CHAPTER 1

INTRODUCTION

1.1. STATEMENT OF THE PROBLEM

Cardiovascular diseases are the leading cause of morbidity and mortality in the United States. They are the clinical expression of advanced atherosclerosis, which begins early in life, but usually takes decades to form the stenotic plaques responsible for the chronic ischemic episode in the arteries (Dalager-Pedersen, et al., 1998). Several risk factors have been identified in the development of atherogenesis, such as high plasma total cholesterol concentration, low physical activity, male sex, hypertension, smoking, advanced age, and high homocysteine level. Particularly, an increasing body of evidence implicates oxidized low-density lipoprotein (LDL) in the development of atherosclerosis.

Low-density lipoprotein is the major carrier of cholesterol in the circulation and the main substrate for oxidation during the development of atherosclerosis. Reactive oxygen species (ROS)/free radicals production in the body can initiate lipid peroxidation in LDL, which leads to the development of atherosclerosis chronically. Cholesterol and its esters in LDL contain unsaturated bonds, which are the most susceptible to the peroxidation reactions induced by ROS. The peroxidation of cholesterol and its esters can lead to the oxidation of LDL, subsequently, resulting in a conformation change and composition of LDL particles (Yuan and Brunk, 1998). Consequently, atherosclerosis can be initiated by these oxidative reactions of cholesterol and its ester in LDL.

The primary function of oxygen is to act as a mitochondrial electron acceptor during the generation of ATP via oxidative phosphorylation. Approximately 2-5 % of the oxygen consumed, however, cannot end up as water and leaks out from the electron transport chain as superoxide anion (O$_2^-$). Subsequent reactions from superoxide can generate hydroxyl
radicals and hydrogen peroxide. All of these compounds are recognized as ROS because each of them has an unpaired electron in its molecular orbits and/or can react with lipids, nucleic acids, and proteins, leading to the damage of cells and tissues. The production of ROS is in proportion to the oxygen consumed in oxidative phosphorylation; therefore, people undergoing exercise or sports may have increased levels of ROS generated in the body compared to those who are sedentary. It has been observed that the urinary excretion of peroxides increased during physical activity in young sportsman (Olinescu, 1995). Furthermore, Wetzstein et al. (1998) showed that 30 minutes of exercise at a moderate intensity and duration was sufficient to induce in vitro LDL oxidation in humans. Although exercise is recommended for improving and maintaining health, ROS generation during the participation in any exercise or sport can cause oxidative stress, possibly leading to developments of chronic diseases in the long term. Therefore, acute exercise is a means to test the protective effect of antioxidant supplementation against ROS, and further establish its antioxidant ability to inhibit oxidative stress and plasma and LDL oxidation.

Usually ROS are quickly scavenged in cells by antioxidant defense systems. When the elevation of ROS generation overcomes the antioxidant defense systems, the situation produced is called oxidative stress. There are two categories of antioxidative defenses, located at sites where ROS are generated: antioxidant enzymes and non-enzymatic antioxidant compounds. Enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidant compounds include vitamin E, vitamin C, beta-carotene, glutathione, uric acid, and albumin. Together, these enzymes and antioxidant compounds convert ROS to safer compounds before ROS can cause severe damage in cells and tissues.

Antioxidant supplementation has been extensively investigated in the prevention of oxidative damage and atherosclerosis development. The reduction of damage induced with concentric exercise by antioxidant supplementation has also been studied. It has been found that vitamin E supplementation in rats reduced thiobarbituric acid reactive substances
(TBARS), which are an indicator of free-radical-mediated lipid peroxidation, in plasma and leg muscle after one-hour exercise on a treadmill (Goldfarb et al., 1994). Bocan et al. (1992) reported that supplementation of vitamin E and vitamin C inhibited atherosclerosis in rabbits. It also has been suggested that increased dietary intake, serum levels, and adipose tissue levels of several dietary antioxidants including vitamin E, vitamin C, and beta-carotene are associated with reduced risk of cardiovascular diseases (Todd, et al., 1995; Street, et al., 1994; and Kardinaal, et al., 1993). Increasing antioxidant intake, from either supplements or foods, can provide protection against oxidative damage, atherosclerotic development, and cardiovascular diseases from oxidative stress.

The incidence of cardiovascular diseases in Asians has been lower than that in Americans. It has been suggested that soy food in Asian diets may play an important role in the prevention of cardiovascular diseases. The beneficial effect of soy on cardiovascular diseases can be attributed to some components of soy, including isoflavones, amino acid composition of protein, fiber, and saponins. Isoflavones have a chemical structure similar to estrogen, which was reported to have a weak antioxidant activity because of the hydroxyl group(s) on their phenolic ring(s) as does vitamin E (Tiidus, 1995). In addition, isoflavones have multiple hydroxyl groups to act as effective antioxidants (Tikkanen et al., 1998) by donating a hydrogen atom(s) from their phenolic hydroxyl group(s) to peroxyradicals. Genistein, one of the isoflavones in soy with three hydroxyl groups, has been found to be a strong antioxidant that can decrease LDL oxidation in vitro (Kapiotis et a., 1997). Additionally, Cai and Wei (1996) observed that dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activities of antioxidant enzymes in the small intestine and skin of mice.

Although the antioxidant activity of genistein has been observed in vitro and in animals, the in vivo antioxidative capability of isoflavones, especially genistein, in animals and humans still lacks solid evidence. In order to investigate the antioxidant activity of genistein in plasma and further investigate its capability to protect LDL and plasma against
oxidation, an \textit{in vivo} oxidative stress must be initiated. Intensive physical activity has been suggested as one means to induce a surge of ROS in the body, leading to oxidative stress because of the imbalance between antioxidants and oxidants (Ji, 1993). Therefore, the combination of dietary isoflavone supplementation and intensive exercise is a way to explore whether isoflavones from soy can counteract the oxidative stress that results from the imbalance between antioxidants and oxidants in the body by their antioxidative capability and, thus, perhaps slow the development of atherosclerosis through long-term isoflavone intake.

\textbf{1.2. HYPOTHESES}

Current literature suggest that moderate to high intensity exercise can induce oxidative stress in the body, because elevated consumption of oxygen can lead to increased generation of ROS. Antioxidant supplementation can provide extra protection against these ROS. Isoflavones were observed to have an antioxidant activity \textit{in vitro} and \textit{in vivo}, and augmented the activities of antioxidant enzymes in rats. It can be hypothesized that isoflavone supplementation can increase total antioxidant defense systems and reduce the impact of ROS generated due to intensive exercise. The following five hypotheses were tested in this study:

- Isoflavone supplementation will increase genistein concentration in tissues.
- Isoflavone supplementation will modulate the activities of antioxidant enzymes in red blood cells pre- and post- exercise.
- Isoflavone supplementation will reduce lipid peroxidation in LDL and plasma both pre- and post- exercise.
- Isoflavone supplementation will prevent the decrease of ascorbic acid in the plasma after exercise.
- Isoflavone supplementation will prevent the increase of oxidized glutathione in the blood after exercise.
CHAPTER 2

LITERATURE REVIEW

2.1. ATHEROSCLEROSIS

In the developed world, cardiovascular diseases are some of the leading causes of morbidity and mortality. Cardiovascular diseases, including coronary heart disease, stroke, and peripheral vascular disease, are the clinical expressions of advanced atherosclerosis. Therefore, it is not a disease in its own right, but a process that principally contributes to the pathogenesis of myocardial and cerebral infarction (Ross, 1993). Atherosclerosis begins early in life, but it usually takes decades to form the stenotic plaques responsible for the chronic ischemic episode in the arteries (Dalager-Pedersen et al., 1998). Simply, atherosclerosis is the result of a dynamic interaction among blood elements, disturbed flow, and the vessel wall, involving several pathologic processes (Fuster and Lewis, 1994). The development of the disease, a relatively complicated and incompletely established process, is shown in Figure 1 below.

2.2. RISK FACTORS OF ATHEROSCLEROSIS

The development of atherosclerosis could be attributed to a collection of “risk factors,” such as, plasma total cholesterol concentration, distribution of cholesterol among lipoproteins, blood pressure, LDL oxidation, smoking status, advanced age, and homocysteine levels (Wood, 1998). In addition to these risk factors, some other factors can also trigger atherosclerosis, such as high alcohol consumption, sedentary lifestyle, male sex, stress, and heredity.
Injury endothelial membrane permeable to LDL
↓
LDL accumulation in intima
↓
Oxidation of LDL by SMC, macrophages, or endothelium
↓
Expression of adhesion molecules on endothelial surface stimulated by oxidized LDL
↓
Adhesion of monocytes to endothelial surface
↓
Monocyte transmigration into intima
↓
Monocyte transformation into macrophages and scavenging of oxidized LDL
↓
Formation of foam cells from macrophages and oxidized LDL
↓
Secretion of cytokines from foam cells, endothelial cells, SMCs
↓
SMC migration into intima
↓
Proliferation of SMC and macrophages
↓
Production of extracellular matrix proteins in intima by SMCs
↓
ATHEROSCLEROTIC PLAQUE

Figure 2.1. Steps in formation of atherosclerotic plaque in vivo. SMC, smooth muscle cell.
(Patel and Kent, 1998)
2.2. LDL OXIDATION

2.2.1. Low density lipoprotein composition

Low-density lipoprotein oxidation is one possible risk factor for cardiovascular diseases. Human LDL can be isolated from the plasma by ultracentrifugation within a density gradient of 1.019-1.063 g/ml (Esterbauer and Ramos, 1996). LDL molecules are large spherical particles with a diameter of 19-25 nm and molecular weights between 1.8 and 2.8 million, with an average of 2.5 million. The mean chemical composition (weight %) of LDL is 22.3% phospholipids, 5.9% triglycerides, 9.6% free cholesterol, 42.2% cholesteryl ester, and 22.0% protein (Esterbauer et al. 1992) (Esterbauer and Ramos, 1996). The total amount of fatty acids in an LDL molecule is roughly 2600, and about one-half of them are polyunsaturated fatty acids (86% linoleic acid 18:2, 12% arachidonic acid 20:4, 2% docosahexaenoic acid 22:6).

2.2.2. Evidence that LDL peroxidation leads to atherosclerosis

Some of the major factors contributing to the evidences that lipid peroxidation is an integral part of the pathogenesis of atherosclerosis are:

1. the existence of oxidized LDL and lipid peroxides in areas of the atherosclerotic plaque (Aviram, 1993)
2. the increased susceptibility of LDL from atherosclerotic patients to undergo in vitro lipid peroxidation (Aviram, 1993)
3. the anti-atherogenicity of antioxidant therapy (Aviram, 1993)
4. elevated plasma titers of autoantibodies to malondialdehyde (MDA)-modified LDL in patients with vascular diseases (Salonen et al., 1992).

In vitro studies, moreover, showed that several physiochemical features of oxidized LDL are different from those of native LDL (Yuan and Brunk, 1998). These include the following findings:

1. complete loss of antioxidants and polyunsaturated fatty acids
2. partial loss of phosphatidyl choline, cholesteryl esters, and free amino groups of apo-B protein
3. increased content of oxysterols, hydroxyl- and hydroperoxy-polyunsaturated fatty acids, conjugated dienes, MDA and other aldehydes
4. enhanced electrophoretic mobility due to increased negative charge
5. fragmentation and conformational rearrangement of the apo-B protein in oxidized LDL.

2.2.3. Lipid peroxidation in the circulating LDL

Although oxidized LDL is accepted as one of the initiators of atherogenesis, the pathways leading to the oxidative modification of LDL in vivo are still unclear (Westhuyzen, 1997). Theoretically, the native LDL particle circulating in the plasma should contain unmodified apoB-100 and no peroxides or aldehydes, and should be rich in antioxidants and polyunsaturated fatty acids (Parthasarathy et al., 1999). In reality, circulating LDL contains oxidized lipids, such as lipid hydroperoxides and other degradation products, depending on the dietary lipids, and pathological conditions. This LDL represents the “seeded” LDL, which can have an increased propensity to undergo further oxidation in the circulation. Some researchers believe that lipid peroxidation of LDL may not take place in the circulation (Esterbauer, et al., 1991) because several potent antioxidative defense mechanisms are highly active in the plasma compartment (Esterbauer et al., 1992 and Steinberg, 1997), and oxidized LDL is rapidly taken up by liver Kupffer cells and sinusoidal endothelial cells (de Rijke and van Berkel, 1994). Furthermore, oxidized LDL is cleared more rapidly from circulation as compared to native LDL (de Rijke and van Berkel, 1994). The result from the sensitive enzyme-linked immunosorbent assay (ELISA), however, suggests that some oxidized LDL can be present in the circulation (Holvoet et al., 1998). LDL oxidation almost certainly involves ROS because of their capability to oxidize LDL, however, the nature of these radicals and their sources are uncertain (Holvoet and Collen, 1994). It has been believed that LDL oxidation in vivo possibly involves ROS, such as superoxide anion, hydroxyl radical, hypochlorite, and peroxynitrite (Yla-Herttuala, 1998) and cells, such as macrophages, endothelial and SMC (Mabile et al., 1997). When such oxidation is carried out to a minimal degree, the resultant LDL particles may represent the “minimally oxidized LDL and, as such, may be physically indistinguishable from the native lipoprotein, except for the expected loss of PUFAS and antioxidants.
2.2.4. *In vitro* LDL oxidation by oxidants

The extent and measurement of oxidation in LDL particles after their isolation from plasma by means of ultracentrifugation depends on the limitations of currently available methods of isolation, detection of oxidized lipids and their degradation products. LDL is extremely oxidation-prone and labile even during the ultracentrifugal isolation. However, Parthasarathy et al (1999) stated that it is unlikely that any measurable degree of oxidation of lipids occurred during the ultracentrifugation, instead loss of antioxidants is more prone to happen, thus affecting further *in vitro* LDL oxidation. Cholesterol and its ester in LDL contain unsaturated bonds, which are the most susceptible to the peroxidation reaction. The peroxidation of cholesterol and its ester can lead to the oxidation of LDL, subsequently, resulting in the changes of conformation and composition of LDL particles (Yuan and Brunk, 1998). The most popular method to determine the susceptibility of LDL to oxidation uses oxidants, such as copper, to induce lipid peroxidation. However, the mechanism by which Cu^{++} induces *in vitro* lipid peroxidation in LDL is still poorly understood. It seems very likely that cupric ions bind to discrete sites of the apo B and form centers for repeated free radical production (Esterbauer and Ramos, 1996). Once bound, Cu^{++} must be reductively activated by a net transfer of one electron, and it is likely that the rate of this reaction is rate-limiting and equal to the rate of initiation of lipid peroxidation. As LDL is usually isolated in phosphate buffer, the required reducing equivalents in lipid peroxidation reaction must be provided either from apo B (e.g. cysteine residues), antioxidants, or from the lipids.

It is well established that oxidation catalyzed by transition metal ions begins in the lipid moiety of the lipoprotein by abstraction of a hydrogen atom from a bisallylic methylene group of a polyunsaturated fatty acid (PUFA) (Dix and Aikens, 1993) (Karten et al., 1997). A large number of kinetic experiments *in vitro* have shown that the chronology of LDL oxidation by Cu^{++} ions can be divided into three consecutive time phases: lag time (or lag phase), propagation phase, and decomposition phase (Esterbauer and Ramos, 1996; Esterbauer et al. 1992). During the lag phase, the LDL becomes progressively depleted of its antioxidants, with alpha-tocopherol as the first and beta-carotene as the last to be decreased. When the LDL is depleted of its antioxidants, the lipid peroxidation rapidly accelerates to a
maximum rate of the uninhibited process. A lipid peroxide peak is reached when about 70%-80% of the LDL PUFAS are oxidized; thereafter, the peroxide content of LDL starts to decrease again, because decomposition reactions become predominant (Parthasarathy et al., 1999). The decomposition of lipid hydroperoxides to aldehydes, such as malondialdehyde, is a general phenomenon in fat autoxidation and lipid peroxidation in biological systems. The major, if not the only, source of malondialdehyde is the fatty acids with more than three double bonds, i.e., 20:4 and 22:6. These aldehydes are able to react with the apolipoproteins so that the native lipoprotein recognition receptor is hindered (Steinbrecher et al., 1987; Karten et al., 1997). Therefore, oxidized LDL is engulfed through a different receptor such as a scavenger receptor in the macrophage.

2.3. EXERCISE AND ROS GENERATION

2.3.1. Free radical formation under normal physiological conditions

Oxygen is a universal electron acceptor that allows aerobic organisms to use energy stored in foodstuffs, such as carbohydrates, fats, and protein. It is widely accepted and experimentally proven that this catabolic process can generate oxygen free radicals and other ROS such as superoxide (O$_2^-$), hydroxyl radical, and hydrogen peroxide (H$_2$O$_2$) (Halliwell and Gutterridge, 1989). Under normal physiological conditions, the majority of free radicals is produced in the mitochondrial electron transport chain (ETC) since 90% of the oxygen consumption by the body is reduced to water in the mitochondria (Ames et al., 1995). Loschen et al. (1973) have demonstrated that the rate of H$_2$O$_2$ formation in mitochondria is linked directly to the energy coupling mechanism. McCord (1979) has estimated that for every 25 oxygen molecules reduced by cytochrome oxidase in the mitochondria, one oxygen molecule is reduced by ubisemiquinone to produce a free radical. Therefore, a normal cell approximately produces $2 \times 10^{10}$ O$_2^-$ and H$_2$O$_2$ per day, which can add up to $3.3 \times 10^{-14}$ moles per day (Ames et al., 1995). In addition to free radicals produced by the mitochondria, some free radicals are produced by other pathways such as respiratory burst by neutrophils to kill bacteria, viruses, and other xenobiotics (Cannon and Blumberg, 1994), fat metabolism via beta-oxidation in the peroxisome (Godin and Wohaieb, 1988), oxidation of D-amino acids, activation of cytochrome P450, degradation of xanthine to uric acid, and catecholamine
autoxidation (Yu, 1994; Chance et al., 1979). Although all these processes are part of normal cell life, ROS have a strong tendency to extract electrons to reach a chemically more stable structure; therefore, they are capable of eliciting oxidative damage to various cellular components (Ames et al., 1995; Yu, 1994).

2.3.2. Free radical-induced oxidation

All ROS are considered highly reactive because their unstable electron configurations allow for the attraction of electrons from other molecules, resulting in other free radicals that are capable of reacting with more molecules. This chain reaction contributes to lipid peroxidation (Hochstein and Ernster, 1963), DNA damage (Kasai et al., 1986), and protein degradation (Griffith et al., 1988) during oxidatively stressful events. The superoxide radical, the most well-known ROS and unlike the other ROS, can lead to the formation of additional reactive species (Harris, 1992), such as hydrogen peroxide. Hydrogen peroxide although not a free radical in itself, is a biologically important oxidant because of its ability to generate the hydroxyl radical, an extremely potent radical (Aruoma et al., 1991). This hydroxyl radical has a very short half-life and is one of the most reactive oxidants because of a strong ability to remove or add hydrogen molecules to unsaturated hydrogen bonds of organic lipids in biological systems. The most well-described consequence of the generation of free radicals and ROS is lipid peroxidation (Hochstein and Ernster, 1963). In vitro, the interaction between free radicals and lipids involves three phases: initiation, propagation, and termination. During initiation, conjugated dienes are formed as a result of the abstraction of a hydrogen atom from a backbone methylene group of a lipid (Alessio, 1993). This is followed by the propagation step which comprises the interaction of molecular oxygen with carbon-centered free radicals to form lipid hydroperoxides. The resultant decomposition of these lipid hydroperoxides produces alkoxy or peroxyl radicals that continue the process of propagation (Ross and Moldeus, 1991). The termination step takes place when two lipid peroxides or one lipid peroxide and an antioxidant react with each other and the propagation phase is stopped. Polyunsaturated fatty acids are particularly vulnerable to this process of initiation and propagation because of the multiple unsaturated points found along their backbone. Most human studies examined by-products of lipid peroxidation, including
conjugated dienes, TBARS, MDA, and lipid peroxides to assess oxidative stress (Clarkson and Thompson, 2000).

2.3.3. Free radical generation during exercise

In humans, exercise is no longer a means of survival, but is increasingly becoming a lifestyle, a recreation, and sometimes, a means of therapeutic treatment. It is well established that regular aerobic exercise has some protective effects against atherosclerosis development (Berlin and Colditz, 1990) by reducing cholesterol levels and high blood pressure (Hollmann, 1994), and enhancing nitric oxide (NO) production (Gattullo et al., 1999) and antioxidant defense systems (Lawler and Powers, 1998). Although certain exercises can benefit health, too much may cause a deleterious effect on the body. The rate of oxygen uptake by the body during exercise may increase by 10- to 15-fold, which results in increased oxygen flux by 100-fold in the active peripheral skeletal muscle tissue with a 30-fold increase in blood flow. Although delivery of increased amounts of oxygen to active tissues during exercise fuels oxidative metabolism, maximizing energy yield per unit substrate and avoiding lactate accumulation, aerobic organisms have to pay a price for such a metabolic advantage (Halliwell and Gutteridge, 1989). An elevated metabolic rate as a result of exercise can dramatically increase free radical/ROS production (Alessio, 1993; Davies et al., 1982; Jenkins, 1988; Ji, 1995a; Kanter et al., 1988; Kanter et al., 1993). The direct detection of ROS in biological systems is difficult because of their high reactivity and low steady-state concentration. Most of the evidence of increased ROS generation has been derived from studies that have monitored the by-products of exercise-induced oxidative tissue damage.

