation that lymphocytes may be more vulnerable to cytotoxic agents during recovery from exercise.

In summary, this study found that HIIT elicits an acute immune and oxidative stress responses. There was a significant increase in circulating leukocytes immediately following exercise and well into the recovery period. The lymphocyte number increased during exercise and was significantly elevated immediately post exercise, yet did not significantly decrease from pre exercise levels during recovery. Thus, no post-exercise lymphopenia occured following exercise of this nature. The number of circulating neutrophils increased immediately post exercise and continued to significantly increase well into the recovery period. The postulation that circulating neutrophils contribute to oxidative stress during exercise cannot be determined from this study. A significant increase in oxidative stress was found following exercise on Days 1 and 2, but not Day 3. Lymphocytes adapted to oxidative stress by increasing their antioxidant enzyme activities. SOD activity significantly increased post exercise and 3hrs post exercise each day. GPX activity significantly increased post exercise on Days 1 and 3 of exercise. CAT activity significantly increased post exercise on Days 1 and 2 of exercise. There were no significant increases in lymphocyte antioxidant gene expression following HIIT. Analysis of lymphocyte cell viability *in vitro* following H_2O_2 exposure showed a significant improvement in cell viability on Days 2 and 3 compared to the first day of exercise. Additionally, there was a significant decrease in lymphocyte cell viability during the 3hr time period compared to both pre exercise and 24hrs post exercise. In conclusion, HIIT did not cause significant post exercise immunosuppression. Additionally, it appeared that the lymphocyte endogenous antioxidant enzyme defenses increased in activity in response to elevated oxidative stress levels. This adaptation appears to occur at the post-transcriptional level, as no significant increases in

antioxidant gene expression were found in this study. It is difficult to assess changes in the adaptive immune system because of the dynamic exchange between the circulation and tissues. While we did not find any significant changes in antioxidant gene expression, the experiments *in vitro* suggest that exercise may fortify lymphocytes by increasing their antioxidant enzyme activities and enable greater resistance to subsequent episodes of oxidative stress. Additionally, the analysis *in vitro* suggests that lymphocytes are more vulnerable to cytotoxic molecules during recovery from exercise. HIIT did not significantly impair the immune system *in vivo*, thus this HIIT model may be an ideal form of exercise to improve health and performance without over stressing the immune system. Future research assessing these same variables over longer training cycles should be performed. Carefully designed experiments measuring both the immune response and oxidative stress over extended periods of time and multiple training sessions are needed. These types of studies would provide data to better understand the link between oxidative stress and the immune system.

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APPENDICES

PAR Q Medical Questionnaire*

Verbal PARQ Interview

1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

2. Do you feel pain in your chest when you do physical activity?

3. Have you ever had chest pain when you were not doing physical activity?

4. Do you lose your balance because of dizziness or do you ever lose consciousness?

5. Do you have a bone or joint problem that could be made worse by a change in your physical activity?

6. Are you currently taking any prescription, over the counter or recreational drugs that may affect your ability to perform physical activity?

7. Are you currently taking any non-steroidal anti-inflammatory drugs (NSAIDS)?

8. Do you have any chronic illnesses which may limit or be complicated by physical activity? (i.e. diabetes, thyroid or endocrine problems, asthma, etc.)

9. Are you not feeling well because of a temporary illness such as a cold or fever?

10. Do you know of any other reason why you should not do physical activity?

I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction.

Investigators Signature_____   Date _____

APPENDIX B

RECENT TRAINING HISTORY QUESTIONNAIRE

Subject code number:_____   Date:_____

Please answer these questions regarding your recent training level. Answer these questions by giving explanations provided, where indicated.