Electron spin resonance and electron paramagnetic resonance (EPR) spectroscopy are the most powerful devices to directly detect and characterize oxygen free radicals. Jackson et al. (1985) demonstrated that 30 min of excessive contractile activity on muscle resulted in a 70% increase in the concentration of the major free radical in the active intact muscle compared with muscle at rest by EPR spectroscopy. In addition, Davies et al. (1982) reported that EPR signals were intensified in the muscle homogenate of rat after an acute bout of treadmill running to exhaustion. The increased catecholamine levels (Cohen and Heikkila, 1974) and lactic acid production (Demopoulos et al., 1986), and an elevated rate of
hemoglobin autoxidation (Mista and Fridovich, 1972) during and after exercise may also increase free radical production. Salo et al (1991) also suggested that exercise-induced hyperthermia may induce ROS production by promoting mitochondrial uncoupling and loss of respiratory control. Furthermore, the transient hypoxia and reoxygenation that may occur in muscles and joints during exercise can enhance ROS generation (Merry et al., 1989). The results of the aforementioned studies have provided reasonable evidence that exercise enhances ROS production in the body.

2.4. Biomarkers of oxidative stress induced by exercise

2.4.1. Introduction

Despite the existence of elaborate antioxidant defenses such as antioxidant enzymes and glutathione, β-carotene, ascorbic acid, and alpha-tocopherol, (Meister and Anderson, 1983), apparently some superoxide radicals and H2O2 molecules remain unscavenged and lead to oxidative stress and possibly tissue damage in humans performing physical activities (Kanter, 1994). Therefore, continued investigation is needed to determine whether ROS produced during the participation in exercise or sport could be scavenged by the enzymatic and non-enzymatic antioxidant systems. It has also been suggested that the “weekend warriors” should ensure that their antioxidant levels are adequate (Brooks et al., 1992). There is, therefore, still speculation about whether the body’s antioxidant systems can provide sufficient protection to cells and tissues proximal to the sites of ROS production.

Although the body is equipped with antioxidant defense systems that protect itself from the potentially deleterious consequences of ROS driven reactions, many reports have shown that exercise causes oxidative stress, e.g., increases in oxidative damage biomarkers such as protein carbonyls and thiobarbituric acid reactive substances (Sen et al., 1997; Sen et al., 1994); defected mitochondrial function (Ravalec et al., 1996; Willis and Jackman, 1994); and decreases in levels of antioxidants and antioxidant enzymes in the heart (Reznick et al., 1982; Somani et al., 1995a), blood (Ji, 1993; Lew et al., 1985; Viguie et al., 1993), lung (Salminen et al., 1984), liver (Brady et al., 1979; Ji, 1993; Lew et al., 1985), brain (Somani et
2.4.2. Lipid peroxidation as a biomarker

The most popular biomarker of oxidative stress is lipid peroxidation. The extent of lipid peroxidation in LDL and plasma by ROS can be monitored by MDA levels (Sumida et al., 1989). Because the products of peroxidation are affected by both the chemical composition of the tissue being studied and the presence or absence of metal ions, there is no single biomarker that is considered the “gold standard” of lipid peroxidation (Clarkson and Thompson, 2000). Results from human and animal studies have suggested that the formation of ROS and urinary excretion of peroxides increase during physical activity (Davies et al., 1982; Packer, 1986; Zerba et al., 1990; Clarkson, 1995; Olinescu, 1995). Increase in blood MDA was found after an 80-km race (Kanter et al., 1988), after a 30-min treadmill test at 60% and 90% VO2pk (Kanter et al., 1993), after downhill running (Maughan et al., 1989), and after incremental cycling tests to exhaustion in sedentary and moderately trained men (Lovlin et al., 1987; Sumida et al., 1989). In contrast, no increases in MDA were found after a half-marathon (Duthie et al., 1990), after 60-min of bench-stepping exercise (Maxwell et al., 1993), after maximal cycle ergometry exercise in elite athletes (Viinikka et al., 1984). Furthermore, MDA amounts were found to decrease immediately after a marathon (Rokitzki et al., 1994) and immediately after a graded exercise test in long-distance skiers (Hubner-Wozniak et al., 1994). The discrepant results may be due to the high inter-subject variability in MDA formation and the non-specificity of the assay used in these studies. However, the above results still showed that strenuous exercise, depending on the mode of exercise, intensity and duration of the activity can lead to harmful effects on the body, especially LDL and plasma oxidation after ROS are released into circulation.

2.4.3. LDL oxidation

The oxidation of LDL has been a topic of great interest in coronary artery disease related research (Goldstein and Brown, 1978; Steinberg et al., 1989; Parthasarathy, 1994) because of many pro-atherogenic properties of oxidized LDL. Paradoxically, aerobic
exercise which may be an important deterrent of coronary artery disease (MacAuley, 1993), is thought to impose an oxidative stress. Aerobic exercise increases oxygen utilization and is accompanied by increased ROS formation (Jenkins, 1988; Sjodin et al., 1990), and increased concentration of myeloperoxidase (MPO) followed by the activation of neutrophils. With exercise increased ROS generation, LDL oxidation could occur (Daughtery et al., 1994; Gray et al., 1993; Pincemail et al., 1990; Pyne, 1994).

If ROS produced during or after exercise can escape from the antioxidant defense systems in cells and plasma, they can have a chance to induce lipid peroxidation in cell membranes and in the lipid moiety of lipoproteins, thus leading to the formation of oxidized LDL. It has been observed that oxidized LDL constitutes a minor part of native LDL in plasma, but its proportion in native LDL increases significantly after long-duration aerobic exercise (Sanchez-Quesada et al., 1995). Furthermore, it has been shown that 30 minutes of exercise at a moderate intensity and duration is a sufficient oxidative stress to increase the susceptibility of LDL to in vitro oxidation in humans (Wetzstein et al., 1998). It can be surmised that low antioxidant contents in LDL and plasma after intensive aerobic exercise may increase oxidative modification of LDL. Optimal amounts of exogenous antioxidant supplementation, therefore, may be necessary to prevent the attack of ROS on LDL and other cells after they are produced during or after physical activity.

2.5. ANTIOXIDANT DEFENSE SYSTEMS AND ACUTE EXERCISE

Higher organisms have developed remarkably efficient antioxidant systems over the course of evolution (Halliwell and Gutteridge, 1989). There are two major antioxidant defense systems against ROS in the body, antioxidant enzymes and non-enzymatic antioxidants. Enzymatic antioxidant systems involve glutathione peroxidase, superoxide dismutase, and catalase (Harman, 1981). Non-enzymatic antioxidants, such as glutathione, vitamin E, vitamin C, uric acid, albumin, and beta-carotene, can reduce ROS and protect tissues against damage by trapping ROS. Generally, regular physical exercise or sport promotes the increase of the antioxidant capacity in the body. However, acute or irregular participation in exercise can result in oxidative stress due to the increased use of antioxidants during the defense against ROS. The extent of oxidative damage during physical exercise is
determined not only by the level of ROS generation, but also by the defense capacity of antioxidant defense systems. However, we still have insufficient knowledge about the interaction of each antioxidant and exercise, which is important in assessing the adequacy of protection against oxidative damage and the necessity of dietary manipulation and/or supplementation.

2.5.1. Antioxidant enzymes

Antioxidant enzymes, which provide the primary defense against ROS generated during exercise, may be activated selectively during an acute bout of strenuous exercise depending on the oxidative stress imposed on the specific tissues as well as the intrinsic antioxidant defense capacity (Jenkins, 1988; Ji, 1995a; Sen, 1995). Superoxide dismutase reduces superoxide to hydrogen peroxide; and catalase and glutathione peroxidase reduce hydrogen peroxide from the SOD reaction to water. In addition, glutathione peroxidase can reduce lipid peroxides directly. There is still insufficient knowledge about the kinetics or molecular regulation of these enzymes in mammalian tissues (Ji, 1995b).

An acute bout of exercise has been shown to increase SOD activity in a number of tissues including the liver (Kuppasamy and Zweier, 1989; Alessio and Goldfarb, 1988b; Ji et al., 1992; Ji and Fu, 1992; Ji et al., 1990; Lang et al., 1987), skeletal muscle (Ji et al., 1992; Ji and Fu, 1992; Ji et al., 1990; Lawler et al., 1993; Quintanilha and Packer, 1983), heart (Ji and Mitchell, 1994; Quintanilha and Packer, 1983), and red blood cells (Mena et al., 1991; Ohno et al., 1988). This activation of SOD is thought to result from increased superoxide production during exercise (Ji, 1993). GPx activity has demonstrated variable responses to an acute bout of exercise. Some studies show no change in this enzyme in skeletal muscle after acute exercise (Leeuwenburgh and Ji, 1995; Ji et al., 1990; Brady et al., 1979; Vihko et al., 1978), whereas others report significant elevation of GPx activity (Ji et al., 1992; Ji and Fu, 1992; Leeuwenburgh and Ji, 1996; Oh-Ishi et al., 1996; Quintanilha, 1984). Most of the previous studies revealed no significant alteration in CAT activity with acute exercise (Meydani and Evans, 1993; Ji, 1995a). However, there are exceptions; CAT activity was found to increase significantly in rat muscle after an acute bout of exercise to exhaustion or at high intensity (Ji et al., 1992; Ji and Fu, 1992). Activation by either allosteric or covalent
modification of the enzyme molecules is possible because for some enzymes, such as SOD and catalase, partial occupancy of the enzyme molecules by their substrates over a wide range of concentrations is known to increase their catalytic activity (Chance et al., 1979). In addition, Blum and Fridovich (1985) and Kono and Fridovich (1982) observed that catalase and glutathione peroxidase are inhibited by superoxides.

The aforementioned results in antioxidant enzymes is based on research on tissues whose cells contain nuclei. The investigations in erythrocyte antioxidant enzymes may show different results because erythrocytes are unable to repair damaged components by resynthesis, and their membranes are made up of components that are vulnerable to peroxidative decomposition (Tauler et al., 1999). The factors that regulate acute changes of antioxidant enzyme activities in erythrocytes “in vivo” are attributable to covalent modification of proteins or other protein interactions, because erythrocytes do not have the machinery to synthesize proteins.

Physical exercise can cause oxidative stress on erythrocytes because of increased generation of ROS. Antioxidant enzymes may need to enhance their activities to reduce ROS to safe compounds during physical activity. However, the increase of activities of erythrocyte antioxidant enzymes by physical exercise is still controversial. A session of acute exhaustive exercise was shown to increase SOD activity, indicating increased superoxide production during exercise in erythrocytes (Somani et al. 1995a). Ohno et al. (1986) and Kaczmarski et al. (1999), however, reported that physical exercise did not increase erythrocyte SOD activity in humans, and Ji et al. (1990) and Alessio and Goldfarb (1988a) observed that one session of acute exercise did not increase SOD activity in rats. Catalase and glutathione peroxidases are the main enzymes that remove H$_2$O$_2$ in erythrocytes. However, most of the literature revealed no significant alternation in catalase activity with acute exercise (Ji et al. 1990; Meydani and Evans, 1993; Ohno et al., 1986), while studies conducted by Somani et al. (1995a) and Kaczmarski et al. (1999) reported that erythrocyte catalase decreased after acute exercise. The decrease in catalase activity was in accordance
with an increase in oxidative stress, probably because the oxidative defenses were overwhelmed (Aguiló et al., 2000). Erythrocyte GPx activity was slightly reduced after a brief (30 min) physical exercise in sedentary students (Ohno et al., 1986). Somani and Husain (1995) noted that the affinity of glutathione peroxidase for glutathione is higher following exercise. These changes might be part of an adaptive mechanism to meet the greater production of free radicals during exercise. There is as yet no clear explanation for these discrepancies (Ji, 1995a).

2.5.2. Antioxidants

Several studies have examined the effect of acute exercise on changes in the amounts of antioxidants in the blood to provide information on oxidative stress induced by exercise (Koshiishi and Imanari, 1997). Changes in blood amounts of vitamin C and glutathione have been used to indicate increased oxidative stress. It is thought that these antioxidants may be mobilized from tissue stores to combat oxidative stress elsewhere in the body.

2.5.3. Vitamin C

Vitamin C, well known as a potent water-soluble antioxidant in cytosol and extracellular fluid, has two forms, ascorbic acid and dehydroascorbic acid. It is found in high concentrations in many tissues; human plasma contains about 60 µM. On interaction with ROS, ascorbic acid is oxidized to dehydroascorbic acid via the intermediate ascorbyl free radical. Dehydroascorbic acid is recycled back to ascorbic acid by the enzyme dehydroascorbate reductase. Thus, dehydroascorbic acid is found in only very low levels compared with ascorbic acid. In addition, the ratio of ascorbic acid and dehydroascorbic acid is suggested as an indicator of oxidative stress (Lykkesfeldt et al., 1995).

Ascorbic acid proved to be superior in vitro to the other water-soluble plasma antioxidants bilirubin, uric acid, and protein thiols as well as to the lipoprotein-associated antioxidants alpha-tocopherol, ubiquinol-10, lycopene, and beta-carotene (Frei, 1991). Although these antioxidants can lower the rate of detectable lipid peroxidation, they do not prevent its initiation. Only ascorbic acid was reactive enough to effectively intercept oxidants in the aqueous phase before they attacked and caused detectable oxidative damage.
to lipids. Not only can ascorbic acid directly participate in the reduction of superoxide, hydroxyl radical, and singlet oxygen to safe compounds and inhibit lipid peroxidation (Beyer, 1994; Bendich et al., 1986), but it can also regenerate tocopherol from the tocopheryl radicals (Packer, 1992).

Gleeson et al. (1987) reported that the plasma concentration of ascorbic acid increased from 52.7 to 67 mM immediately after a 21-km running race. The increase in ascorbic acid significantly correlated with an increase in cortisol. The authors suggest that the increase in ascorbic acid is a result of a concomitant release of cortisol from the adrenal glands. However, Camus et al. (1994) examined plasma ascorbic acid concentration after 35 min of treadmill downhill running and found that AA concentration decreased 20 min into the run, decreased further immediately after exercise, and approached resting values at 20 min after exercise. Furthermore, decreased levels of plasma ascorbic acid have been reported in physically active men (Hemila, 1996).

2.5.4. Glutathione homeostasis

GSH (gamma-glutamylcysteinylglycine) is the most abundant non-protein thiol source in the cell and serves multiple functions in protecting tissues from oxidative damage and keeping the intracellular environment in the reduced state (Meister and Anderson, 1983). GSH reduces hydrogen- and organic-peroxides via a reaction catalyzed by glutathione peroxidase; it serves as a scavenger of hydroxyl radical and singlet oxygen; and GSH also reduces tocopherol radicals, either directly, or indirectly by reducing semidehydroascorbic acid, thereby preventing free radical chain reaction and lipid peroxidation (Niki et al., 1985). After GSH is oxidized to GSSG, GSH can be recycled by glutathione reductase (GR) at the expense of NADH or NADPH. The ratio of the concentrations of GSH and TGSH is often considered as an indication of oxidative stress because GSH is oxidized to GSSG in cells in response to an increase in free radicals (Lew and Quintanilha, 1991; Ji, 1995b). The exercise-associated decline in the GSH redox ratio may therefore diminish GSH-dependent antioxidant protection and impair the antioxidant chain reaction as well (Sen et al., 1994). It also has been observed that severe GSH depletion results in a significant down-regulation of
liver GPX (Leeuwenburgh and Ji, 1995). The liver synthesizes GSH from endogenous or dietary amino acids de novo, and supplies most of the circulating GSH (Meister and Anderson, 1983). Exercising skeletal muscles appear to increase GSH import from plasma via the gamma-glutamyl cycle (Ji et al., 1992; Leeuwenburgh and Ji, 1995; Ji and Fu, 1992; Lew et al., 1985).

During prolonged exercise, hepatic GSH efflux is increased due to the stimulation of elevated plasma glucagons and vasopressin levels (Lu et al., 1990). This ensures plasma GSH homeostasis despite enhanced tissue GSH use (Leeuwenburgh and Ji, 1995; Lew et al., 1985; Sen et al., 1992). However, plasma and muscle GSH content may be decreased eventually during prolonged exercise when hepatic GSH reserve is diminished and GSH use exceeds GSH uptake (Sen et al., 1992; Durarte et al., 1993; Duthie et al., 1990). A decreased liver GSH content after exercise has been reported in several rodent studies (Lew et al., 1985; Sen et al., 1992; Leeuwenburgh and Ji, 1995), where GSSG was found to be either unchanged (Ji and Fu, 1992) or decreased (Leeuwenburgh and Ji, 1995).

Mammalian erythrocytes are rich in GSH (~2 mM) and account for most of the blood GSH. At rest, 15~20% of the total GSH (TGSH; GSH+GSSG) in human blood is present in the oxidized form (Gohil et al., 1988; Ji et al., 1993). It is reported that human blood GSH is increased possibly from hepatic efflux during progressive intensity exercise (Sahlin et al., 1991) and during prolonged exercise (Ji et al., 1993). However, during heavy exercise with long duration, such as marathon running, hepatic GSH supply may not be sufficient to match the enhanced peripheral utilization, and this may result in a net reduction of blood GSH (Duthie et al., 1990). Marin et al. (1993) observe a decrease in plasma GSH in dogs following a treadmill exercise due to an increased utilization of GSH by skeletal muscles.

An exercise-associated increase in blood GSSG reflects GSH oxidation in the erythrocytes and other tissues. Sastre et al. (1992) and Gohil et al. (1988) observed that trained men who were exercised to exhaustion on a treadmill had increased blood amounts of GSSG immediately after exercise, but values returned to rest within 1 hour. Tessier et al.
(1995) also reported that blood GSSG increased in response to a maximal aerobic capacity test, thereby reducing the ratio of GSH to GSSG. Gohil et al. (1988), Laires et al. (1993), and Viguie et al. (1993) found that prolonged submaximal exercise resulted in an increase in GSSG, and a decrease in GSH. It has been suggested that decreased plasma GSH after exercise reflects its consumption by skeletal muscle (Kretzschmar and Muller, 1993). In contrast to the previous studies, Camus et al. (1994) and Marin et al. (1990) reported that treadmill running for 30 and 35 minutes, respectively, leads to no change in blood GSH and GSSG. Currently, it is difficult to explain these equivocal results.

TGSH is defined as the sum of GSH plus two times the amount of GSSG. After subjects exercised at 70% of VO$_2$max, Ji et al. (1993) observed a consistent, non-significant increase in blood TGSH up to the first 120 min. Furthermore, Sen et al. (1994) did not observe any significant increase in blood TGSH after either maximum capacity or aerobic threshold exercise, indicating that there was no increase of either GSH or GSSG from other tissues into the blood. Therefore, they suggested that the remarkable increase in blood GSSG after maximum capacity and aerobic threshold exercise was dependent on the oxidation of GSH in the blood itself. Because the plasma level of GSH is almost undetectably low in humans (<0.01 µM) (Gohil et al., 1988) and quite low in rats (22-27 µM) (Anderson and Meister, 1980), the oxidation of GSH may have taken place mostly in the erythrocytes. On the other hand, Sen et al. (1992) reported that skeletal muscle-derived cells experience a rapid loss in intracellular TGSH after exposure to oxidant stress. They explained that exposure of the muscle cells to oxidant challenge resulted in a rapid oxidation of intracellular GSH, which in turn was followed by a rapid efflux of GSSG. Therefore, the researchers assumed that during prolonged strenuous exercise, when the total VO$_2$ of the body is sufficiently large, substantial amounts of GSH in active peripheral tissues such as the skeletal muscle may be oxidized to GSSG and exported to the general circulation. From the aforementioned literature, there are two sources of blood GSSG, GSH oxidation in erythrocytes and active skeletal muscle tissue. It is possible that increased efflux of GSSG from active skeletal muscle and increased GSSG formation and GSH utilization in erythrocytes all contribute to no significant change of TGSH.
2.5.5. Uric acid

Uric acid is the end product of enzymatic purine catabolism from the xanthine oxidase pathway in man and higher primates (Becker, 1993). In plasma, uric acid is in the form of its monovalent anion, urate. The plasma or serum concentration of urate in man is normally 160-450 µmol/l (Hochstein et al., 1984) and about three to four times higher than plasma levels of ascorbate. The well-known antioxidative function of urate (Sevanian et al., 1991) has been proposed to be partly responsible for the relatively long human life span (Halliwell and Grutteridge, 1990). Uric acid is not only a primary radical scavenger, but it also recycles DHAA. A more positive redox potential of urea (0.26 V) in comparison with that of ascorbate (0.015 V) makes it feasible to recycle oxidized ascorbic acid (Sevanian et al., 1991). Thus, uric acid could serve as a protective antioxidant for ascorbic acid and save ascorbate in biological systems.

High-intensity exercise may produce a cellular environment in favor of activating the XO pathway (Hellsten, 1994) leading to the accumulation of hypoxanthine and uric acid in tissues (Norman et al., 1987; Hellsten-Westing et al., 1993). Uric acid concentration increases exponentially with workload indicating a rapid degradation of purine products. Therefore, plasma uric acid concentration may be an index of exercise intensity.

2.6. ANTIOXIDANTS SUPPLEMENTATION AND EXERCISE

There has been much interest in the effects of antioxidant supplementation both in terms of promoting performance and in terms of preventing tissue damage, which occurs with exercise, particularly in those who undertake irregular and strenuous activity.

Packer (1991) suggested that there is an increased vitamin E requirement during endurance training. The Emory researchers found that people who have participated in an aerobic exercise program for one year actually have a greater rate of LDL oxidation than a similar group who do not exercise (Shern-Brewer et al., 1998). Researchers did suggest that individuals just starting an exercise program should pay careful attention to their diets.
because the antioxidant-rich foods (fruits and vegetables) can help offset the increase in free radicals during or after exercise (Shern-Brewer et al., 1998).

Numerous studies have demonstrated that antioxidant vitamin supplementations can be beneficial in lowering markers indicative of oxidant stress and lipid peroxidation (Packer, 1986; Kanter et al., 1993; Brown et al., 1997; Sürmen-Gür et al., 1999). However, an almost equal number of studies have failed to show protective effects of antioxidants in preventing tissue damage and lipid peroxidation (Sürmen-Gür et al., 1999).

It has been reported that vitamin C supplementation enhances total antioxidant activity and reduces plasma MDA levels after submaximal exercise (Sen et al., 1994; Alessio et al., 1997). Vitamin E is the primary lipid-soluble antioxidant in cell membranes and can quench singlet O$_2$ radicals (Gaby and Singh, 1991). It has been found that 250 IU vitamin E /Kg diet fed to rats can reduce TBARS and lipid peroxide levels in plasma and leg muscles, after one hour of exercise on the treadmill, compared to rats fed a normal diet (Goldfarb et al., 1994). Kanter et al. (1993) observed that subjects given the vitamin combination (vitamin C 1000mg/day, vitamin E 800IU/d, and beta carotene 30 mg/d) for 6 wk before undergoing 60% VO2pk and 90% VO2pk running exercise sessions had significantly lower levels of breath pentane and serum MDA both at rest and after each exercise bout. However, the supplementation did not prevent an exercise-induced increase in lipid peroxidation.

2.7. ISOFLAVONES

2.7.1. Introduction

The growing concerns for general health, chronic disease prevention, and aging have fueled consumer interest in phytonutrients because of their potential curative, preventative, and nutritive value (Guhr and Lachance, 1997). Phytonutrients have demonstrated functionality as antioxidants, as well as in enzyme modulation, cell proliferation, and apoptosis (Balentine et al., 1999).
Soybeans represent 20-60% of the daily protein intake in people from Asia. They are rich in the phytonutrients which contain isoflavones (Brandi, 1997). It has been surmised that soy consumption, one of the major differences between Asian and Western diets, may be a factor in the low incidence of cardiovascular diseases (Beaglehole, 1990; Knight and Eden, 1996; Aguiló et al., 2000) and hormone-related cancers (Coward et al., 1993; Gotoh et al., 1998). Epidemiologists have long noted that Asian populations who consume soy foods as a dietary staple have a lower incidence of cardiovascular disease (CVD) than those who consume a typical Western diet (Beaglehole, 1990). Soy protein consumption in Japan is reported to be as much as 55 g/d (Nagata et al., 1998), compared with <5 g/d in the United States (Messina et al., 1994a). The soy consumption may be responsible for different CVD incidence rates in Asia and the United States. In 1998, deaths from CVD per 100,000 people between the ages of 35 and 74 years were as follows: 401 for US men, 201 for Japanese men, 197 for US women, and 99 for Japanese women (American Heart Association, 1998). Isoflavones may play the major beneficial role of soy in lowering the incidence of diseases (Brandi, 1997). Therefore, increased soy protein consumption may contribute to a lower incidence of heart disease/cardiovascular disease and cancers.

2.7.2. Metabolism of isoflavones

There are many varieties of isoflavones extracted from soy protein, especially daidzein and genistein (Figure 2). Daidzein, genistein, and their corresponding glycosylated forms account for the major isoflavones in soy. Most isoflavones existing in soy products are present in conjugated forms. After the ingestion of soy isoflavones by humans, microflora in the large intestine are needed to convert conjugated forms, daidzin and genistin, to aglycone forms, daidzein and genistein, respectively. After absorption from the large intestine, isoflavones are transported to the liver via the portal vein, where aglycone forms are conjugated with glucuronic acid, and to a much smaller extent with sulfate (Messina et al., 1994a). Most isoflavones are transported as conjugated forms (glucuronidation) in the blood after being secreted from the liver. Subsequently, genistein is transported to tissues, such as the mammary gland, prostate, testes, ovaries, thyroid gland, liver, skeletal muscle, and brain in rats (Chapter 3; Chang et al., 2000). The peaks of daidzein and genistein in the blood are observed from six to 8 hours after ingestion (King and Bursill, 1998; Watanabe et al. 1998).
Like endogenous estrogen, these conjugated isoflavones are excreted through both urine and bile and undergo enterohepatic circulation (Adlercreutz et al., 1995; Kurzer and Xu, 1997).

![Daidzein](image1)
![Genistein](image2)

**Figure 2.1.** Skeletons of classical isoflavones, daidzein and genistein.

### 2.7.3. Bioavailability of isoflavones

Lichtenstein (1998) reported that a variety of factors influence the bioavailability of soybean isoflavones. Xu et al. (1995) and Zhang et al. (1999) suggested that gut microflora may play an important role in the bioavailability of isoflavones in humans. Moreover, plasma genistein concentration appears to vary even though the same amount of genistein is consumed in humans. Apparently, the bioavailability of isoflavones in some humans may be relatively low because the extent of the degradation of conjugated isoflavones into aglycone forms for absorption by gut microflora is varied.

Different isoflavones may have varied absorption and excretion rates. Xu et al. (1994) assessed the bioavailability of soymilk daidzein better than genistein in adult females. Other components in foods, as well as food processing may also affect isoflavone bioavailability. Tew et al. (1996) reported that ingestion of wheat fiber with soy protein reduced plasma genistein by 55% but had no significant effect on daidzein concentrations. Furthermore, fermentation decreases isoflavone content of food items but increases their bioavailability (Hutchins et al. 1995).

### 2.7.3. Consumption of isoflavones in humans

The daily consumption of isoflavones is relatively different in Asian and Western populations. Dwyer et al. (1994) suggested that the intake of isoflavones depends upon the
types and amounts of food consumed and the levels of various compounds in each food in the diet. In Asian countries, the average consumption of isoflavones was reported to range from 50 to 100 mg a day (Adlercreutz et al., 1993; Coward et al., 1993), while British intake was estimated in data collected in the 1980s to be less than 1 mg a day (Jones et al., 1989). Furthermore, Adlercreutz et al (1993) reported that isoflavone consumption per capita among the Japanese was estimated to be up to 200 mg/d, consequently leading to a possible health advantage in the long term.

A dramatic rise in plasma isoflavone concentrations is observed after soy protein supplementation to a typical Western diet, the levels reaching 901±245 for genistein and 498±102 nmol/L for daidzein. Adlercreutz et al. (1993) also observed that the average genistein concentration in the plasma in Japanese men was 86.9 ng/ml, 44-fold more than in Finnish men. These results demonstrate that isoflavones are bioavailable to humans.

2.7.4. Antioxidative effect of genistein

Wiseman (1996b) suggests that dietary isoflavones have a potential to reduce the risk for cardiovascular disease by inhibiting LDL oxidation. Isoflavones have a chemical structure similar to estrogen, which was reported to have a weak antioxidant activity because of the hydroxyl group on its “A” ring which is in the same location as in vitamin E (Tiidus, 1995), leading to the membrane stabilization which provides protection against lipid peroxidation due to decreased membrane fluidity (Wiseman et al., 1993; Wiseman, 1996a). In addition, isoflavones also have multiple hydroxyl groups which increase their effectiveness as antioxidants (Tikkanen et al., 1998; Wang, 2000) by donating a hydrogen atom(s) from their phenolic hydroxyl group(s) to peroxy radicals. The differential potency between genistein and daidzein can be attributed to the presence of the 5-hydroxyl group of genistein (Bickoff et al., 1992).

Genistein with the highest antioxidant characteristic among isoflavones has been shown to prevent LDL oxidation initiated by oxidizing agents (cupric ions or azo-induced oxidants) in vitro (Bakhit and Potter, 1995; Kapiotis et al., 1997; Kerry and Abbey, 1998) in a concentration-dependent manner (Wang, 2000), as measured by thiobarbituric acid-reactive
substance formation, altered electrophoretic mobility, and lipid hydroperoxides. Meng et al. (1999) and Yamakoshi et al. (2000) reported that in vitro incorporation of isoflavone oleic acid esters into LDL increases the oxidation resistance of LDL. Additionally, genistein inhibits bovine aortic endothelial cell- and human endothelial cell-mediated LDL oxidation and protects vascular cells from damage by oxidized LDL (Kapiotis et al., 1997).

The antioxidant activity of genistein was also tested in different systems. Pratt et al. (1981) observed that genistein prohibited hemolysis of red blood cells by dialuric acid or H$_2$O$_2$; Jha et al. (1985) found that genistein protected microsomal lipid peroxidation induced by an Fe$_2^+$-ADP complex; Record et al. (1995) observed that genistein was effective against UVA- and UVB- or peroxyl radical-induced liposomal lipid peroxidation; and Wei et al. (1993) reported that genistein suppresses H$_2$O$_2$ production by 12-O-tetradecanoylphorbol-13-acetate stimulated human polymorphonuclear leukocytes and HL-60 cells in a dose-dependent manner over the concentration range 1-150 µM.

Soy isoflavones, especially genistein, act as antioxidants in in vitro models. However, most of the main isoflavoids of soy, genistein and daidzein, are conjugated in the liver and then released into the circulation. The antioxidant activity of isoflavone in in vivo animal and human studies may have a different mode of performance because isoflavones are present as conjugated forms in tissues and blood. Larner et al. (1993), and Shwaery et al. (1997) have suggested that endogenous human estrogens can be converted to lipid-soluble esters in human tissues and become incorporated into LDL. However, only minute amounts of unmodified isoflavone, corresponding to approximately one-isoflavone molecule/500 LDL molecules, are found in LDL (Tikkanen et al. 1998; Yamakoshi et al., 2000). LDL isolated from subjects after the intake of soy-containing isoflavones was less susceptible to oxidation than LDL isolated from individuals consuming a soy-free diet. Alternatively, some lipid-soluble isoflavones such as those esterified at different hydroxyl groups may have been incorporated into LDL, thereby inhibiting LDL oxidation (Meng et al. 1999; Yamakoshi et
However, there is currently no evidence that isoflavones are converted to such lipid metabolites in vivo.

The in vivo antioxidant activity of genistein in a mouse model with singlet oxygen-induced cerebral stroke was evaluated by Trieu et al. (1999). At a dose of 16 mg/kg genistein administered every 6 h from 24 h prior to irradiation until 24 h after irradiation, the average size of the cerebral lesion of genistein-treated mice (8.1 mm$^2$) was significantly smaller than that in control mice (14.6 mm$^2$) treated with the carrier dimethylsulfoxide (DMSO). Genistein in this study was administrated to provide protection against irradiation, was not in the conjugated form that reaches the tissues after ingestion. In the study of Cai and Wei (1996), dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activities of antioxidant enzymes in the skin and small intestine of SENCAR mice. In contrast, Breinholt et al. (1999) observed that dietary genistein administration at 0.1 g/kg body weight for 2 weeks consistently inhibited the activity of GR, CAT, SOD, and GPx in RBC of female rats, while there was no effect of genistein on liver antioxidant enzymes. The effect of isoflavones on antioxidant enzymes may have tissue specific characteristic, furthermore, the varied dose administered to animals may also lead to discrepant results in different studies. Even though isoflavones are considered as good antioxidants, an assumption predominantly based on in vitro studies, very little is known about their actual in vivo capability to function as antioxidants.

2.7.5. Other activities of isoflavones

In addition to its antioxidant activity, genistein has a number of other properties that suggest its potential as an antiatherogenic agent. Isoflavones have weak antiestrogenic and estrogenic effects in both animals and humans through the interaction of these molecules with estrogen receptors (Martin et al., 1978; Setchell et al., 1984; Kondo et al., 1990). Among the isoflavones, genistein is the most active phytoestrogen with the highest binding affinity for the estrogen receptor (Shutt and Cox, 1972). The beneficial effects of estrogen include lowering LDL cholesterol and increasing HDL cholesterol. Isoflavone-rich soy protein lowered cholesterol significantly more than soy protein without isoflavones in humans (Cassidy et al., 1995; Pelletier et al., 1995; Crouse et al., 1999). Crouse et al (1999)
concluded that the cholesterol-lowering effect of soy protein was entirely due to isoflavones. However, Nestel et al. (1997) found no changes in plasma lipid levels in women consuming extracted soy isoflavones (without soy protein). Therefore, both soy protein and isoflavones may be needed in combination for the maximal cholesterol-lowering effect of soy.

In addition to its effect on lipids, genistein is a potent inhibitor of tyrosine kinase activity (Akiyama et al., 1987) and thus it blocks the action of growth factors implicated in the growth of the atherosclerotic lesion, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor and other growth factors that work through tyrosine kinase action (Wilcox and Blumenthal, 1995). In addition, genistein may reduce overall thrombosis associated with atherosclerosis by interfering with platelets and thrombin action through this inhibition of tyrosine kinase (Ozaki et al., 1993; Sargeant et al., 1993), further preventing thrombin-induced platelet activation and aggregation (Asahi et al., 1992). Genistein also inhibits the proliferation of many vascular cells, including vascular endothelial cells (Raines and Ross, 1995) and smooth-muscle cells (Fugio et al., 1993), and inhibits the atherosclerotically important process of angiogenesis (Raines and Ross, 1995). The expression of leukocyte adhesion molecules (B2-integrins) after activation is likely to be of importance in the initiation of lesion formation (Gimbrone et al., 1990). Genistein has also been shown to inhibit cell adhesion (Raines and Ross, 1995).

2.8. REFERENCES


CHAPTER 3

High-Genistin Isoflavone Supplementation Modulated Erythrocyte Antioxidant Enzymes and Increased Running Endurance in Rats Undergoing One Bout of Exhausting Exercise

3.1. ABSTRACT

Genistein putatively acts as an antioxidant in vitro. To investigate the in vivo antioxidative activity of genistein, forty-eight male rats were divided into four groups and fed diets with or without 598 mg isoflavone extract per kg of diet for four weeks. On the final day of the study, twenty-four rats were exercised to exhaustion (22 meters/minute at 10% inclination on the treadmill) and then all the rats were sacrificed. The high-genistin isoflavone extract (HGI) diet significantly increased the running time (GE vs. CE: 54 vs. 48 min) and genistein concentrations in the plasma, liver, and gastrocnemius muscle (GE vs. GS: 730.3 vs. 348.5 ng/ml, 529.3 vs. 216.9, and 59.0 vs. 24.9 ng/g, respectively). Exercise doubled genistein concentrations in all tissues and significantly enhanced liver malondialdehyde (MDA). The HGI supplementation did not prevent the increase of MDA; instead, it substantially increased MDA levels in the muscle (HGI vs. control: 0.46 vs. 0.29 mg/kg). The HGI supplementation maintained the decreased activities of catalase and glutathione peroxidase (GPx) due to exercise (GE vs. GS: 0.113 vs. 0.101 and 0.412 vs. 0.403, respectively). It can be concluded that even though HGI modulates erythrocyte antioxidant enzymes against oxidative stress and increases endurance capacity, the supplemented level of HGI does not seem to be optimal for defending the liver and skeletal muscles against oxidative stress.

Key words: genistein, oxidative stress, malondialdehyde, antioxidant enzymes, exercise

3.2. INTRODUCTION

The role of exercise and physical activity in the prevention of chronic disease and promotion of optimal health has drawn the attention of the public (Singh 1992). However,
research on dietary intervention that protects body tissues from damage during vigorous exercise is in its infancy. This damage is mostly attributed to the sharply increased reactive oxygen species (ROS) in the body during exercise (Davies et al. 1982; Packer 1997). Olinescu et al. (1995) reported that the increase of urinary excretion of peroxides demonstrated the presence of ROS during exercise. These highly reactive free radicals are known to cause damage to mitochondrial membranes and cytoplasmic structures through peroxidation of phospholipids, proteins, and nucleotides, resulting in tissue damage (Jenkins 1993; Packer 1997). Fortunately, endogenous and exogenous antioxidant defense systems in the body can cope with ROS, including vitamin E, vitamin C, betta-carotene, and antioxidant enzymes (SOD, catalase, and GPx). However, an imbalance occurs when ROS, generated during exercise, overcome antioxidant defense systems, a state known as oxidative stress.

Generally, regular physical activity or participation in a sport will increase the total antioxidant capability of the body. According to Ji (1995), the activities of antioxidant enzymes provide the first line of defense against ROS increase in the heart, liver, lung, blood platelet, and skeletal muscle, in order to cope with oxidative stress induced by acute or exhausting exercise. However, it is possible that acute or irregular participation in exercise will result in oxidative stress due to the elevated use of antioxidants during the defense against ROS. Therefore, more investigations have focused on whether dietary antioxidant supplementation will boost the antioxidant defense systems and overcome oxidative stress. Goldfarb et al. (1994) found that rats fed a 250 IU vitamin E/ kg diet for five weeks had lower thiobarbituric acid reactive substance (TBARS) and lipid peroxide levels in plasma and leg muscles after one hour of treadmill exercise, than rats fed a control diet. This finding suggests that antioxidant supplementation in humans and animals may be needed to protect tissues against ROS attack induced by exercise.

Since isoflavones from soy clearly exhibit antioxidant activity in vitro and in vivo (Kurzer and Xu 1997), Brandi (1997) surmised that isoflavones in soy might be responsible for the beneficial effect of lowering the incidence of diseases in Asians. There are many varieties of isoflavones found in soy protein. Genistein and daidzein, and their
corresponding glucose conjugated forms, genistin and daidzin, account for the majority of isoflavones in soy.

Isoflavones have a chemical structure similar to estrogen, which has been reported to have a weak antioxidant activity because of the hydroxyl group on its “A” ring in the same location as in vitamin E (Tiidus, 1995). In addition, isoflavones are effective antioxidants, because their phenolic rings have multiple hydroxyl groups that reduce peroxyradicals by donating hydrogen atoms (Tikkanen et al. 1998). With the highest antioxidant characteristic among isoflavones, genistein has been shown to prevent LDL oxidation initiated by oxidizing agents in vitro (Bakhit and Potter 1995; Kapiotis et al. 1997) and to protect microsomal lipid peroxidation induced by a Fe$^{2+}$-ADP complex (Jha et al. 1985). Additionally, Cai and Wei (1996) have observed that dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activities of antioxidant enzymes in various organs of SENCAR mice.

Most prior studies have used in vitro measures to evaluate the antioxidative ability of isoflavones. However, it is not completely understood if isoflavones can defend against ROS or boost up total antioxidant defenses in vivo. In order to initiate the maximum impact of oxidative stress on rats, one single exhaustive exercise, without exercise training, was given to rats in an exercised group. We hypothesized that exercise would initiate oxidative stress in the rats, further resulting in changes of erythrocyte antioxidant enzymes and increased products of lipid peroxidation. We also hypothesized that four-week HGI supplementation would modulate activities of erythrocyte antioxidant enzymes and decrease products of lipid peroxidation in tissues after the rats were exercised on the treadmill. The objective of this study was to investigate the in vivo antioxidant ability of the isoflavones, especially genistein, in rats undergoing acute exhausting exercise.

3.3. MATERIALS AND METHODS

This study was approved by the Virginia Tech Institutional Review Board.
3.3.1. Animals

Forty-eight one-year-old Sprague-Dawley male rats were purchased from Harlan Industries (Indianapolis, IN). The rats were housed at 25°C with a 12-hr light/dark cycle with free access to feed and water throughout the study. Upon delivery to the Virginia Tech animal research facility, the rats were acclimatized on a chow diet for one week.

3.3.2. Experimental design

The rats were randomly divided into four treatments of equal number. The four treatments were control diet and exercise (CE), control diet and sedentary (CS), high-genistin isoflavone extract (HGI) diet and exercise (GE), and HGI diet and sedentary (GS). All semipurified ingredients were purchased from ICN (ICN Pharmaceuticals Inc., Costa Mesa, CA). The control diet was prepared according to the formula of the American Institute of Nutrition (AIN 93 M). The HGI diet was identical to control diet except that 598 mg isoflavone extract per kg diet was added to replace an equal amount of cornstarch. The composition of isoflavones added in this diet was 84.4% genistein, 14.8% daidzein, and 0.8% glycitein. Each rat’s feed intake was measured daily throughout the study. All the rats were given the experimental diets for 28 days.

On the final day of the study, all the rats in the CE and GE groups were exercised vigorously in one session until they were exhausted. The exercise protocol was 22 meters/minute at 10% inclination on a treadmill (Exer-4/8 treadmill, Columbus, OH). In order to familiarize the rats with the treadmill, they all were trained to walk on the treadmill before the final acute exercise session. The protocol for the walking training was ten meters/minute for five minutes twice a week for the first three weeks. The treadmill was equipped with an electric shocking grid on the rear barrier to motivate the rats to exercise. A rat was considered exhausted when it would not be prompted to run any more. The rats would stop running and when overturned would continue to lie on their backs disregarding gentle prods and electric shock. The running time of each rat in the CE and GE groups was recorded.
3.3.3. Sacrifice and sample collections

Immediately after acute exercise, all the rats were anesthetized using a halogen and nitrogen gas mixture and the maximum possible volume of blood was drawn via the heart puncture. Blood samples were immediately kept in ice. Plasma and red blood cells (RBC) were harvested following centrifugation at 1000 x g for 20 minutes at 4°C. After blood collection, the rats were sacrificed by cervical dislocation and the liver and legs were both removed. In order to diminish tissue exposure to heat and air, the liver and legs were immediately wrapped in aluminum foil and instantly frozen in liquid nitrogen. All samples, including the liver, plasma, RBC, and muscle were stored at –80°C until further analyses.