    1. On average, how many days per week do you perform resistance exercise?
_____

    2. On average, how many days per week do you perform endurance exercise?
    _____

    3. How long have you been exercising on a regular basis?
_____

    4. Did you do any interval training during this time? If yes, please describe.
_____
_____
_____
_____

APPENDIX C

Auburn University

Auburn University, Alabama 36849-5323

Department of Kinesiology	Telephone: (334) 844-4483
2050 Memorial Coliseum	Fax: (334) 844-1467
Thermal Lab (Room 2118)	Thermal Lab: (334) 844-1479

Informed Consent for a Research Study Entitled

"Oxidative Stress and Antioxidant Defense Mechanisms Following High Intensity Interval Training"

Project Overview: You are invited to participate in a research study that will examine the oxidative stress response following high intensity interval training in recreationally active individuals. We are recruiting participants to complete one week of high intensity interval training using a cycle ergometer protocol.

Purpose: The purpose of this investigation is to examine oxidative stress blood bio-markers and antioxidant responses following high intensity interval training.

Participation Requirements: To be eligible, you must be:

1. Male participant of 19 to 35 years of age
2. VO_2 Peak below ACSM 50th percentile, as determined by preliminary testing.
3. Low risk for medical complications (as determined by physical activity readiness questionnaire (PARQ)).
4. Currently engaging in no more than three days per week of moderate strength and/or endurance training (as determined by recent training questionnaire).
5. Currently not taking any antioxidant supplementation or Non Steroidal Anti-inflammatory Drugs (NSAIDS)

 You must meet all of the requirements to be eligible for participation in this study.

Participant's initials _____

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"Oxidative Stress and Antioxidant Defense Mechanisms Following High Intensity Interval Training"

Testing Procedures:

Visit 1: On the first visit to the lab, you will complete the PARQ Questionnaire, complete the training log, and read and sign the University-approved informed consent form. Either Gordon Fisher or David D. Pascoe will be present for all informed consent briefings.

Descriptive data will be obtained (age, height, weight, 3-site skin fold body composition assessment). You will be familiarized with the Monark, Computrainer, and Quinton cycle ergometers. After familiarization, you will perform a 30-second Wingate anaerobic power test. This is a 30-second all-out cycling exercise against a resistance which is determined by your bodyweight. We will monitor you closely and ensure that you complete a thorough cool-down by cycling for several minutes against a very low resistance after the test is complete.

The total time for the exercise testing will be approximately ten minutes (including a warm-up and cool down) while descriptive data will not take more than 50 minutes, making total time commitment for the entire visit approximately one hour.

A one week diet record will be given to you to complete over the course of the week.

Visit 2: On the second visit, you will perform a peak VO_2 test on an electronically-braked cycle ergometer (separated by at least 48 hours from your first visit). Each participant will pedal against 100 watts for five minutes, 150 watts three minutes, 200 watts for three minutes, and then wattage will be increased at 25 watts per minute until volitional exhaustion. Oxygen uptake will be measured with an automated metabolic testing system Heart rate and oxygen uptake will be measured throughout the testing. Individuals will be monitored closely during recovery to ensure that a complete and thorough cool-down is performed. Total time for the exercise testing will be 30 minutes.

Participant's initials _____

"Oxidative Stress and Antioxidant Defense Mechanisms Following High Intensity Interval Training"

Following preliminary testing you will perform high intensity interval training (HIIT).

Pre-Exercise: You will need to consume at least 500 mL (about 1 full glass) of water before arriving at the lab. During the first trial you will be able to drink as much water as you would like, this will be matched during each of the subsequent trials in order to match for changes in fluid volume. Upon arrival to the lab, you will provide a small urine sample which we will test to determine your hydration status. If the test indicates that you are dehydrated, you will be given a chance to re-hydrate by consuming water before beginning the trial or the trial will be rescheduled. A polar heart rate monitor will be placed on your chest for heart rate analysis.