3.3.4. Biochemical measurements

Genistein concentrations in the plasma, gastrocnemius muscle, and liver were determined by a HPLC method, modified from methods of Wang and Murphy (1994) and Xu et al. (1994). In vivo thiobarbituric acid reactive substances (TBARS) method was used to measure MDA concentrations in the liver and gastrocnemius muscle tissues (Pikul et al. 1989). One gram of tissue, 5 ml of 5% trichloroacetic acid, and 200 µl of 0.15 % butylated hydroxyltoluene were homogenized for one minute. Following homogenization, the mixture was spun at 17,000 x g for 15 minutes at 4°C. One ml of supernatant was filtered by using 9 cm GF/C filter paper and small funnels, and then was mixed with one ml of 0.67% thiobarbituric acid (TBA) solution. The absorbance was read on a spectrophotometer (532 nm) within 1 hour after incubation in a 95°C water bath for 40 minutes. The concentration of the MDA product was calculated by comparison with a standard curve established from different concentrations of tetraethoxypropane. RBCs were used to measure the activities of antioxidant enzymes, SOD, catalase, and GPx. The activities of antioxidant enzymes were standardized by erythrocyte protein. Superoxide dismutase (EC 1.15.11.) was determined by the method of Xin et al. (1991). The activities of GPx (EC 1.11.1.9) and catalase (1.11.1.6) were determined according to the methods developed by Agergaard and Jenson (1982) and Aebi (1983), respectively.
3.3.5. Statistical analysis

A two-way ANOVA was used to compare the effects of exercise and diet, and their interactions on the means of MDA concentrations in tissues and activities of antioxidant enzymes in the rats (Sokal and Rohlf 1995). The significant level was set at p<0.05. When the interactions between exercise and dietary supplementation were statistically significant, Tukey’s HSD procedure at the 0.05 level experimentwise was used to compare group means. A Student’s t-test was used to contrast mean genistein concentrations between the GE and GN groups and the running time between the NE and GE groups (Sokal and Rohlf 1995). The computer software program JMP (SAS Institute Inc., Cary, NC) was used for all computations.

3.4. RESULTS

3.4.1. Body weight, feed intake, and running ability

At the time of sacrifice, mean body weights among the CE, CS, GE, and GS groups were not significantly different, 545.3, 549.9, 583.8, and 544.8 gm, respectively (n =12, SE = 12.7, 20.2, 15.6, and 16.6 gm). High-genistin isoflavone supplementation did not affect body weight gain or feed intake. The average running time of the rats in the GE group, 54 minutes (n = 12, SE = 2 minutes), was significantly longer than that of the rats in the CE group, which was 48 minutes (n = 12, SE = 1.4 minutes) (P<0.05).

3.4.2. Genistein concentrations

High-genistin isoflavone supplementation led to significant (P<0.001, 0.001, and 0.002, respectively) increases in genistein concentrations in plasma, liver, and gastrocnemius muscle in sedentary rats (Table 3.1; Fig 3.1). After the rats underwent one session of acute exhausting exercise on the treadmill, all genistein concentrations in plasma, liver, and gastrocnemius muscle were significantly higher than in the sedentary rats. Exercise more than doubled the concentrations of genistein in plasma, liver, and gastrocnemius muscle (109%, 144%, and 137% increase, respectively) as compared to sedentary HGI fed rats. There was no significant correlation between genistein concentration and other biochemical parameters.
3.4.3. Products of lipid peroxidation

Malondialdehyde is an index of the extent of lipid peroxidation. MDA concentration in the liver and gastrocnemius muscle were approximated by the TBARS method.

According to the results of a two-way ANOVA (Table 3.2; Fig 3.2), the interactions between exercise and diet were not significant (P = 0.10) and the main effects of exercise led to significant increase of MDA concentrations in the liver (P = 0.008), while the main effects of diet did not (P = 0.37). Multiple comparisons of the means suggested synergy of exercise and HGI supplementation on the increase of liver MDA, but the relationship was weak, with HGI and exercise explaining only $R^2 = 20\%$ of the variation in the liver MDA concentration.

The two-way ANOVA revealed no significant interaction effects between diets and exercise levels (P = 0.47) on MDA concentrations in the gastrocnemius muscle, and one session of acute exhausting exercise did not result in a significant augmentation of MDA concentrations in rats fed control diet and HGI diet (P = 0.75). Yet, genistein supplementation caused a slightly significant increase of MDA (P = 0.044) in both sedentary and exercised rats (Table 3.3; Fig 3.3). Mean MDA concentration was 0.454 mg/kg ($n = 24$, SE = 0.049 mg/kg) with HGI supplementation, and was 0.322 mg/kg ($n = 24$, SE = 0.040 mg/kg) with no supplementation.

3.4.4. Activities of antioxidant enzymes in erythrocytes

Two-way ANOVA analysis showed that one session of exercise significantly increased SOD activity in rats fed control and HGI diets (P=0.037); however, HGI supplementation led to an inhibition of SOD activity in rats (P=0.031) (Table 3.4; Fig 3.4). There was no significant interaction between exercise and dietary supplementation on SOD activity (P = 0.19).

The main effects of HGI supplementation and exercise on catalase activity (Table 3.5; Fig 3.5) were not significant (P = 0.42 and 0.18, respectively), while the interactions between diet and exercise were significant (P = 0.003). Comparing means by Tukey’s HSD
procedure, one session of acute exhaustive exercise significantly decreased catalase activities in the rats fed the control diet compared to those in sedentary rats, but exercise did not result in a reduction in erythrocyte catalase activities in rats fed the HGI diet.

The interactions between diet and exercise were significant (P = 0.018) in GPx activities of RBC, and the main effects of exercise were significant (P = 0.038), but the main effects of diet were not statistically significant (P = 0.12) (Table 3.6; Fig 3.6). The mean activity of GPx in rats that underwent one session of acute exhaustive exercise was 0.353 µmol/s/mg RBC protein (n = 24, SE = 0.018 µmol/s/mg RBC protein), and was 0.415 µmol/s/mg RBC protein (n =24, SE = 0.026 µmol/s/mg RBC protein) in sedentary rats. One session of acute exhausting exercise significantly decreased GPx activities in RBC in rats fed the control diet, based on multiple comparisons of means by Tukey’s HSD procedure. However, GPx activities in the GE rats did not change with the acute exhausting exercise.

3.5. DISCUSSION

This study assessed the effects of dietary high-genistin isoflavone supplementation on immediate post-exercise indices of tissue oxidative damage and modulations antioxidant enzymes (SOD, GPx, and catalase) in erythrocytes. It is the first study to examine the antioxidant potential of isoflavones using an in vivo animal exercise model. It was shown that significant accumulation of genistein in the plasma, skeletal muscle, and liver occurred after dietary HGI supplementation for four weeks but this did not increase the antioxidative capability of the liver and skeletal muscle in these rats. Nor did an increased genistein concentration in the liver provide significant protection against the oxidative stress due to acute exhaustive exercise. However, the HGI supplementation significantly extended the rats’ running time to reach exhaustion on the treadmill. Furthermore, in regard to the antioxidant enzymes in erythrocytes, HGI, while it significantly prevented the decrease of GPx and catalase activities by exercise, did not significantly influence the activities of GPx and catalase in the sedentary rats. In addition, the HGI supplementation significantly decreased erythrocyte SOD activity in both exercised and sedentary rats.
The major isoflavones in soy are the conjugated forms genistin and daidzin, which are glycosylated genistein and daidzein, respectively. It has been suggested that conjugated isoflavones derived from foods could not be absorbed from the small intestine (Hollman 1997). Hydrolysis of the mostly beta-glucosidic bonds that attach isoflavones to sugars by the gut microorganisms is necessary for absorption of genistin (Hollman 1997; King and Bursill 1998; Zhang et al. 1999). Significantly increased genistein concentrations in the plasma, skeletal muscle, and liver in rats fed at 500 mg genistin containing compounds per kg diet indicated that isoflavones from food supplementation could lead to a significant accumulation of genistein in tissues. However, genistein concentrations varied among each of sedentary rats fed the HGI diet. It has been suggested that high inter-individual variation of isoflavone bioavailability in humans may be attributed to existence of gut bacteria, which are necessary for the degradation of conjugated isoflavones into aglycone forms for absorption (Xu et al. 1994; Wiseman 1999). The current results support such a suggestion in rats. It may be that degradation of conjugated isoflavones in the colon possibly plays a role in their bioavailability in rats. Aglycone forms of isoflavones are conjugated with glucuronic acid and to a much smaller extent with sulfate after absorption from the large intestine (Messina et al. 1994). Subsequently, genistein is secreted from the liver and transported as inactive conjugated forms in the blood (Messina et al. 1994).

Genistein concentrations in the plasma, skeletal muscle, and liver of rats fed the HGI diet doubled after acute exhausting exercise. Exercise can significantly enhance antioxidants in tissues such as vitamin E in plasma and erythrocytes (Pincemail et al. 1988; Vasankari et al. 1997) and vitamin C in plasma (Maxwell et al. 1993). Furthermore, Jakeman and Maxwell (1993) observed that plasma total antioxidant capacity increased after exercise, representing a net efflux of antioxidants into plasma. Pincemail et al. (1988) hypothesized that exercise moved vitamin E from other tissues into the plasma and that skeletal muscle utilized circulating vitamin E for protection against oxidative damage. Like vitamin E, genistein might be mobilized from some tissues other than the liver and skeletal muscle because both the above tissues and plasma had significantly higher genistein concentrations in exercised rats than in sedentary rats in this study. Chang et al. (2000) reported the
presence of genistein in the prostate gland, testes, thyroid gland, and brain of rats fed a genistein diet. Therefore, it can be hypothesized that genistein may be transported from these tissues to the plasma, liver, and muscle tissue. In addition to mobilization from storage sites, acute exhausting exercise might also decrease the clearance of genistein from the liver. Like endogenous estrogen, glucuronidated genistein is excreted through urine and bile and undergoes enterohepatic circulation (Kurzer and Xu 1997). Several reports have noted an increase in plasma estrogen concentrations following acute exercise in women (Bonen et al. 1981). It has been suggested that this increase is primarily due to the decreased metabolic clearance of estrogen as a direct consequence of exercise induced reduction in hepatic blood flow during exercise (Sutton et al. 1990). Therefore, the increased genistein concentrations in the plasma, liver, and gastrocnemius muscle may be attributed to a movement of genistein from other tissues and low clearance from the liver.

Genistein also has a structure similar to estrogen and has been suggested to have estrogenic and anti-estrogenic responses in mammalian tissues (Kurzer and Xu 1997). Estrogens were suggested to act as antioxidants in a manner similar to vitamin E to terminate peroxidation chain reactions (Burton and Ingold 1989), however, they were reported to act as prooxidants and elevate plasma TBARS concentrations in rats when administrated in the form of pharmacological contraceptives (Kose et al. 1993; Pizzichini et al. 1993). In the present study, 500 mg genistin-containing compounds per kg diet from isoflavone extract given to rats may be too high to act as an antioxidant. Thus, a significantly increased MDA concentration in the gastrocnemius muscle in rats fed HGI diet means that genistein at high levels may act as a prooxidant rather than an antioxidant. Unpublished data from our lab showed that 399-ppm HGI supplementation led to a slightly lower plasma MDA concentration measured by the TBARS method than 598-ppm HGI supplementation in rats.

Oxidative stress induced by acute exercise can significantly elevate markers of tissue peroxidative damage such as MDA, and possibly elevate tissue antioxidant enzyme activities (Gee and Tappel 1981; Davies et al. 1982; Ji 1995; Sen 1995; Somani and Arroyo 1995) because physical exercise promotes the production of ROS due to a substantial increase in
oxygen consumption (Davies et al. 1982; Packer 1986; Ayres et al. 1998). The present study showed that exercise at 22 meters/min at 10% inclination for 1 hour significantly elevated MDA levels in the livers of rats fed control and HGI diets. However, one session of acute exhausting exercise did not result in a significant accumulation of MDA in the gastrocnemius muscle. Ji and Fu (1992) also observed that exhausting exercise (20 meters/min and 0% inclination) did not significantly elevate the MDA level in muscle, but significantly increased it in the liver. Therefore, acute exercise might not lead to lipid peroxidation in all tissues equally. It is plausible to speculate that products of lipid peroxidation may be transported from the muscle into the circulation, and possibly to the liver.

One session of acute exhaustive exercise significantly increased MDA levels in the liver; however, HGI supplementation did not diminish the increase of MDA. Although genistein has been strongly suggested as an antioxidant in vitro and in vivo, in this study, genistein did not perform as an antioxidant in the liver. Further research is necessary to explore whether there a dose-dependent response of isoflavones on increased liver MDA initiated by exhaustive exercise.

Every antioxidant, including vitamin antioxidants, is a redox agent, protecting against ROS in some circumstances, but also promoting ROS generation in others (Herbert 1996). Herbert (1994, 1995) reported that antioxidant vitamin supplements at pharmacological levels might promote heart disease, cancer, and liver and kidney disease. The present study’s use of genistin-containing compounds from isoflavone extract in the diet may have been too high, thereby promoting ROS generations in tissues. The results of this study showed genistein not only enhanced MDA in the gastrocnemius muscle of exercised and sedentary rats, but also provided no significant protection against increased MDA in the liver due to exercise.

Although dietary HGI supplementation did not prevent MDA increase in the liver of rats run to exhaustion on the treadmill, the running time was significantly longer than rats fed the control diet. Because oxidative damage may occur with exercise, antioxidant
administration has drawn much attention both in terms of preventing damage and in terms of affecting performance. Packer (1997) suggested that oxidative stress might play a role on the fatigue process, and antioxidant administration might reduce the fatigue, leading to an increase in performance. Yet, the reports from most studies showed that antioxidant supplementation exhibited no effect on performance in humans (Clarkson 1995). Although antioxidant supplementation may not bolster the performance in humans, exogenous glutathione supplementation was found to increase swimming time in mice (Novelli et al. 1991). Furthermore, Balakrishnan and Anuradha (1998) reported that exogenous glutathione influenced the endurance capacity of athletes. Studies conducted in our laboratory, demonstrated that isoflavones increased GSH concentration in the blood. In addition, Appelt and Reick (1999) observed that feeding rats 810-PPM soy isoflavones led to increased GSH concentration and decreased GSSG concentration in plasma. Therefore, isoflavones may increase endurance capacity in rats by their influence on glutathione concentrations. The present study suggests that HGI supplementation increases endurance capacity in one-year old rats. The further study is needed to investigate the effect of isoflavones on running endurance.

It was reported that genistein may modulate antioxidant enzyme activities. Genistein, administrated to female rats at 0.1 g/kg BW levels a day, inhibited the activity of glutathione reductase (GR), catalase, SOD, and GPx in RBC (Breinholt et al. 1999). These researchers observed that the activities of antioxidant enzymes in RBC decreased concurrently with an increase in the antioxidant potential due to administered flavonoids. Others observed that genistein could suppress 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated H2O2 production in vitro and in vivo and inhibit superoxide anion formation by the xanthine-xanthine oxidase system (Wei et al. 1993; Wei et al. 1995). Hence, Breinholt et al. (1999) hypothesized that antioxidant enzymes in RBC were down-regulated by genistein in response to an improved antioxidant status of the RBC due to the increase of high antioxidant potential from genistein supplementation. This study confirms the hypothesis of Breinholt et al. (1999), because it showed that HGI supplementation decreased SOD activities in RBC.
Physical exercise can cause oxidative stress in erythrocytes because of increased generation of ROS, which may trigger antioxidant enzymes to enhance their activities and reduce ROS to safe compounds. However, the increase of activities of antioxidant enzymes by physical exercise is still controversial. A session of acute exhaustive exercise was shown to increase SOD activity, indicating increased superoxide production during exercise (Ji 1993) in a number of biological tissues including heart (Ji 1993), liver, (Alessio and Goldfarb 1988; Ji et al. 1988; Ji et al. 1990), lung (Reddy et al. 1992), blood platelets (Buczynski et al. 1991), skeletal muscle (Ji et al. 1990; Lawler et al. 1993), and erythrocytes (Somani et al. 1995). Ohno et al. (1986) and Kaczmarski et al. (1999), however, reported that physical exercise did not increase erythrocyte SOD activity in humans, and Ji et al. (1990) and Alessio and Goldfarb (1988) observed that one session of acute exercise did not increase SOD activity in muscle from rats. In contrast, most of the literature revealed no significant alternation in catalase activity with acute exercise (Ji et al. 1990; Meydani et al. 1993), while studies conducted by Somani et al. (1995) and Kaczmarski et al. (1999) reported that erythrocyte catalase activity decreased after acute exercise. The effect of an acute session of exercise on GPx activity in various tissues has not been reported consistently in the literature (Ji 1995). Erythrocyte GPx activity was slightly reduced after a brief (30 min) physical exercise in sedentary students (Ohno et al. 1986), yet, Ji et al. (1990) observed that acute exercise did not affect GPx activity in the livers of rats. There is yet no clear explanation for these discrepancies (Ji 1995). This study agrees with Somani et al. (1995) that erythrocyte catalase activities were significantly decreased by one session of acute exhausting exercise in the rats fed the control diet, while erythrocyte SOD activity significantly increased. In addition, RBC GPx activity was significantly decreased by exercise. In general, an increased generation of ROS during an acute session of strenuous exercise, causing the activation of antioxidant enzymes, remained the most viable explanation (Ji et al. 1988; Ji et al. 1990; Ji 1993). In the present study, only erythrocyte SOD in rats reflected the increased ROS production, because its activity was significantly enhanced by acute exercise. However, this explanation could not effectively account for the decrease of erythrocyte GPx and catalase activities in the study.
Erythrocyte SOD activity was significantly increased by acute exercise in the rats, while the activity of the GE rats was maintained by HGI administration at the same level as that of the NS rats. As Wei et al. (1993, 1995) observed that genistein could suppress H$_2$O$_2$ production and inhibit superoxide formation, genistein may diminish superoxide anion generated by one session of acute exhaustive exercise. In regard to GPx and catalase activities in erythrocytes of exercised rats, HGI prevented the decrease of GPx and catalase activities due to acute exercise. Although the physiological justification in the decrease of enzymes’ activities is not clear, the homeostasis of antioxidant defense is disturbed by oxidative stress due to one session of acute exhaustive exercise. The HGI supplementation could prevent this disturbance of oxidative stress due to exercise, further leading to maintainance of a more reductive environment in erythrocytes. Therefore, we can speculate that HGI administration in the diet of the rats maintained redox status, because genistein acts as an antioxidant or exerts its estrogenic effect to modulate antioxidant enzyme activities.

3.6. SUMMARY

This study demonstrated that HGI supplementation modulated erythrocyte antioxidant enzyme activities in response to acute exhaustive exercise maybe through antioxidative ability or estrogenic effect of genistein. Isoflavones could enhance endurance capacity in rats by their influence on glutathione homeostasis. In addition, acute exhausting exercise also can change the distribution of genistein in the liver, gastrocnemius muscle, and plasma. The increased genistein in the liver, skeletal muscle, and plasma may possibly come from the prostate, testes, thyroid gland, and brain during exercise or reduced clearance from the liver. However, elevated genistein concentrations in the liver did not provide an extra protection against oxidative stress due to acute exhaustive exercise. The dose of HGI administered in this study might be too high, and consequently genistein may act as both an antioxidant and a prooxidant. It can be concluded that genistein may elevate antioxidant defense in erythrocyte against oxidative stress and increase endurance capacity by its antioxidative effect directly or by its estrogenic effect indirectly, while the amount of HGI may not be optimal to defend the liver and skeletal muscle from oxidative stress. Further study is necessary to explore the optimal dose of dietary genistein supplementation for its best beneficial effect on antioxidant defense systems and its disadvantage effect on the increased MDA in skeletal muscle.
3.7. REFERENCES


Table 3.1. Genistein concentrations in plasma, liver, and gastrocnemius muscle in rats.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>Plasma (ng/ml)</th>
<th>Liver (ng/g)</th>
<th>Muscle (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>12</td>
<td>730.3</td>
<td>a</td>
<td>70.6</td>
</tr>
<tr>
<td></td>
<td>sedentary</td>
<td>12</td>
<td>348.5</td>
<td>b</td>
<td>41.0</td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Means with different letters were significantly different, using a student’s t-test, P<0.05.
Figure 3.1. Genistein concentrations in plasma, liver, and gastrocnemius muscle in rats. Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise, using a student’s t-test.
Table 3.2. Mean liver concentration of malondialdehyde by dietary supplement and exercise.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>MDA (mg/kg)</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>11*</td>
<td>0.640ab</td>
<td>0.640a</td>
<td>0.111</td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td>0.449ab</td>
<td>0.449ab</td>
<td>0.071</td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td>0.366ab</td>
<td>0.366ab</td>
<td>0.062</td>
</tr>
</tbody>
</table>

*One sample was deleted from statistical analysis because it was an outlier.

Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995). Exercise effect was significant, P=0.008.
Figure 3.2. Mean liver concentration of malondialdehyde by dietary supplement and exercise. Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995). Exercise effect was significant, P=0.008.
Table 3.3. Malondialdehyde (MDA) concentrations in the gastrocnemius muscle.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>MDA (mg/kg)</th>
<th>Mean(^a)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>12</td>
<td></td>
<td>0.441</td>
<td>0.052</td>
</tr>
<tr>
<td>HGI</td>
<td>sedentary</td>
<td>12</td>
<td></td>
<td>0.468</td>
<td>0.086</td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td></td>
<td>0.356</td>
<td>0.066</td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td></td>
<td>0.228</td>
<td>0.046</td>
</tr>
</tbody>
</table>

\(^a\)There was a statistical difference due to diets (P = 0.044).
Figure 3.3. Malondialdehyde (MDA) concentrations in the gastrocnemius muscle. There was a statistical difference due to diets ($P = 0.044$).
Table 3.4. The activities of superoxide dismutase (SOD) in RBC in rats.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>SOD (unit/mg RBC protein)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>12</td>
<td>0.091$^{a b}$</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>HGI</td>
<td>sedentary</td>
<td>12</td>
<td>0.080$^a$</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td>0.138$^b$</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td>0.092$^{a b}$</td>
<td>0.012</td>
<td></td>
</tr>
</tbody>
</table>

$^{a,b}$Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).
Figure 3.4. The activities of superoxide dismutase (SOD) in RBC in rats. Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).
Table 3.5. The activity of catalase in RBC.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>Catalase (unit/g RBC protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>12</td>
<td>0.113&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGI</td>
<td>sedentary</td>
<td>12</td>
<td>0.101&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td>0.086&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td>0.116&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).*
Figure 3.5. The activity of catalase in RBC. Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).
Table 3.6. The activity of glutathione peroxidase (GPx) in RBC.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Exercise level</th>
<th>n</th>
<th>GPx (µmol/s/mg RBC protein)</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGI</td>
<td>exercised</td>
<td>12</td>
<td></td>
<td>0.412</td>
<td>0.024</td>
</tr>
<tr>
<td>HGI</td>
<td>sedentary</td>
<td>12</td>
<td></td>
<td>0.403</td>
<td>0.029</td>
</tr>
<tr>
<td>control</td>
<td>exercised</td>
<td>12</td>
<td></td>
<td>0.294</td>
<td>0.010</td>
</tr>
<tr>
<td>control</td>
<td>sedentary</td>
<td>12</td>
<td></td>
<td>0.428</td>
<td>0.043</td>
</tr>
</tbody>
</table>

*Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).*
Figure 3.6. The activity of glutathione peroxidase (GPx) in RBC. Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey’s HSD, Sokal and Rohlf, 1995).
CHAPTER 4

Dietary Isoflavone Supplementation Decreased Plasma Malondialdehyde and Increased Blood Total Glutathione, but did not Affect Lipid Profiles in Rats

4.1. ABSTRACT

The purpose of this study was to investigate the optimal amount of isoflavone necessary to increase antioxidation and reduce plasma cholesterol levels in rats. A five-week study was conducted using 30 one-year-old male Sprague-Dawley rats which were randomly assigned to chow and four experimental AIN diets supplemented with 0, 209, 522, and 1045 PPM high genistin isoflavones extract (HGI), respectively. Genistein in the plasma, liver, and gastrocnemius muscle was determined by HPLC. The antioxidative effect of the HGI diet was detected by measurement of total glutathione (TGSH) and glutathione (GSH) concentrations in whole blood and measurement of malondialdehyde (MDA) by means of Thiobarbituric Acid Reactive Substances (TBARS) in plasma, liver and muscle. Plasma lipid profile was measured. Genistein concentrations were significantly higher (P<0.05) in tissues of rats fed the 1045 PPM HGI diet than in rats fed 522 or 209 PPM HGI diets (plasma: 301.98, 167.19, and 45.92 ng/ml; liver: 200.25, 107.29, and 36.98 ng/g; muscle: 76.60, 18.35, and 48.5 ng/g; respectively). The ratio of GSH and TGSH was significantly higher (P<0.03) than 0 PPM (0.38) only for rats fed 1045 PPM HGI (0.66). Significant reduction of MDA concentration (P<0.05) was observed only in the plasma of rats fed 522 and 1045 PPM HGI diets compared to those fed 0 PPM (-1.08, -0.82, and 0.03 μM, respectively). Plasma lipid profiles were not significantly different. It can be concluded that isoflavones at 522-1045 PPM HGI diet have antioxidative effects. The results imply that the reduction of cholesterol by soy protein may not be attributed to isoflavones.

Key words: isoflavones, malondialdehyde, glutathione, lipid profiles, rats
4.2 INTRODUCTION

There is abundant research on the health benefits of soy in ameliorating cardiovascular disease and cancers. The lower incidence of these diseases in Asia has been attributed to the significantly high consumption rate of soy foods in this area as compared to people in the United States (Beaglehole, 1990). However, in spite of the obvious correlation between soy consumption and good health, its mechanism of action remains elusive. For example, the hypolipidemic effect of soy protein has been repeatedly demonstrated and was recognized by the US Food and Drug Administration in their 1999 Health Claim. However, the actual mechanism of such an effect is still unknown. There is even an indication that the hypolipidemic effect may be in part due to the presence of soy isoflavones with the soy protein. This is because upon extraction of soy protein from soybeans, the isoflavones are usually also extracted in the same process and are therefore reserved in soy protein (Huff et al., 1977; Anthony et al., 1996; Balmir et al., 1996; Lovati et al., 1991). Several isoflavones are present in soy but the two predominant ones are genistein and daidzein which are mostly found in their glycosylated forms: genistin and daidzin. Upon ingestion, intestinal bacteria release the aglycones from their glycoside link, after which they are absorbed into the bloodstream. The soy isoflavones genistein and daidzein are bioavailable to both animals and humans (Rowland et al., 1999).

There are several proposed mechanisms for the effects of isoflavones on lowering blood cholesterol. One proposed mechanism is based on the estrogen-like structure of these isoflavones. Due to this structure, genistein can bind to estrogen receptors, inducing a weak estrogenic response that in turn influences LDL receptor activity leading to a hypocholesterolemic effect (Martin et al., 1978). Kirk et al. (1998) reported that isoflavone-rich soy protein reduces cholesterol levels in mice by increasing LDL receptor activity. Genistein has also been shown to increase LDL receptor gene expression in Hep-G2 cells (Kanuck and Ellsworth, 1995). Therefore, the contention that soy isoflavones may play a role in the hypolipidemic effect of soy is a valid one.
Soy isoflavones also have antioxidant effects that may have antiatherogenic and anticancer implication. Isoflavones exhibit antioxidant activity in vitro and in vivo (Kurzer and Xu, 1997). This is attributed to their phenolic ring structure and multiple hydroxyl groups, especially on the “A” ring. These multiple hydroxyl groups reduce peroxyradicals by donating hydrogen atoms (Tikkanen et al., 1998). Genistein has the highest antioxidant characteristic of all the soy isoflavones. It was shown to prevent LDL oxidation initiated by an oxidizing agent (Kapiotis et al., 1997) and to protect against microsomal lipid peroxidation induced by a Fe$_{2}^{+}$-ADP complex (Jha et al., 1985). In addition, Cai and Wei (1996) reported that dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activity of antioxidant enzymes in the skin and small intestine of SENCAR mice. Therefore, there is a good indication that soy isoflavones prevent the development of disease by means of protecting cellular components against oxidative damage.

Although several studies investigating the antioxidant effect of soy isoflavones were performed, the evidence was by no means conclusive. Most of the studies were performed in vitro and to our knowledge, there was no investigation of dose dependent response to isoflavones in rats. We hypothesized that soy isoflavones have a dose-dependent response due to their antioxidative and cholesterol-lowering effects. The objective of this study, which was approved by the Virginia Tech Institutional Review Board, was to investigate the in vivo antioxidant and cholesterol lowering effects of a high-genistin isoflavone (HGI) extract fed to rats for 5 weeks.

4.3. MATERIALS AND METHODS

4.3.1. Animals

Thirty one-year-old Sprague-Dawley male rats were purchased from Harlan Industries (Indianapolis, IN). All rats were housed at 25°C with a 12-hr light/dark cycle and given free access to feed and water for the duration of the study. Before the rats were fed the study diets, they were acclimatized on a chow diet for one week.
4.3.2. Experimental design

The rats were randomly divided into five groups of six rats each. Each group was fed either the baseline chow diet or one of four experimental diets. All semipurified ingredients were purchased from ICN (ICN Pharmaceuticals Inc., Costa Mesa, CA). The experimental diets were designated as 0 PPM high-genistin isoflavone (HGI), 209 PPM HGI, 522 PPM HGI, and 1045 PPM HGI. The 0 PPM HGI diet was prepared according to the formula in Table 4.1; and other three HGI diets were made based on the 0 PPM HGI diet by replacing the corresponding amount of corn starch with HGI (Table 4.2). The cholesterol and butter were added to induce hypercholesterolemia. The rats in the baseline group were fed chow for 7 days. However, the rats in the experimental groups were fed the different HGI diets for 35 days.

4.3.3. Sacrifice, sample collections, and blood processing

On the first day of study, all rats in the baseline group were anesthetized using a halogen and nitrogen gas mix. The maximum possible volume of blood was then drawn from the heart. The blood samples were kept in ice until further processing. The rats were sacrificed by cervical dislocation; and the liver and legs were removed, immediately wrapped in aluminum foil and instantly frozen in liquid nitrogen to diminish tissue exposure to heat and air. On the 35th day of study, the same procedures of blood collection, sacrifice of rats, and tissue sample collection were applied to the rats in the experimental groups.

Within 3 hours after blood collection, one ml from each blood sample was mixed with 2 ml of 10% 5-sulfosalicycyclic acid containing bathophenanthrolinedisulfonic acid (BPDS, final concentration 1 mmol/L), and two ml of blood were mixed with 1 ml of 10% 5-sulfosalicycyclic acid containing BPDS. Each mixture was centrifuged at 10,000 x g at 4°C for 15 minutes. The supernatant was retained for further total glutathione and oxidized glutathione measurements, respectively.

All samples were stored at –80°C until further analysis.
4.3.4. Biochemical measurements

Genistein concentrations in the plasma, gastrocnemius muscle, and liver were determined by a HPLC method, modified from methods of Wang and Murphy (1994) and Xu et al. (1994). The gastrocnemius muscle was isolated from the leg after thawing.

Thiobarbituric acid reactive substances (TBARS) method was used to measure \textit{in vivo} malondialdehyde (MDA) concentrations in the liver and gastrocnemius muscle tissues (Pikul et al. 1989). One gram of tissue, 5 ml of 5% trichloroacetic acid, and 200 µl of 0.15% butylated-hydroxyltoluene were homogenized for one minute. The mixture was then spun at 17,000 x g, 4°C for 15 minutes. The supernatant solution was mixed with 0.67% thiobarbituric acid (TBA) solution and incubated at 95°C in a water bath for 40 minutes. Absorbance was read at 532 nm within 1 hour after incubation. Plasma MDA was measured according to the method developed by Fukunaga et al. (1998). According to this method, an aliquot of 50 µl plasma or standards was added to 1 ml thiobarbituric acid reagent (0.2% (w/v) TBA in 0.1 mol/L sodium acetate buffer, pH 3.5) in a polypropylene microtube. The mixture was heated in a water bath at 95°C for 60 minutes. After cooling to room temperature, the reaction mixture was spun at 10,000 x g for 15 minutes. The TBA-MDA adduct was measured in the resulting supernatant by means of HPLC with a fluorometric detector. Standard curves were plotted for varying concentrations of 1,1,2,2-tetraethoxypropane (TEP) and then MDA concentrations in plasma, liver and muscle samples were determined by comparison to the standard curves.

Total glutathione and oxidized glutathione were measured by the methods according to Griffith (1980). These methods comprise a DTNB-oxidized glutathione reductase recycling assay. Briefly, DTNB oxidized all glutathione in a sample to GSSG and then glutathione reductase plus NADPH reduced GSSG to GSH. For total GSH measurement, absorbance at 412 nm was measured for 66 seconds and concentrations determined by comparison against a standard GSH curve. For GSSG measurement, GSH in the samples was first derivatized by means of 2-vinylpyridine. The derivatized sample was assayed as described above.
Total Cholesterol, HDL-cholesterol, and total triglyceride in plasma were measured by means of Stanbio Laboratory Inc. kits (San Antonio, TX).

4.3.5. Statistical analysis

A one-way ANOVA was used to compare the effect of dietary HGI supplementation on the mean concentration of plasma genistein, lipid profile, tissue MDA and total glutathione and oxidized glutathione (Sokal and Rohlf 1995). This is in addition to ANOVA of the reduction of plasma MDA after 35-day HGI supplementation. Statistical significance was set at p<0.05. When dietary supplementation was statistically significant, Tukey-Kramer Honestly Significant Difference (HSD) was used to compare group means at P=0.05 (Sokal and Rohlf, 1995). The computer software program JMP (SAS Institute Inc., Cary, NC) was used for all computations.

4.4. RESULTS

4.4.1. Feed intake and body weight

At onset of supplementation, there were no significant differences among the mean body weights of the different groups. Feed intakes of rats were not significantly different among rats throughout the study. However, 522 PPM HGI diet led to a significant increase in body weight.

4.4.2. Genistein concentrations

High-genistein isoflavone supplementation led to significant (P<0.0001, P<0.0001, and P=0.0009, respectively) increases in genistein concentrations in the plasma, liver, and gastrocnemius muscle in rats according to a one-way ANOVA test (Table 4.3; Fig 4.1). 522 and 1045 PPM HGI supplementation significantly increased plasma and liver genistein, however, only 1045 PPM HGI significantly increased muscle genistein.

4.4.3. Plasma lipid profile

A hypercholesterolemic diet high in cholesterol and saturated fat led to increases of triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol. However, HGI
supplementation at 209, 522, and 1045 PPM did not lead to a significant reduction of the lipid profiles of the rats (Table 4.4; Fig 4.2).

4.4.4. Lipid peroxidation in the plasma, liver, and muscle

MDA is a lipid peroxidation product used to measure the extent of lipid peroxidation. Plasma MDA and liver and muscle MDA were determined by a HPLC method and a spectrophotometric TBARS method, respectively (Table 4.5 and 4.6; Fig 4.3 and 4.4). Plasma MDA was significantly decreased in rats fed 522 and 1045 PPM HGI. However, no significant change in MDA was observed in liver and muscle tissues.

4.4.5. Total Glutathione and oxidized glutathione in whole blood

HGI supplementation at 1045 PPM significantly increased GSH concentration and the GSH/TGSH ratio (Table 4.7; Fig 4.5 and 4.6). Also TGSH was slightly increased at 1045 PPM supplementation but not significantly. There was no effect on GSSG.

4.5. DISCUSSION

This is the first study to examine the effect of dietary isoflavone supplementation on tissue lipid peroxidation and plasma lipid profiles in Sprague-Dawley rats. Our results showed that significant accumulation of genistein in the liver, plasma, and skeletal muscle was dose-dependent. The HGI supplement fed to the rats was mostly composed of genistin and daidzin. These two conjugated isoflavones are not absorbed as such from the intestine (Hollman, 1997). Gut microorganisms hydrolyze the beta-glucosidic linkage between aglycone and glucose, therefore, allowing absorption of the corresponding aglycone (Hollman, 1997). The work of Chang et al. (2000) and a previous study (Chapter 3) performed in our laboratory are both in agreement with the results of the current study that genistein accumulates in the tissues when the conjugated form is ingested. In the aforementioned experimental work, genistein levels also significantly increased in the plasma, liver, and muscle at 598 PPM (500 PPM genistein containing compounds). Such a significant increase was seen in the plasma and liver but not in the muscle tissue at 522 PPM supplementation. Therefore, it seems that the extent of isoflavone accumulation in tissues is dose dependent.
The cholesterol lowering effect of soy in animals and humans has been attributed to the estrogenic activity of genistein (Anderson et al., 1999). Studies in monkeys fed a soy protein- and isoflavone-rich diet led to an increase in high density lipoprotein (HDL), and a decrease in both serum cholesterol and triglyceride (Anthony et al., 1996). A study by Crouse et al. (1999) of moderately hypercholesterolemic men and women showed a dose dependent effect of isoflavone-rich soy protein intake on reduction of low density lipoprotein (LDL) cholesterol. However, abundant evidence exists supporting the cholesterol lowering effect of soy protein with isoflavones, yet a purified isoflavone extract administrated to humans and monkeys did not achieve the same effect (Nester et al., 1997; Hodgson et al., 1998; Greaves et al., 1999). The results of this study, in accordance with the current literature suggested that soy isoflavones at concentrations ranging from 209-1045 PPM did not affect rat total cholesterol levels.

Isoflavones in addition to having estrogenic properties (Kurzer and Xu 1997) also function as antioxidants. Genistein has the highest antioxidant activity among isoflavones and can inhibit the primary reaction leading to ROS production, as well as directly inhibit agonist-stimulated ROS production (Peterson, 1995). Both animal and human studies demonstrated that isoflavone consumption led to positive antioxidant effects in the body. Such effects included a decrease in LDL oxidation in rats fed a genistein-rich soy protein diet (Anderson et al., 1998) and increase of lag time of LDL oxidation by copper in human subjects fed soy protein bars (Tikkanen et al., 1998). However, the mechanism of such action is attributed to the estrogenic property of genistein rather than to its ability to act as an antioxidant by donating hydrogen (Mitchell et al., 1998). Furthermore, the circulating forms of isoflavones are predominantly glucuronidated on the 7 position of the A ring, which removes the most readily available hydrogen at that position (Mitchell et al., 1998). In order to analyze the extent of lipid peroxidation and hence the effectiveness of antioxidants in any subject, an easily meaningful indicator is needed such as \textit{in vivo} plasma MDA. In this study, HGI supplementation at 522 and 1045 PPM for 35 days significantly reduced plasma MDA in rats. Supplementation at 209 PPM had no effect. This is in agreement with Kerry and Abbey (1998) who concluded that the \textit{in vitro} antioxidant effect of genistein was
concentration dependent, and also demonstrated that lag time of copper mediated LDL oxidation was extended by higher than 0.5 µmol/L genistein.

In contrast, there was no effect on MDA levels in the liver and muscle tissues although the significant elevation of genistein concentrations was observed in these tissues. Both genistein and MDA concentrations in the liver were comparable to those in an earlier study performed in our laboratory (Chapter 3). It is not clear why genistein accumulation in the liver and muscle did not reduce the formation of MDA. However, this could be attributed to the predominant occurrence of genistein in the tissues in its glucuronide form (Nestel et al., 1997) which may have less antioxidant activity than the free form (Mitchell et al., 1998) because of losing one hydroxyl group. Kapiotis et al (1997) reported that genistin, which losses the hydroxyl group from the same position as glucuronidated genistein, was less active in inhibiting LDL oxidation in vitro. In addition, the inherent antioxidant defense systems in the liver and muscle may override the effect of dietary isoflavones.

Glutathione, one of endogenous antioxidants, directly reduces any toxic aldehydes and other oxidative products. It has been demonstrated that increased blood levels of GSSG and decreased GSH/GSSG ratio are indices of oxidative stress (Loft et al., 1992; Stephen et al., 1995; Erhola et al., 1997; McIntyre et al., 1997). Mizutani, et al., (2000) reported that 1 µmol/L genistein incubated with vascular smooth muscle cells of rats can result in increased TGSH (GSH+GSSG) concentration and elevated expression of gamma-glutamylcysteine synthetase mRNA, the rate-limiting enzyme for GSH synthesis. In addition, Appelt and Reick (1999) observed that feeding rats 810-PPM soy isoflavones led to increased GSH concentration and decreased GSGS concentration in plasma. Similarly, in this study HGI supplementation increased the GSH/TGSH ratio and slightly (but not significantly) increased TGSH in blood, indicating that HGI supplementation protected the body against oxidative insult by sparing GSH.

4.6. SUMMARY

This study demonstrated that HGI supplementation mediates plasma oxidative stress by means of its direct antioxidative ability and by modulating the antioxidant capacity of
glutathione. However, dose administration into rats is critical in determining HGI antioxidant activity. A dose-response effect of HGI was observed in plasma MDA, suggesting that at least 522 PPM HGI is necessary to protect against oxidative stress in rats. The lack of similar effects in the liver and muscle tissues suggests that the isoflavone antioxidant activity may be tissue specific. Although 25 gm soy protein intake (containing isoflavones) is accepted as a cholesterol lowering diet, this study implies that isoflavones may not be the hypercholesteremic factor in soy. Further research is necessary to explore the antioxidant effect mechanisms of soy isoflavones.

4.7. REFERENCES


Table 4.1. The composition of the control diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage, %</th>
</tr>
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<td>Casein</td>
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</tr>
<tr>
<td>Dextrinized starch</td>
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</tr>
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<td>Sucrose</td>
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</tr>
<tr>
<td>Butter</td>
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</tr>
<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Cholesterol</td>
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<td>Cholic acid</td>
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</tr>
<tr>
<td>Alphacel</td>
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</tr>
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<td>Mineral mix (AIN 93M)</td>
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</tr>
<tr>
<td>L-cystine</td>
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<tr>
<td>Vitamin mix (AIN 93M)</td>
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</tr>
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<td>Choline bitartrate</td>
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</tr>
<tr>
<td>hydroquinone</td>
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</tr>
<tr>
<td>Total</td>
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</tr>
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Table 4.2. The composition of isoflavone extract used in HGI diet.

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</tr>
<tr>
<td>Genistein</td>
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</tr>
<tr>
<td>Daidzin</td>
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</tr>
<tr>
<td>Daidzein</td>
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<td>Glycitin</td>
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<td>Glycitein</td>
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<tr>
<td>Total</td>
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</table>
Table 4.3. Genistein in tissues after five weeks of HGI supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Plasma, ng/ml</th>
<th>Liver, ng/g</th>
<th>Muscle, ng/g</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Baseline</td>
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<td>0</td>
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</tr>
<tr>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>209 PPM</td>
<td>6</td>
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<td>ab</td>
</tr>
<tr>
<td>522 PPM</td>
<td>6</td>
<td>167.2</td>
<td>b</td>
</tr>
<tr>
<td>1045 PPM</td>
<td>6</td>
<td>302.0</td>
<td>c</td>
</tr>
</tbody>
</table>

abc Means in the same column with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Figure 4.1. Genistein in tissues after five weeks of HGI supplementation. Means in the same tissue with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Table 4.4. Lipid profiles in plasma after five weeks of HGI supplementation.