HIIT: You will perform the 20-minute HIIT protocol. This is a type of interval training performed on a stationary cycle in which the intensity will vary from high to low within the protocol.These workloads will be based upon pre-trial Wingate tests. This cycle will be repeated four times within each protocol, ending with two minutes at 15% of maximum anaerobic power. HIIT training will consist of three sessions for one week. Each session will last about 45 minutes. You will be asked to refrain from other physical activity during the course of the study and to continue similar dietary practices (using the preliminary diet recall log, which will be provided to each of you). You will be tested at the same time of day during the course of the study. Total time commitment will be about 20 hrs over the course of two weeks.

Participant's initials _____

"Oxidative Stress and Antioxidant Defense Mechanisms Following High Intensity Interval Training"

Blood Sampling: A venous butterfly catheter will be inserted into the antecubital vein region of the arm on days 1, 3, and 5 (which will remain in place for the entire time you remain in the lab), while days 2, 4, and 6 will only require a single butterfly needle sample. Blood samples will be taken pre-exercise, immediately post-exercise, three hours, and 24 hours post exercise. Blood will be used for analysis of oxidative stress markers and antioxidant defense markers in the body.

Potential Risks:

1. While performing any exercise there is a chance of muscle strains, sprains, pulls, and even death. The American College of Sports Medicine estimates the risk of 0.5 per 10,000 individuals.

2. Due to the high intensity nature of the Wingate cycle ergometer test, many participants feel nauseous and/or light-headed after completing the test.

3. With any blood draw procedure there is a risk of infection, bleeding, bruising, irritation at injection site, and/or fainting.

"Note" It is important for you to realize that you are responsible for any costs incurred in the event of an injury.

Precautions:

1. Although the training for this trial is of higher intensity, it is of short duration and at a comfortable environmental temperature and humidity level. Heart rate will be recorded throughout the trial. We have additionally employed the use of a modified PARQ to assist in eliminating participants that have potential medical or orthopedic identified risks. During the trials you will always be accompanied by either Gordon Fisher or David Pascoe, both maintain current CPR Certifications. You will not be allowed to begin trials if our pre-trial testing indicates that you are dehydrated. Your heart rate will be recorded throughout the trial.

2. After completion of the test you will be monitored and be given a chance to warm-down. Specifically, you will be instructed to continue pedaling against a very light resistance for as long as needed (typically 4 or 5 minutes).

2. Both investigators participating in data collection (Gordon Fisher and David Pascoe) are certified in phlebotomy. Only new, sterile blood-gathering equipment and aseptic techniques will be utilized throughout all data collection and analysis processes.

Benefits: You will receive a Wingate assessment of your maximum anaerobic power, VO_2 peak assessment of aerobic capacity, and body composition assessment.

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"Oxidative Stress and Antioxidant Defense Mechanisms Following High Intensity Interval Training"

Your participation is completely voluntary. If you change your mind about participating, you can withdraw at any time during the study. If you choose to withdraw, you can request to have your data withdrawn. Your decision about whether or not to participate or to stop participating will not jeopardize your future relations with Auburn University, the Department of Kinesiology, or the Thermal Lab.

Your privacy will be protected. Any information obtained in connection with this study will remain anonymous.

If you have any questions, we invite you to ask us now. If you have questions later, you can contact Gordon Fisher (fishego@auburn.edu or call 334-844-1479). You will be provided with a copy of this document to keep. For more information regarding your rights as a research participant, you may contact the Auburn University Office of Human Subjects Research or the Institutional Review Board phone number (334) 844-5966 or email at hsubjec@auburn.edu or IRBChair@auburn.edu.

HAVING READ THE INFORMTION PROVIDED, YOU MUST DECIDE WHETHER OR NOT YOU WISH TO PARTICIPATE IN THIS RESEARCH STUDY. YOUR SIGNATURE INDICATED YOUR WILLINGNESS TO PARTICIPATE.

_____   _____   _____   _____

Participant's Signature   Date   Investigator obtaining consent   Date

_____   _____

Print Name   Print Name

                                                                  _____   _____

                                                                  Co-Investigator   Date

                                                                  _____

                                                                  Print Name

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