<table>
<thead>
<tr>
<th></th>
<th>TG, mg/dl</th>
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<th>LDL-C, mg/dl</th>
<th>HDL-C, mg/dl</th>
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</thead>
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<tr>
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<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Baseline</td>
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<td>8.6</td>
<td>105.6</td>
<td>8.5</td>
</tr>
<tr>
<td>Control</td>
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<td>27.7</td>
<td>176.8</td>
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<tr>
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<td>140.0</td>
<td>18.1</td>
<td>163.1</td>
<td>17.8</td>
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<tr>
<td>522 PPM</td>
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<td>25.2</td>
<td>194.6</td>
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</tr>
<tr>
<td>1045 PPM</td>
<td>139.3</td>
<td>20.3</td>
<td>199.4</td>
<td>17.4</td>
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</table>

*Means in the same column with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).*
Figure 4.2. Lipid profiles in plasma after five weeks of HGI supplementation. Means in the same measurement with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Table 4.5. Plasma MDA measured by HPLC after five weeks of HGI supplementation\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
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<th>5 week</th>
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<td></td>
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<td>Mean</td>
</tr>
<tr>
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<td>1.54</td>
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<tr>
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<td>209 PPM</td>
<td>6</td>
<td>1.72</td>
<td>0.21</td>
<td>1.08</td>
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<td>-0.64</td>
</tr>
<tr>
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<td>6</td>
<td>2.25</td>
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<td>1.17</td>
<td>0.11</td>
<td>-1.08</td>
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<td>1045 PPM</td>
<td>6</td>
<td>1.81</td>
<td>0.12</td>
<td>1.00</td>
<td>0.09</td>
<td>-0.82</td>
</tr>
</tbody>
</table>

\(^1\)MDA was expressed as micromolar.

\(^{ab}\)Means in the same column with the different letters were significantly different (\(P<.05\)), using Tukey’s HSD (Sokal and Rohlf, 1995).
Figure 4.3. The difference of plasma MDA between week 0 and 5. Means with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Table 4.6. MDA concentrations in the liver, and gastrocnemius muscle in rats fed HGI diet.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
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<th>Muscle</th>
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<tr>
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<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
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<td>0.73&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Control</td>
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<td>0.42&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>0.06</td>
<td>0.08&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>1045 PPM</td>
<td>6</td>
<td>0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04</td>
<td>0.10&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>1</sup>MDA was expressed as microgram/g.

<sup>ab</sup>Means in the same column with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Figure 4.4. MDA concentrations in the liver, and gastrocnemius muscle in rats fed HGI diet. Means in the same tissue with the different letters were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Table 4.7. Glutathione, total glutathione, and oxidized glutathione concentrations in whole blood after 5-week HGI supplementation.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>TGSH</th>
<th>GSH</th>
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<th>GSH/TGSH</th>
</tr>
</thead>
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<td>SE</td>
<td>Mean</td>
</tr>
<tr>
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<td>664.5a</td>
<td>53.0</td>
<td>373.5ab</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>442.7a</td>
<td>43.7</td>
<td>165.7b</td>
</tr>
<tr>
<td>209 PPM</td>
<td>6</td>
<td>491.4a</td>
<td>64.6</td>
<td>168.3b</td>
</tr>
<tr>
<td>522 PPM</td>
<td>6</td>
<td>495.5a</td>
<td>98.4</td>
<td>198.1ab</td>
</tr>
<tr>
<td>1045 PPM</td>
<td>6</td>
<td>635.1a</td>
<td>74.7</td>
<td>446.3a</td>
</tr>
</tbody>
</table>

\(^1\)TGSH, GSH, and GSSG were expressed as nmol/ml.

\(^{ab}\)Means in the same column with the different letter were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Figure 4.5. Glutathione, total glutathione, and oxidized glutathione concentrations in whole blood after 5-week HGI supplementation. Means in the same measurement with the different letter were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
Figure 4.6. 1 The ratios of GSH/TGSH and GSH/GSSG. Means in the same measurement with the different letter were significantly different (P<.05), using Tukey’s HSD (Sokal and Rohlf, 1995).
5.1. ABSTRACT

Exercise has become a lifestyle, a recreation, and sometimes, a means of therapeutic treatment. However, it is widely accepted and experimentally proven that increased whole body oxygen flux during exercise may elicit potentially toxic perturbations in cellular homeostasis via increased reactive oxygen species (ROS) production. The aim of this study was to investigate whether 30 minutes of 80% VO\textsubscript{2}pk exercise on cycle ergometer could affect total antioxidant defense systems and increase products of lipid peroxidation in 32 moderately active male college students. Blood samples from each subject were taken pre- and post-exercise. Glutathione homeostasis was determined in the whole blood, enzymatic antioxidants were measured in red blood cells, and all other tests were measured in the plasma and/or LDL. Oxidized glutathione (GSSG) was significantly increased; and total glutathione (TGSH), glutathione (GSH), and the GSH/TGSH ratio were significantly decreased by exercise. Superoxide dismutase (SOD) activity was increased 16.5% and glutathione peroxidase (GPx) activity was decreased 10.9% by exercise. Plasma uric acid and ferric reducing/antioxidant ability (FRAP) were significantly increased 4.9% and 7.8%, respectively. In addition, there was an 88% partial correlation between uric acid and FRAP. Exercise increased plasma malondialdehyde (MDA) by 12.6%. However, it decreased \textit{in vitro} LDL-MDA by 10.3% but almost decreased \textit{in vivo} LDL-MDA by 9.0% (P = 0.055). Glutathione homeostasis, SOD, GPx, and plasma MDA responded more sensitively to oxidative stress induced by exercise. However, plasma FRAP value may not be a good index of total non-enzymatic antioxidant defense. It can be concluded that 30 minutes at 80% VO\textsubscript{2}pk exercise induces oxidative stress in moderately active college men.

\textit{Key words:} exercise, oxidative stress, antioxidant defense, lipid peroxidation, men
5.2. INTRODUCTION

Oxygen is an electron acceptor that allows aerobic organisms to generate energy from foodstuffs such as carbohydrates, fats, and protein. However, oxygen reduction to water during energy generation in the mitochondrial electron transport chain (ETC) leads to the production of reactive oxygen species (ROS) (Ames et al., 1995; Chance et al., 1979; Ji, 1995). Reactive oxygen species are defined as any species containing an unpaired electron that is capable of independent existence such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical (Gutteridge, 1994). They have a strong tendency to extract electrons from other compounds in order to reach a chemically more stable structure; therefore, they are capable of eliciting oxidative damage to the various cellular components such as lipid, protein, and DNA (Ames et al., 1995; Yu, 1994).

Remarkably efficient antioxidant defense systems have evolved to protect the body against ROS (Halliwell and Gutteridge, 1989). There are two defense systems in the body against ROS, enzymatic antioxidants and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT); and non-enzymatic antioxidants include vitamins E and C, betta-carotene, glutathione, uric acid, and albumin. Although antioxidant defense systems are very efficient against ROS, the surge of ROS generation can overwhelm these systems. An imbalance favoring ROS over antioxidant defense systems is referred to as oxidative stress, which leads to oxidative damage. The extent of oxidative damage is determined not only by the amount of free radical generation, but also by the capability of antioxidant defense systems.

Exercise has become a lifestyle, a recreation, and sometimes, a means of therapeutic treatment. However, it is widely accepted and experimentally proven that increased whole body oxygen flux during participation in aerobic exercise may elicit potentially toxic perturbations in cellular homeostasis via increased ROS production in the locomotive muscles and heart as well as other tissues (Ashton et al., 1999; Halliwell and Gutteridge, 1989; Jenkins, 1988; Ji, 1995; Meydani and Evans, 1993; O’Neill et al., 1996; Sen, 1995; Sjodin et al., 1990). Therefore, it has been hypothesized that exercise may cause oxidative
stress, leading to further tissue damage (Alessio, 1993; Ji, 1995; Ji, 1996; Ji, 1999). There have been many reports showing that exercise caused oxidative stress, e.g., the direct detection of free radical generation in rat muscle and liver (Davies et al., 1982; Somani and Arrcayo, 1995); increase in oxidative damage biomarkers such as protein carbonyls and thiobarbituric acid reactive substances (TBARS) (Sen et al., 1997); effects on mitochondrial function (Ravalec et al., 1996; Willis and Jackman, 1994); and changes in antioxidant defense systems.

Human erythrocytes are well equipped with antioxidant enzymes SOD, catalase and GPx, that protect the cells against the accumulation of superoxide radical and/or hydrogen peroxide (Ohno et al., 1986). The changes in the activities of these enzymes in response to exercise in both animal and human tissues have been reported. An acute bout of exercise increased SOD activity in a number of tissues including the liver (Alessio and Goldfarb, 1988b; Ji et al., 1990; Ji et al., 1992; Ji and Fu, 1992; Kuppasamy and Zweier, 1989; Lang et al., 1987), skeletal muscle (Ji et al., 1990; Ji et al., 1992; Ji and Fu, 1992; Lawler et al., 1993; Quintanilha and Packer, 1983), heart (Ji and Mitchell, 1994; Quintanilha and Packer, 1983), and red blood cells (Mena et al., 1991; Ohno et al., 1988). This activation of SOD possibly resulted from increased superoxide production during exercise (Ji, 1993). Ohno et al. (1986), however, reported no increase in erythrocyte SOD activity after a brief physical exercise in sedentary students. GPx activity showed variable responses to an acute bout of exercise. Some studies have shown no change in this enzyme in skeletal muscle (Brady et al., 1979; Ji et al., 1990; Leeuwenburgh and Ji, 1995; Vihko et al., 1978) and erythrocytes (Duthie et al., 1990) after acute exercise, whereas others have reported significant elevation of GPx activity (Ji et al., 1992; Ji and Fu, 1992; Leeuwenburgh and Ji, 1996; Oh-Ishi et al., 1996; Quintanilha, 1984). In addition, decreased GPx activity in erythrocytes after exercise was reported by Ohno et al (1986), Paduraru et al (1997), and in our laboratory (chapter 3). Most of the previous studies have revealed no significant alteration in CAT activity with acute exercise (Duthie et al., 1990; Meydani and Evans, 1993; Ohno et al., 1986). However, CAT activity increased after an acute bout of exercise in rat muscle (Ji et al., 1992; Ji and Fu, 1992); and
CAT activity in erythrocytes decreased after exercise as reported by Paduraru et al (1997) and in our laboratory (Chapter 3).

Vitamin C, well known as a potent water-soluble antioxidant in the cytosol and extracellular fluid, has two forms, ascorbic acid and dehydroascorbic acid. Not only can ascorbic acid directly participate in the reduction of ROS to safe compounds and inhibit lipid peroxidation (Bendich et al., 1986; Beyer, 1994), but it can also regenerate tocopherol from tocopheryl radicals. Importantly, decreased levels of plasma ascorbic acid have been reported in physically active men (Hemila, 1996). Furthermore, the ratio of ascorbic acid and dehydroascorbic acid was suggested as an indicator of oxidative stress (Lykkesfeldt et al., 1995). GSH is the most abundant non-protein thiol source in the cell and serves multiple functions in protecting tissues from oxidative damage by scavenging ROS directly or indirectly via glutathione peroxidase reaction (Meister and Anderson, 1983). GSH also reduces tocopherol radicals, either directly or indirectly by reducing dehydroascorbate to ascorbate (Niki et al., 1985). Liver synthesizes GSH from endogenous or dietary amino acids de novo and supplies most of the circulating GSH (Meister and Anderson, 1983). Exercise has been found to decrease reduced GSH and TGSH, and increase GSSG in tissues in animal and human studies (Durarte et al., 1993; Duthie et al., 1990; Gohil et al., 1988; Ji et al., 1993; Marin et al., 1993; Michelet et al., 1995; Sen et al., 1992). Oxidation of GSH to GSSG is also a sensitive marker of oxidative stress (Lew and Quintanilha, 1991). Uric acid is the other strong antioxidant; it is a purine metabolite by means of the xanthine oxidase pathway that is activated by high-intensity exercise (Hellsten, 1994; Radak et al., 1995; Rasanen et al., 1996). Therefore, exercise results in increased uric acid in plasma (Hellsten-Westing et al., 1993; Norman et al., 1987; Westing et al., 1989).

In addition to the changes in antioxidant defense systems by exercise, increased products of lipid peroxidation have been reported in animals and humans. Polyunsaturated fatty acids, which have multiple double bond points, are very susceptible to ROS attack. Malondialdehyde is a decomposition product of lipid peroxidation, thus justifying its use as an indirect determinant of free radical-mediated oxidative damage (Ashton et al., 1999).
Several studies reported that plasma MDA was elevated by means of exercise (Lovlin et al., 1987; Wade and Van Rij, 1988). The change of plasma MDA after exercise is controversial because of the variability in the type of exercise and conditions studied and methods used to measure MDA (Atalay et al., 1997; Duthie et al., 1990; Marzatico et al., 1997; Mena et al., 1991; Ortenbland et al., 1997). It seems that the level of products of lipid peroxidation in blood plasma depends on the duration of the exercise as well as on the endurance fitness of the subject. The determination of the susceptibility of LDL to in vitro oxidation is an alternative in the evaluation of extent of oxidative stress. Moreover, LDL oxidation has been suggested to play a key role in the development of atherosclerosis. The difference between plasma and LDL-MDA was that the former was performed under an environment of complete antioxidant defenses, especially water-soluble antioxidants in plasma, urate (300 µM) and ascorbate (20-60 µM) (Esterbauer et al., 1997; Sevanian et al., 1991). According to a previous study, LDL susceptibility to CuSO$_4$-induced oxidation was enhanced after exercise (Sánchez-Quesada et al., 1995). This increase in the susceptibility of LDL to in vitro oxidation was suggested to be a consequence of a “seeding” of LDL with lipid peroxides during exercise (Sánchez-Quesada et al., 1998). Furthermore, decreased lag phase time and increased conjugated diene production after exercise was observed (Sánchez-Quesada et al., 1995).

Although the modulation of antioxidant defense systems and increased products of oxidative damage were often reported in animal and human studies, there are still a large quantity of discrepancies and inconsistencies in the observed results. The lack of consistency in the results may be attributed to experimental design, methods of biochemical measurements, exercise type, and subject condition. In order to understand which biomarker in the blood would be the most suitable and convenient to measure oxidative stress, untrained college students were used in the study. We hypothesized that thirty-minute 80% VO$_2$pk exercise could result in oxidative stress in young men, further leading to changes of erythrocyte antioxidant enzyme activities, the disturbance of glutathione and vitamin C homeostasis, and increased products of lipid peroxidation. The objectives of this study were to determine whether 80% VO$_2$pk exercise induced oxidative stress and to identify
biomolecules for oxidative stress induced by intensive exercise in moderately active young men.

5.3. MATERIALS AND METHODS

5.3.1. Subjects

A total 32 non-smoking healthy men, aged 20±1.94 years old, were recruited from the Virginia Tech campus area (Table 5.1). Men were selected as subjects due to the stability of their hormonal status. Hormone fluctuations found in women, such as estrogen with antioxidative activity, can affect the extent of oxidation of LDL and plasma (Tiidus, 1995). In order to induce the maximum oxidative stress due to exercise, physically inactive men (less than 4 hrs exercise/week), according to their own physical activity questionnaire, were invited to participate in the study. In addition, the subject did not take any antioxidant supplements, such as vitamin A, C, or E, one month before the study. All subjects signed an informed consent form before the beginning of the study.

5.3.2. Exercise protocol

Before performing 80% submaximal exercise, peak oxygen consumption (VO2pk) and dietary records were collected from subjects. Dietary records were analyzed by Nutritionist V (First DataBank Inc., San Bruno, CA) (Table 1). Following the VO2pk test, each subject had at least a 3-day rest before undergoing the 80% VO2pk exercise. The progressive exercise test assessed VO2pk of each subject and maximum watts on a cycle ergometer when the subject reached maximum exercise capacity. A Borg scale (Borg, 1982) was also used to monitor subject exhaustion during the progressive exercise test. The peak oxygen consumption and respiratory rate during the progressive exercise test were measured on the Monark cycle ergometer (Monark 818E, Monark, Stockholm, Sweden), using a MedGraphics CPX/D VO2 System (St Paul, MN) automated metabolic cart. The value of VO2pk is the definitive laboratory indicator for individual aerobic exercise capacity (Wareham et al., 1998). Respiratory exchange ratio (R > 1.1), rate of perceived exertion (Borg Scale), and heart rate (90% of predicted maximum heart rate) at peak exercise provided objective evidence of maximal effort endpoints for this test. Pedal speed was held
constant at 60 rpm. Subjects warmed up on the cycle ergometer for two minutes to familiarize themselves with the cycle ergometer and the cycling speed of 60 rpm. The workload was gradually increased by 30-60 watts every 1-2 min, depending on the subjects’ heart rate response and estimated exercise capacity, until the subject was unwilling to continue or unable to maintain 55 rpm. Heart rate was measured by using an ECG system (Quinton: ECG: Models Q5000) with CC5 lead. Total duration of VO\textsubscript{2}pk test was 8-12 minutes long.

Two days before the submaximal exercise, all subjects were instructed not to participate in any exercise or sport. Upon reporting to the laboratory, each subject rested for 10 minutes before a venous blood sample was drawn from an antecubital vein. Subsequently, each subject warmed up to reach his own 80% VO\textsubscript{2}pk on the cycle ergometer and then exercised for 30 minutes. During the submaximal exercise, heart rate and ratings of perceived exertion were monitored continuously throughout the exercise session. In addition, oxygen consumption was measured every five minutes in order to maintain the subject exercised at 80% VO\textsubscript{2}pk. The workload on the cycle ergometer was adjusted according to the subjects’ 80% VO\textsubscript{2}pk; and oxygen consumption was recorded. The average exercise intensity throughout the submaximal exercise was calculated from the recorded oxygen consumption of each subject.

5.3.3. Sample Collection

Before and within 5 minutes after submaximal exercise, 28 ml of blood were drawn from an antecubital vein into EDTA-containing tubes. The blood samples were then immediately kept in ice. Two small aliquots of blood were removed from one tube and prepared for total glutathione and oxidized glutathione measurements, according to the method of Griffith (1980). Both mixtures were kept in ice and within 30 minutes were centrifuged at 10,000 x g at 4°C for 15 minutes. One of the tubes, designated for vitamin C analysis, was immediately covered with aluminum foil and kept in ice. Plasma was separated and aliquots were treated for ascorbic acid, total ascorbic acid, and uric acid analyses, according to the method of Rümelin et al. (1999). The blood in the remaining three
tubes was separated into plasma and RBC by means of centrifugation at 2000 x g for 20 minutes at 4°C. Subsequently, RBC were washed with precooled 0.9% saline solution three times before storage at -80°C. LDL were separated from fresh plasma according to the method developed by Havel et al. (1995). The density of the plasma was adjusted by means of crystalline potassium bromide and the mixture was overlaid with 0.154 M NaCl in a Beckman centrifuge tube. Subsequently, all tubes were centrifuged at 800,000 rpm (Optima L-90K, Beckman Inc., Palo Alto, CA) at 7°C for 45 minutes. After centrifugation, LDL were carefully isolated. Approximately one ml was removed by means of a Pasteur pipette and stored for MDA measurements. Plasma, RBC, and LDL samples were stored in 1.5-ml microtubes and frozen at -80°C until further analyses.

5.3.4. Biochemical measurements

5.3.4.1. Erythrocyte antioxidant enzymes

Red blood cells were used to measure the activities of antioxidant enzymes: superoxide dismutase, catalase, and glutathione peroxidase. The amount of hemoglobin in RBC, used to standardize the activities of enzymes, was measured by a kit (Total Hemoglobin, Sigma 525-A). Superoxide dismutase was determined by a modified method of Xin et al. (1991). Activities of superoxide dismutase in samples were determined by comparison with the calibration curve of SOD standards containing 20.8, 10.4, 5.2, 2.6, and 1.3 U/ml. Catalase activity was measured by the procedure developed by Aebi (1983). The activity of glutathione peroxidase was determined according to the method developed by Agergaard and Jenson (1982). The absorbance of the solution was monitored at 340 nm for 100 seconds after tertiary butyl hydroperoxide was added. The activity of glutathione peroxidase was determined by the change of absorbance and calculated as follows: mmole/l/s=2813* (A_sample - A_blank).

5.3.4.2. Ferric reducing/antioxidant ability in plasma

Ferric reducing/ antioxidant ability (FRAP) in plasma was measured according to the methods of Benzie and Strain (1996) and Langley-Evans (2000). Antioxidant power of
plasma was determined against a standard curve of ferrous sulfate (250, 500, 1000, 1500, and 2500 µM).

5.3.4.3. Vitamin C and uric acid analyses

The method developed by Rümelin et al. (1999) was used for the measurement of plasma ascorbic acid, total ascorbic acid, and uric acid by HPLC. Unknown concentrations of ascorbic acid, total ascorbic acid, and uric acid in plasma were calculated from standard curves. Dithiothreitol freshly made on a daily basis was used to reduce dehydroascorbic acid to ascorbic acid. The reaction mixture was kept in the dark at 25°C for 30 minutes before HPLC analysis.

5.3.4.4. Glutathione determination in blood

Total glutathione and oxidized glutathione concentrations in whole blood were measured by the method developed by Griffith (1980). Unknown concentrations of glutathione in the samples were calculated from standard curve created by using 200, 400, 600, and 800 nmol/ml of glutathione.

5.3.4.5. Malondialdehyde amounts in LDL and plasma

Lipid resistance to oxidation was assessed by means of oxidizing LDL by incubation with cupric ion according to the method modified from Jialal and Grundy (1991) and Naito et al. (1993). The resulting MDA was measured by HPLC (Fukunaga et al., 1998). LDL protein was measured by a kit (Protein Assay Kit, Sigma 5656) in order to determine LDL amount after dialysis and to standardize LDL-MDA in samples. In this method, LDL was dialyzed in 10 µM EDTA phosphate buffer solution at 4°C for 24 hours. Two hundred microliters of 100 µM CuSO₄ and 600 µl distilled water were added to all tubes containing 200 µl dialyzed LDL. The mixture was incubated at 37°C for 24 hours. Seventy-five microliters of oxidized LDL mixture was mixed with 1 ml thiobarbituric acid (TBA) reagent (0.2% (w/v) thiobarbituric acid in 0.1 M sodium acetate buffer, pH 3.5) in a 1.5 ml polypropylene microtube. The TBA and LDL mixture was capped and heated in a water bath at 95°C for 60 minutes. After cooling with tap water, the reaction mixture was centrifuged at 10,000 x g at ambient temperature for 15 minutes. Then, five microliters of supernatant were injected into a HPLC for MDA determination. The thiobarbituric acid-
malondialdehyde adduct was monitored by a fluorescence detector with excitation at 515 nm and emission at 553 nm.

The method developed by Fukunaga et al. (1998) was used to measure MDA in plasma and LDL. LDL-MDA determination used 50 µl dialyzed LDL mixed with 100 µl 0.2% TBA solution. The subsequent steps for measuring in vivo LDL-MDA were the same as those described above for in vitro LDL-MDA determination by HPLC. The concentrations of MDA in LDL and plasma were calculated from the peak area, based on a calibration curve prepared from 1,1,2,2-tetraethoxypropane as standard.

5.3.4.6. Plasma volume change

A hematocrit, analyzed by microcentrifugation, was used to account for plasma volume change (ΔPV(%)) after exercise. The following formula from Van Beaumont et al. (1973) was used to estimate plasma volume change based on the pre-exercise (Hct₁) and post-exercise hematocrit (Hct₂).

\[ \Delta PV(\%) = \frac{100}{(100-Hct_1)} \times 100 \frac{(Hct_2 - Hct_1)}{Hct_2} \]

The concentrations of plasma vitamin C, uric acid, and MDA were adjusted according to plasma volume change before statistical analyses.

5.3.5. Statistical analyses

Results were reported as means and standard error; statistical significance was determined at P<0.05. The JMP IN 4 statistical software package (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses. A paired t-test was applied to test the significance of changes of all measured parameters due to exercise. Partial correlation among biochemical parameters was determined by a multivariate analysis of variance (MANOVA) model in which exercise was considered as a factor.

5.4. RESULTS

The average intensity of submaximal exercise maintained for 30 minutes was 80.0% VO₂pk (n=32, SE=0.8). This observed variation among subjects from the target 80%VO₂pk was due to individual exercise capability on cycle ergometer and/or unwillingness to exert more effort to maintain or elevate exercise intensity. Low fitness level in some physically
inactive participants may be the cause of their inability to approach the goal exercise intensity of 80% VO₂pk.

Mean hematocrit values before and after exercise were 45.87 (n=32, SE= 0.38) and 47.07% (n=32, SE= 0.42) respectively. Plasma volume reduction was 3.94% (n=32, SE= 1.15).

The activities of two erythrocyte antioxidant enzymes were significantly influenced (Table 5.2; Fig 5.1). Superoxide dismutase activity was increased by 16.5%, glutathione peroxidase activity was decreased by 10.9%, and catalase activity was not significantly changed.

Total glutathione, oxidized glutathione, reduced glutathione concentrations, and GSH/TGSH ratio in whole blood were significantly affected by submaximal exercise (Table 5.3; Fig 5.2 and 5.3). Oxidized glutathione was significantly increased and TGSH, GSH and GSH/TGSH ratio were significantly decreased by one bout of acute exercise.

Vitamin C is a water-soluble antioxidant. Submaximal exercise did not have a significant effect on plasma ascorbic acid and total ascorbic acid, which were adjusted by plasma volume change (Table 5.4; Fig 5.4). The ratio of AA/TAA was similar from 1.01 to 0.99 (P = 0.12). Plasma uric acid concentration after adjustment with plasma volume change was significantly elevated by 4.9% due to submaximal exercise (Table 5.4; Fig 5.5). Ferric reducing/antioxidant activity of plasma which is an index of antioxidant power in plasma was significantly increased by exercise by 7.8% (Table 5.4; Fig 5.5). There was 88% partial correlation between FRAP and uric acid in the plasma. The other antioxidants measured in the study, vitamin C and glutathione did not have a significant influence on FRAP value.

In vivo plasma MDA measured by HPLC was increased by 12.6% in participants after exercise (Table 5.5; Fig 5.6). LDL oxidation induced by cupric ion was significantly
decreased by 10.3% (Table 5.5; Fig 5.7). However, in vivo LDL-MDA was insignificantly decreased by 9.0% after submaximal exercise (P = 0.055) (Fig 5.6).

5.5. DISCUSSION

Exercise is considered as a means of improving health. However, strenuous or exhaustive exercise especially may lead to deleterious impact on the body because of increased generation of ROS from all sources during and/or after exercise. The debate between beneficial or deleterious effects of participation in exercise or sports still exists. The extended question from this debate is whether antioxidant supplementation provides the body with extra protection against ROS, and further prevent a disadvantage of exercise. Although oxidative stress due to exercise has been recognized, the magnitude of this oxidative stress depends on type, intensity, and duration of exercise; furthermore, biochemical methods are also important to determine if oxidative stress can be detected. In order to assess whether 80% VO$_2$pk exercise for 30 minutes induces oxidative stress, different methods were applied to measure products of lipid peroxidation, antioxidant homeostasis, and antioxidant enzyme activities.

Physical exercise induces oxidative stress on erythrocytes because of increased generation of ROS. Consequently, it is expected that antioxidant enzymes enhance their activities to reduce ROS to safe compounds. However, whether physical exercise increases the activities of antioxidant enzymes is still controversial. A session of acute exhaustive exercise was shown to increase SOD activity, indicating increased superoxide production during exercise (Ji, 1993) in a number of biological tissues. Kaczmarski et al. (1999) and Ohno et al. (1986), however, reported that physical exercise did not increase erythrocyte SOD activity in humans. Most of the literature revealed no significant alternation in catalase activity with acute exercise (Ji et al., 1990; Meydani and Evans, 1993), while studies conducted by Kaczmarski et al. (1999) and Somani et al. (1995) reported that erythrocyte catalase decreased after acute exercise. The effect of an acute session of exercise on GPx activity in various tissues has not been reported consistently in the literature (Ji, 1995). Erythrocyte GPx activity rose following exercise (Laaksonen et al., 1999); however, Ohno et al. (1986) observed that erythrocyte GPx activity was slightly reduced after a brief (30 min)
physical exercise in sedentary students. Ji et al. (1990) observed that acute exercise did not affect GPx activity in the livers of rats. There is as yet no clear explanation for these discrepancies in changes of enzyme activities after exercise (Ji, 1995). In general, an increased generation of ROS during an acute session of strenuous exercise causing the activation of antioxidant enzymes remained the most viable explanation (Ji et al., 1990; Ji, 1993). SOD activity in our study reflected the increased ROS production was in accordance to observations in the literature because its activity was significantly enhanced by exercise. Increased ROS generation by exercise could not effectively account for the decrease of erythrocyte GPx activity in the study. It has been reported that severe GSH depletion resulted in a significant down-regulation of liver GPx (Leeuwenburgh and Ji, 1995). The reduction of blood GSH, thus, may inhibit GPx activity after exercise. There is also the possibility that GPx activity was inactivated by a large production of ROS (Toskulkao and Glinsukon, 1996). With regard to CAT activity, our results agreed with studies of Ji et al. (1990) and Meydani and Evans (1993) that erythrocyte CAT was not significantly changed by exercise.

GSH concentrations in most tissues are in the millimolar range, far exceeding the levels of most other antioxidants (Ji and Leeuwenburgh, 1996); therefore, it is one of the major water-soluble antioxidants in whole blood. Gohil et al. (1988) were the first to report that even submaximal exercise induced blood GSH oxidation. Furthermore, blood glutathione homeostasis has been suggested as a determinant of resting and exercise-induced oxidative stress (Laaksonen et al., 1999). After oxidant challenge, GSH is transformed to GSSG within the cell. A considerable body of evidence showed that exercise increased GSSG and reduced TGSH and GSH in tissues and blood (Dufaux et al., 1997; Durarte et al., 1993; Duthie et al., 1990; Leeuwenburgh et al., 1997; Lew et al., 1985; Sen et al., 1992). The results of the current study confirmed that 30 minutes 80% VO_{2}pk exercise changed glutathione homeostasis in blood. GSH and TGSH were reduced by 21% and 8.8% respectively, and GSSG increased by 29%. The mechanism is explained in the literature that exercising skeletal muscles increases GSH import from blood via the gamma-glutamyl cycle (Ji et al., 1992; Leeuwenburgh and Ji, 1995; Ji and Fu, 1992; Lew et al., 1985), resulting in
the decreased GSH in the blood. The liver is the major site for GSH production de novo. An 8.8% reduction of blood TGSH suggested that more GSH entered the muscle tissue than hepatic GSH and muscle GSSG entered the blood during exercise. GSSG can be reduced to GSH by glutathione reductase using NADPH as reducing power. With a more severe oxidative stress, the rate of GSSG reduction cannot match the rate of its formation, resulting in GSSG accumulation in tissues (Sen and Packer, 2000). Consequently, GSSG efflux from muscle tissue results in the increase of blood GSSG. The ratio of GSH/TGSH is an indicator of oxidative stress (Laaksonen et al., 1999). The result of the current study confirmed that this ratio is sensitive to oxidative stress induced by exercise.

Vitamin C, a potent water-soluble antioxidant, directly reduces ROS, inhibits lipid peroxidation, and regenerates vitamin E after it is oxidized (Bendich et al., 1986). In the plasma, ascorbate is the first antioxidant to be depleted on exposure to ROS (Frei et al., 1988; Frei et al., 1989). There are two forms of vitamin C in the body, reduced form ascorbic acid (AA) and oxidized form dehydroascorbic acid (DHAA). The significance of AA and DHAA as an antioxidant couple in a biological system is well established (Meister, 1992; Niki, 1987). It has been suggested that the ratio of ascorbic acid to total ascorbic acid (AA plus DHAA) is a useful biomarker reflecting ROS level and an index of oxidative stress (Koyama et al., 1994; Koz et al., 1992; Robertson et al., 1991). Based on the results of the current study, AA/TAA ratio may not be an appropriate index of exercise-induced oxidative stress. This ratio may be useful in clinical studies linking oxidative stress to common human illnesses such as cancer (Weitzman and Gordon, 1990), diabetes mellitus (Chatterjee and Banerjee, 1979) and hepatic diseases (Maellaro et al., 1990; Martensson et al., 1991), other than exercise. The relative DHAA and AA reference concentrations for healthy adults are found with DHAA contents ranging from zero to a few percent of the TAA (Lykkesfeldt et al., 1995). However, many investigators have reported the absence of DHAA in human plasma (Levine et al., 1991; Lopez-Anaya and Mayersohn, 1987; Okamura, 1980). The results of the current study also showed that DHAA was not detected in plasma before exercise; however, exercise insignificantly enhanced DHAA level. The oxidative stress induced by physical exercise can decrease AA and TAA and increase DHAA in plasma.
Exercise enhances vitamin C efflux from the adrenal gland mediated through increased plasma cortisol (Bieri et al., 1979). Vitamin C efflux from the adrenal gland and utilization of vitamin C may explain why no change occurred in plasma vitamin C levels even though the body was confronted with oxidative stress induced by exercise. DHAA reduction to AA by glutathione as a reducing equivalent (Orringer and Roer, 1979) may also account for the insignificant increase of DHAA after exercise. Furthermore, urate has been demonstrated to regenerate ascorbate from the ascorbyl radical, the primary oxidation product of ascorbate, because a more positive redox potential of urate (0.26 V) in comparison with that of ascorbate (0.015 V) makes this reaction possible (Karten et al., 1997; Sevanian et al., 1991).

In humans, uric acid is the end-product of enzymatic purine catabolism by means of the xanthine oxidase pathway (Becker, 1993). The results of the current study were within the range of plasma concentration of urate 160-450 µmol/l reported previously (Hochstein et al., 1984). The increase of uric acid due to exercise is also in agreement with previous studies (Hellsten-Westling et al., 1993; Norman et al., 1987; Westing et al., 1989). It can be suggested that uric acid may be a good index of oxidative stress because exercise significantly enhanced urate concentration in the plasma by 4.9%.

Ferric reducing/antioxidant ability was an index of total antioxidant power in plasma (Benzie and Strain, 1996 and Langley-Evans, 2000). It has been observed that 70% antioxidant power measured by the FRAP method was accounted for by the effect of uric acid. In the current study, there was 89% partial correlation (r=0.89) between uric acid and FRAP value. Vitamin C and glutathione account for a portion of the FRAP measured antioxidant power (Benzie and Strain, 1996), however, that was not supported by the results of the current study. Sub-maximal exercise significantly increased antioxidant power by 7.8%, mostly attributed to the increased uric acid concentration after exercise. The increased FRAP power did not indicate a better antioxidant defense against oxidative stress because of increased plasma MDA due to exercise. Therefore, using FRAP value to represent total antioxidant defense in plasma when exercise is involved should be taken with precaution.
The direct measurement of free radicals in biological systems is difficult because of their high reactivity and low steady-state concentration (Ashton et al., 1999). Despite the theoretical soundness, there is little direct evidence that mitochondrial superoxide production is increased during exercise because of the difficulty with direct detection of ROS generation in the body. The method using electron spin resonance or electron paramagnetic resonance (EPR) spectroscopy directly detects ROS surge after exercise (Balke et al., 1984; Jackson et al., 1985). However, most evidence regarding ROS generation has been derived from studies that monitored by-products of exercise-induced oxidative damage (Sen, 1995). Lipid peroxides are considered to be the major initial reaction products of free radical attack on the cell membrane, whereas MDA is formed as a decomposition product of lipid peroxides, thus justifying MDA measurement as an indirect determinant of free radical-mediated oxidative damage (Ashton et al., 1999). The level of MDA has been estimated indirectly by using thiobarbituric acid-reacting substances (TSARS) (Alessio, 1993; Kanter, 1994; Stringer et al., 1989). The results of human and animal studies showed that physical activity is known to lead to a significant increase in the levels of free radicals and the concentration of end-products of lipid peroxidation in different tissues (Davies et al., 1982; Kanter et al., 1986; Kihlström et al., 1989; Lovlin et al., 1987). In the current study, the sensitivity and specificity in the measurement of MDA was improved by utilizing a TBARS method with HPLC. The exercise protocol applied led to a significant increase of in vivo plasma MDA. Therefore, the intensity, mode, and duration of exercise used in the study were sufficient to overcome delicate antioxidant defense systems and further to induce lipid peroxidation in plasma in vivo.

Lipoproteins, such as very low, low, and high-density lipoproteins, chylomicrons and chylomicron remnants, can be oxidatively modified in vitro by the challenge of oxidants (Karten et al., 1997; Maziere et al., 1993; Mohr and Stocker, 1994; Staprans et al., 1994). LDL oxidation has been suggested to play a key role in the development of atherosclerosis (Esterbauer et al., 1997; Goldstein and Brown, 1978; Parthasarathy, 1994; Steinberg et al., 1989) because oxidized LDL has many pro-atherogenic properties (Parthasarathy, 1994). Intense aerobic exercise enhanced both the susceptibility of LDL to oxidation and the
proportion of LDL(-) (Sánchez-Quesada et al., 1995) because of increased generation of ROS by means of activated neutrophils (Gray et al., 1993; Pincemail et al., 1990; Pyne, 1994) and increased oxygen consumption (Jenkins, 1988; Sjodin et al., 1990) during or after exercise. These aforementioned observations suggested that exercise exerts a deleterious effect on LDL particles that potentially enhances their atherogenicity. The results from the current study that LDL oxidation upon 24 hour incubation with an oxidant was decreased by exercise, was opposite to the theoretically expected increase after exercise. Steinburg et al. (1997) also reported that the amount of conjugated dienes in LDL formed in vitro decreased slightly after exercise. The authors speculated that the decreased in vitro LDL lipid peroxides might be due to lower amounts of polyunsaturated fatty acids present in native LDL (Reaven et al., 1991), which was in agreement with the results of Sumikawa et al. (1993) that exercise decreased the proportion of PUFAs in RBC phospholipids of untrained subjects. Furthermore, the overnight fast the subjects underwent can decrease triglycerides which are substrates for oxidation (Lewis, 1976). The amount of in vitro MDA in LDL is also determined by the seeding of lipid peroxides in native LDL (Sánchez-Quesada et al., 1998). The more in vivo lipid peroxides in LDL, the more susceptible the LDL particle to oxidant challenge. It was believed that very trivial or undetectable amount of in vivo MDA exist in LDL because the antioxidant defense systems in plasma protect LDL against possible oxidative attack (Abuja, 1999). In the current study, a sensitive HPLC-TBARS method detected in vivo LDL-MDA, which was reduced by 9.0% after exercise. This reduction in vivo MDA in LDL can partially explain post-exercises in vitro LDL-MDA 10.3% decrease from pre-exercise in vitro LDL-MDA. The reduction in vivo post-exercise LDL-MDA is in contrast to the theoretically expected increase of MDA. It can be speculated that increased blood flow may accelerate the clearance of mildly oxidized LDL by the liver. Bowry et al. (1992) reported that HDL was the principal vehicle for lipid hydroperoxides and could be efficiently detoxified by hepatocytes, thus suggesting HDL could help to clear oxidized LDL from the circulation.

5.6. CONCLUSIONS

Currently, the evidence for oxidative stress and tissue damage due to exercise remains incomplete because of the complexity of the exercise models and the characteristics of
subjects (Liu et al., 2000). In our study, 30 minutes 80% VO$_2$pk exercise on the cycle ergometer was sufficient to initiate oxidative stress, further leading to the disturbance of erythrocyte antioxidant enzymes and the increased formation of products of lipid peroxidation.

A considerable amount of evidence demonstrated that acute exercise could modulate antioxidant enzyme activities. The best explanation for these modulations was that increased ROS generation activates enzyme activities. Post-exercise increase of erythrocyte SOD activity might be up-regulated by enhanced ROS generation; however, post-exercise decrease of glutathione peroxidase activity cannot be explained by oxidative stress. The decreased GPx activity after exercise may be down-regulated by decreased glutathione as its substrate.

Non-enzymatic antioxidant homeostasis, such as glutathione and ascorbic acid, and products of lipid peroxidation, such as plasma MDA and LDL-MDA, can be regarded as biomarkers of oxidative stress. The most apparent changes of glutathione ratio and in vivo plasma MDA after exercise showed that they both are good biomarkers of oxidative stress induced by exercise applied in the study. Vitamin C may not be as sensitive as glutathione on exposure to oxidative stress. In contrast to the increase of plasma MDA, neither in vivo nor in vitro LDL-MDA increased with oxidative stress, suggesting that LDL-MDA may not be a good indicator of oxidative stress and further research is needed to determine how exercise decreased or increased LDL-MDA.

This is the first study to use FRAP value in determining post-exercise oxidative stress. FRAP value did not reflect plasma MDA in vivo although it has been suggested as a means for determining total plasma antioxidant power.

It can be concluded that 30 minutes 80% VO$_2$pk exercise on cycle ergometer can induce oxidative stress in moderately active college men.

5.7. REFERENCES


Table 5.1. Subject characteristics and dietary intake.

<table>
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<th>n</th>
<th>Mean</th>
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<tr>
<td>Age, years</td>
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<td>20.7</td>
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<tr>
<td>BMI, kg/m²</td>
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<td>VO₂pk, ml/min/kg</td>
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<td>Protein, g/d</td>
<td>32</td>
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<td>Fat, g/d</td>
<td>32</td>
<td>89.2</td>
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Table 5.2. Activities of antioxidant enzymes in red blood cells.

<table>
<thead>
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<th>Post-exercise</th>
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<th>P-value*</th>
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<tr>
<td></td>
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<td>SOD, U/g Hb</td>
<td>32</td>
<td>4.84</td>
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<td>GPx, mmol/s/mg Hb</td>
<td>32</td>
<td>254.8</td>
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<td>226.7</td>
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<td>CAT, U/mg Hb</td>
<td>32</td>
<td>52.4</td>
<td>1.7</td>
<td>53.0</td>
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*A paired t-test was applied to test the significance of changes of all measured parameters due to exercise.*
Figure 5.1. Activities of antioxidant enzymes in red blood cells. SOD, U/g Hb; Gpx, mmol/s/mg Hb; CAT, U/mg Hb. Means in the same measurement with the different letter were significantly different (P<.05), using paired t-test.
<table>
<thead>
<tr>
<th></th>
<th>Pre-exercise</th>
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<th>Change, %</th>
<th>P-value*</th>
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<tr>
<td></td>
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<td>Mean</td>
</tr>
<tr>
<td>TGSH, nmol/ml</td>
<td>32</td>
<td>1134</td>
<td>36</td>
<td>1034</td>
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<tr>
<td>GSH, nmol/ml</td>
<td>32</td>
<td>858</td>
<td>39</td>
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<tr>
<td>GSSG, nmol/ml</td>
<td>32</td>
<td>138</td>
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<td>178</td>
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<td>GSH/TGSH</td>
<td>32</td>
<td>0.75</td>
<td>0.02</td>
<td>0.65</td>
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*A paired t-test was applied to test the significance of changes of all measured parameters due to exercise.*
Figure 5.2. Glutathione homeostasis in whole blood. Means in the same measurement with the different letter were significantly different (P<.05), using paired t-test.
Figure 5.3. Glutathione ratios. Means in the same measurement with the different letter were significantly different (P<.05), using paired t-test.
Table 5.4. Ascorbic acid and uric acid concentrations, and FRAP value in plasma*.

<table>
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<th>P-value¹</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>Mean</td>
<td>SE</td>
<td>Mean</td>
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<tr>
<td>Ascorbic acid,</td>
<td>32</td>
<td>6.44</td>
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<td>6.43</td>
</tr>
<tr>
<td>microgram/ml</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ascorbic acid,</td>
<td>32</td>
<td>6.44</td>
<td>0.47</td>
<td>6.46</td>
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<tr>
<td>microgram/ml</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>AA/TAA</td>
<td>32</td>
<td>1.00</td>
<td>0.01</td>
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<tr>
<td>Uric acid, microgram/ml</td>
<td>32</td>
<td>53.6</td>
<td>1.8</td>
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<td>FRAP, micromolar</td>
<td>32</td>
<td>889</td>
<td>22</td>
<td>958</td>
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*Vitamin C and uric acid concentrations were adjusted according to plasma volume change.

¹A paired t-test was applied to test the significance of changes of all measured parameters due to exercise.
Figure 5.4. Ascorbic acid and total ascorbic acid concentrations in plasma and AA/TAA ratio. A paired t-test was applied to test the significance of changes of all measured parameters due to exercise.
Figure 5.5. Uric acid concentration in plasma and FRAP value. Uric acid, microgram/ml; FRAP, µM. Means in the same measurement with the different letter were significantly different (P<.05), using paired t-test.
Table 5.5. Malondialdehyde concentrations in plasma and LDL*.

<table>
<thead>
<tr>
<th></th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
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<th>P-value&lt;sup&gt;1&lt;/sup&gt;</th>
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<td></td>
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<td>Mean</td>
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<tr>
<td>In vivo plasma MDA, micromolar</td>
<td>32</td>
<td>0.95</td>
<td>0.04</td>
<td>1.07</td>
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<td>In vivo LDL-MDA, micromol/g protein</td>
<td>32</td>
<td>0.080</td>
<td>0.007</td>
<td>0.073</td>
</tr>
<tr>
<td>In vitro LDL-MDA, micromol/g protein</td>
<td>32</td>
<td>54.0</td>
<td>2.4</td>
<td>48.4</td>
</tr>
</tbody>
</table>

*Plasma MDA was adjusted according to plasma volume change.

<sup>1</sup>A paired t-test was applied to test the significance of changes of all measured parameters due to exercise.
Figure 5.6. *In vivo* malondialdehyde concentrations in plasma and LDL. Means in the same measurement with the different letter were significantly different (P<.05), using paired t-test.
Figure 5.7. *In vitro* LDL-MDA. Means with the different letter were significantly different (P<.05), using paired t-test.
CHAPTER 6
Isoflavone Supplementation Modulated Erythrocyte Antioxidant Enzyme Activities and Glutathione Homeostasis in Healthy Young Men Undergoing 80% VO$_2$pk Exercise

6.1. ABSTRACT

The low incidence of cardiovascular diseases and hormone-related cancers in Asians has motivated scientists to investigate the functionality of soy isoflavones. The antioxidant activity of isoflavones, especially genistein, has been observed in both in vitro studies and in vivo animal studies, but the antioxidative potency of isoflavones when they defend against in vivo oxidative stress induced by exercise has not been investigated. The aim of this experiment was to investigate if there is an antioxidant effect of soy isoflavones against oxidative stress produced in moderately active young college men undergoing 80% VO$_2$pk exercise. The study was a double-blind dietary intervention with soy isoflavone or placebo control tablets. Thirty subjects were paired by their fitness levels and then each pair was randomly assigned to either high-genistein isoflavones extract (HGI) group or placebo group. The subjects in HGI and placebo groups were given 150-mg HGI tablets and placebo tablets, respectively, daily for 28 days. All subjects exercised at 80% VO$_2$pk on a cycle ergometer for 30 minutes before and after the supplementation. A total of four blood samples was collected from each subject before and within five minutes after each exercise session. After four-weeks of HGI supplementation, plasma genistein and daidzein concentrations were 499 and 415 ng/ml, and they were significantly increased to 633 and 539 ng/ml by exercise (P=0.04 and P=0.05). Isoflavones significantly decreased pre-exercise plasma MDA (P<0.05), however, the HGI supplementation did not prevent the increase of MDA due to exercise. HGI did not have any significant effect on LDL-MDA. Isoflavones also resulted in significant increase of pre-exercise blood TGSH (P=0.01) and led to a significantly higher utilization of blood TGSH and GSH during exercise. Regarding erythrocyte antioxidant enzymes, HGI supplementation significantly increased pre-exercise erythrocyte SOD activity (P=0.0006) and maintained the decreased activities of GPx due to exercise as pre-exercise
levels. Isoflavone administration led to the decrease of pre-exercise plasma MDA probably by their influence on blood TGSH and SOD activities, and not directly by reducing free radicals or lipid peroxides. Our results demonstrated that isoflavones had antioxidant activity under normal physiological conditions in healthy young men. Isoflavone supplementation also maintained GPx activity which was decreased due to exercise; however, supplementation did not show evidence that they diminish all oxidative stress initiated by 80% VO₂pk exercise.

**Key words:** isoflavones, malondialdehyde, reactive oxygen species, exercise, humans

### 6.2. INTRODUCTION

In the developed world, cardiovascular diseases are some of the leading causes of morbidity and mortality. Cardiovascular diseases are the clinical expressions of advanced atherosclerosis, which begins early in life, but which usually takes decades to form the stenotic plaques responsible for the chronic ischemic episode in the arteries (Dalager-Pedersen et al., 1998). The development of atherosclerosis can be attributed to a collection of “risk factors,” such as, plasma total cholesterol concentration, distribution of cholesterol among lipoproteins, blood pressure, LDL oxidation, smoking status, advanced age, and homocysteine levels (Wood, 1998). Particularly, an increasing body of evidence implicates oxidized low-density lipoprotein (LDL) as one of the triggers of atherogenesis.

Low-density lipoprotein is the major carrier of cholesterol in the circulation and the main substrate for oxidation during the development of atherosclerosis. Even though it is believed that lipid peroxidation of LDL may not take place in the plasma (Esterbauer, et al., 1991), the result from the sensitive ELISA assay suggests that some oxidized LDL is present in the circulation (Holvoet et al., 1998). *In vivo* LDL oxidation involves reactive oxygen species (ROS) (Yla-Herttuala, 1998) and cells, such as macrophages, endothelial and SMC (Mabile, 1997).
It is widely accepted and experimentally proven that catabolic process in cells can generate reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide (Halliwell and Gutteridge, 1989). All of these compounds have an unpaired electron on their molecular orbits, and can react with lipids, nucleic acids, and proteins, leading to the damage of cells and tissues (Ames et al., 1995; Yu, 1994). Under normal physiological conditions, approximately 2-5% of the oxygen consumed, however, cannot end up as water, and leaks out from the electron transport chain as superoxide. Therefore, a normal cell produces approximately $2 \times 10^{10}$ O$_2^-$ and H$_2$O$_2$ per day, which can be added up to $3.3 \times 10^{-14}$ moles per day (Ames et al., 1995).

It is well established that regular aerobic exercise has some protective effects against atherosclerosis development (Berlin and Colditz, 1990) by reducing cholesterol levels and high blood pressure (Hollmann, 1994), and enhancing nitric oxide (NO) production (Gattullo et al., 1999) and antioxidant defense systems (Lawler and Powers, 1998). Although exercise can benefit health, it may cause a deleterious effect on the body because the rate of oxygen uptake by the body during exercise may increase by 10- to 15-fold. Therefore, an elevated metabolic rate because of exercise can dramatically increase ROS production (Alessio, 1993; Davies et al., 1982). Despite the existence of elaborate antioxidant defenses such as antioxidant enzymes, glutathione, β-carotene, ascorbic acid, and alpha-tocopherol (Meister, 1983), apparently some superoxide anion radical and H$_2$O$_2$ molecules remain un-scavenged and lead to tissue damage in humans performing physical activities (Kanter, 1994). When the elevation of ROS generation overcomes the antioxidant defense systems, the ensuing situation is called oxidative stress. There have been many reports showing that exercise causes oxidative stress, e.g., increases in oxidative damage biomarkers such as protein carbonyls and thiobarbituric acid reactive substances (TBARS) (Sen et al., 1994; Sen et al., 1997); defected mitochondrial function (Ravalec et al., 1996; Willis and Jackman, 1994); and decreases in levels of antioxidants (Criswell et al., 1993; Jenkins and Goldfarb, 1993; Ji, 1993; Lew et al., 1985; Powers et al., 1994; Reznick et al., 1982; Sen et al., 1992; Sen et al., 1994; Somani et al., 1995). It has also been observed that the urinary excretion of peroxides increased during physical activity in young sportsmen (Olinescu, 1995). Furthermore,
Wetzstein et al. (1998) reported that 30-minute exercise at a moderate intensity and duration was sufficient to induce in vitro LDL oxidation in humans. Although exercise has been recommended for improving and maintaining health, ROS generation during the participation in any exercise or sport can cause oxidative stress.

There has been much interest in the effects of antioxidant supplementation on improving performance and preventing tissue damage due to exercise, as well as on diminishing the development of atherosclerosis. Numerous studies demonstrated that antioxidant vitamin supplementations can be beneficial in lowering biomarkers of oxidative stress and lipid peroxidation (Brown et al., 1997; Kanter et al., 1993; Sürmen-Gür et al., 1999). It has been reported that vitamin C supplementation enhances total antioxidant activity and reduces plasma MDA levels after submaximal exercise (Alessio et al., 1997; Sen et al., 1994). Goldfarb et al. (1994) found that 250 IU vitamin E/Kg diet fed to rats can reduce TBARS and lipid peroxide levels in plasma and leg muscles, after one hour of exercise on the treadmill, compared to rats fed a normal diet.

The growing concerns for general health, chronic disease prevention, and aging have fueled consumer interest in phytonutrients because of their curative, preventative, and nutritive value (Guhr and Lackance, 1997). The incidence of cardiovascular diseases among Asians has always been lower than among Americans. It has been surmised that soy consumption, one of the major differences between Asian and Western diets, may result in low incidence of cardiovascular diseases (Aguiló et al., 2000; Beaglehole, 1990; Knight and Eden, 1996) and hormone-related cancers (Coward et al., 1993; Gotoh et al., 1998). The beneficial effect of soy on cardiovascular diseases can be attributed to some components of soy, including isoflavones, amino acid composition of protein, fiber, and saponins. However, Brandi (1997) stressed that isoflavones in soy may play the major beneficial role of soy in lowering the incidence of diseases. Adlercreutz et al. (1993) reported that isoflavone consumption per capita among Japanese was estimated to be up to 200 mg/d, consequently leading to a possible health advantage in the long term. Therefore, increased isoflavone ingestion may contribute to low incidences of heart disease/cardiovascular diseases and cancers.
Isoflavones have a chemical structure similar to estrogen, which is reported to have a weak antioxidant activity because of the hydroxyl group(s) on their phenolic ring(s) as in vitamin E (Tiidus, 1995). In addition, isoflavones have multi-hydroxyl groups to act as effective antioxidants by donating a hydrogen atom(s) from their phenolic hydroxyl group(s) to peroxynitrides (Tikkanen et al., 1998). Genistein, one of the isoflavones in soy with three hydroxyl groups, has been found to be a strong antioxidant that can decrease LDL oxidation \textit{in vitro} (Kapiotis et al., 1997). The \textit{in vivo} antioxidant activity of genistein in a mouse model of singlet oxygen-induced cerebral stroke was observed by Trieu et al. (1999). Additionally, Cai and Wei (1996) observed that dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activities of antioxidant enzymes in the small intestine and skin of mice.

Although the antioxidant activity of genistein has been observed \textit{in vitro} and in animals, the \textit{in vivo} antioxidative capability of isoflavones in humans still lacks solid evidence. In order to investigate the antioxidant activity of genistein in plasma and further investigate its capability to protect LDL and plasma against oxidation, an \textit{in vivo} oxidative stress either must be pre-existent or must be initiated. Intensive physical activity has been suggested as one means to induce a surge of ROS in the body, leading to oxidative stress because of the resulting imbalance between antioxidants and oxidants (Ji, 1993). Therefore, the combination of dietary isoflavone supplementation and intensive exercise is a way to explore whether isoflavones from soy can counteract the oxidative stress that results from the imbalance between antioxidants and oxidants in the body. A positive finding implies that long term intake of isoflavones will slow the development of atherosclerosis and other chronic diseases by means of their antioxidative capability.

It is hypothesized that four-weeks of 150 mg HGI supplementation daily in humans would increase erythrocyte antioxidant enzyme activities, reduce plasma- and LDL-MDA, improve glutathione homeostasis, decrease plasma vitamin C and enhance ferric reducing/antioxidant ability (FRAP) value. The overall implication is that isoflavone
administration would protect the body against oxidative stress induced by means of intensive exercise. There were two objectives of this study, which was approved by Virginia Tech Institutional Review Board. First, we investigated whether the supplementation of an isoflavone extract from soy would modulate total antioxidant defense systems in subjects under normal physiological conditions. Second, we investigated whether isoflavones would provide antioxidative protection when subjects underwent oxidative stress induced by 80% peak oxygen consumption (VO$_{2pk}$) exercise for 30 minutes.

6.3. MATERIALS AND METHODS

6.3.1. Subjects

Thirty non-smoking healthy men, aged 20±1.94 years old, were recruited from the Virginia Tech campus (Table 6.1). They were the same population group as in chapter 5. Men were selected as subjects due to the stability of their hormonal status. Hormone fluctuations found in women, such as estrogen with its antioxidative activity, can affect the extent of oxidation of LDL and plasma (Tiidus, 1995). Before beginning the study, all recruited subjects were screened according to the following criteria, non-smoking, apparently healthy, and moderately active (<4 hr exercise a week). All subjects were informed that they cannot take any antioxidant supplements, such as vitamin A, C, or E, one month before and during the study; and were asked to eliminate all soy products, beans, peas, nuts (peanut and peanut butter included), sprouts, seeds and any food that contains any of these components (Tikkanen et al., 1998). All subjects signed an informed consent form before the beginning of the study. During the study, all subjects were asked to maintain their regular dietary and physical lifestyles.

6.3.2. Study design, dietary intervention, and study timeline

The study was a double blind dietary intervention with either soy isoflavone or placebo control tablets. Each isoflavone tablet contained 75 mg of a high-genistein isoflavone extract. Both isoflavone and placebo tablets were supplied by Archer Daniels Midland (ADM). The subjects were paired according to fitness level (VO$_{2pk}$) and each pair randomly assigned to one of the treatments. Three-day dietary and physical records were
collected from the subjects on the week prior to and at the end of the supplementation period. Dietary intakes were analyzed by Nutritionist V (First DataBank Inc., San Bruno, CA).

Each subject was instructed to take two tablets of HGI or placebo with plenty fluid, one in the morning and one in the evening, each day during a four-week supplementation period. Therefore, subjects in the HGI group consumed 150-mg isoflavone extract daily. The isoflavone tablet composition was 50.8% genistein, 39.1% daidzein, and 10.1% glycitein. Supplementation was initiated at the same day as the first submaximal exercise was conducted. Each participant’s compliance of tablet intake was estimated by means of a questionnaire that was filled out by the subjects on the last day of the experiment. The compliance was 97.2%.

The duration of whole study was 35 days for each subject, including orientation, VO2pk test, two sessions of submaximal exercise and 4-week dietary supplementation (Fig 6.1). The schedule for subjects was staggered to have the VO2pk test and submaximal exercise.
Start Date
Day 1

Start of Study
Orientation of subjects

Day 3
First 3-day dietary and physical activity records

VO₂pk exercise test

Day 6
First 80% VO₂pk exercise for 30 minutes and three blood draws:
1. 5 minutes before exercise
2. within 5 minutes after exercise

Isoflavone supplementation for 28 days

Day 35
Second and last 3-day dietary and physical activity records

Second and last 80% VO₂pk exercise for 30 minutes and three blood draws:
1. 5 minutes before exercise
2. within 5 minutes after exercise

End of the study

Figure 6.1. Time of the study.
6.3.3. Peak oxygen consumption and submaximal exercise

Peak oxygen consumption (VO\(_2\)pk test) and respiratory rate were measured on the Monark cycle ergometer (Monark 818E, Monark, Stockholm, Sweden), using a Med Graphics CPX/D VO2 system (St. Paul, MN) automated metabolic cart. This test assessed VO\(_2\)pk of each subject and maximum watts on the cycle ergometer when the subject reached maximum exercise capacity. According to the plot of VO\(_2\)pk, watts, and time from the VO\(_2\)pk test, 80% VO\(_2\)pk exercise protocol for each subject was designated. The cycle ergometer was used in the study instead of treadmill because of the advantages of the cycle ergometer, which causes less stress and burden on subjects’ muscle and less risk of tripping than treadmill. Each subject’s peak oxygen consumption was measured before isoflavone supplementation and the first 80% VO\(_2\)pk exercise. Each subject exercised at his 80% VO\(_2\)pk twice on a stationary ergometer for 30 minutes in order to induce oxidative stress (Gohil et al., 1988; Ohno et al., 1986; Wetstein et al., 1998) before and after the supplementation.

The value of VO\(_2\)pk is the definitive laboratory indicator for individual aerobic exercise capacity (Wareham et al., 1998). The respiratory exchange ratio (R > 1.1), rate of perceived exertion (Borg Scale (Borg, 1982)), and heart rate (95% of predicted maximum heart rate) were obtained from each subject and used to provide objective evidence of maximal effort endpoints for this test. Before the peak oxygen consumption test, the subjects were interviewed to establish an estimated exercise capacity for cycling, so that a progression of workloads on the ergometer can be provided such that the peak oxygen consumption may be achieved within 8-12 min before muscle fatigue. Pedal speed was held constant at 60 rpm. Subjects pedaled on a cycle ergometer for two minutes as a warm-up to familiarize themselves with the test and the speed of 60 rpm. The workload was increased by 30-60 watts every 1-2 min depending on the subjects’ heart rate response and estimated exercise capacity. The workload was gradually increased until the subject was unwilling to continue or unable to maintain the 55 rpm. The MedGraphics VO\(_2\) system was used to determine oxygen consumption and respiratory rate. Subjects were fitted with a breathing apparatus (mouthpiece, nose clip and three-way breathing tube). One end of the breathing tube was
open to room air so the subject could breathe freely. The other end was connected to the MedGraphics machine to analyze expired air during exercise. Software programs that drive the MedGraphics metabolic cart via desktop computer automatically stored and recorded the responses for peak oxygen consumption and the respiratory exchange ratio, all of which were used to verify the attainment of the subjects’ aerobic exercise capacity. Heart rate was measured by using an ECG system (Quinton: ECG: Models Q5000) with CC5 lead. This ECG system involves three electrodes: LL = at the fourth left intercostals space, below and lateral to the nipple (positive electrode); RA = at the fourth right intercostals space and lateral to the nipple (negative electrode); and RL = at the lower right quadrant of the anterior thorax.

All subjects were instructed not to participate in any exercise or sport two days before the submaximal exercise. Upon reporting to the laboratory, each subject rested for 10 minutes before a venous blood sample was drawn from an antecubital vein. Subsequently, the subject warmed up for 3-5 minutes and then exercised for 30 minutes at his 80% VO₂pk on the cycle ergometer. During the submaximal exercise, heart rate was monitored continuously throughout the exercise by using an ECG system. In addition, oxygen consumption was obtained every five minutes of the exercise to carefully monitor whether the subject exercised at 80% VO₂pk. The workload on the cycle ergometer was increased or decreased if oxygen consumption was not at 80% VO₂pk for the specific subject.

6.3.4. Sample collection

There were four 28-ml blood draws during this study. The four blood draws were scheduled during the two sessions of 80% VO₂pk exercise. The schedule for each two blood samples obtained during the submaximal exercises was 5 minutes before exercise and within 5 minutes after exercise. Subjects arrived at the lab after overnight fasting (12 hours) for all blood draws.

Blood samples were drawn from an antecubital vein into EDTA containing tubes. The blood samples were then immediately kept on ice. Two small aliquots of blood were removed from one tube and prepared for further total glutathione and oxidized glutathione
measurements, according to the method of Griffith (1980). Both mixtures for TGSH and GSSG analyses were kept in ice and within 30 minutes were centrifuged at 10,000 x g at 4°C for 15 minutes. One of the tubes, designated for vitamin C analysis, was immediately covered with aluminum foil and kept in ice. Plasma was separated and aliquots were treated for ascorbic acid, total ascorbic acid, and uric acid analyses, according to the method of Rümelin et al. (1999). The blood in the remaining three tubes was separated into plasma and RBC by means of centrifugation at 2000 x g for 20 minutes at 4°C. Subsequently, RBC was washed with precooled 0.9% saline solution three times before storage at -80°C. LDL was separated from fresh plasma according to the method developed by Havel et al. (1995). The density of the plasma was adjusted by means of crystalline potassium bromide and the mixture was overlaid with 0.154 M NaCl in a Beckman centrifuge tube. Subsequently, all tubes were centrifuged at 800,000 rpm (Optima L-90K, Beckman Inc., Palo Alto, CA) at 7°C for 45 minutes. After centrifugation, approximately one ml LDL was carefully isolated by means of a Pasteur pipette and stored for in vivo and in vitro MDA measurements. Plasma, RBC, and LDL samples were stored in 1.5-ml microtubes and frozen at -80°C until further analyses.

6.3.5. Biochemical measurements (the same methods as in chapter 5)

6.3.5.1. Erythrocyte antioxidant enzymes

Red blood cells were used to measure the activities of antioxidant enzymes: superoxide dismutase, catalase, and glutathione peroxidase. The amount of hemoglobin in RBC, used to standardize the activities of enzymes, was measured by a Sigma Kit 525-A (St Louis, MO). Superoxide dismutase was determined by a modified method of Xin et al. (1991). Concentrations of superoxide dismutase in samples were determined by comparison with the calibration curve of SOD standards containing 20.8, 10.4, 5.2, 2.6, and 1.3 U/ml. Catalase activity was measured by the procedure developed by Aebi (1983). The activity of glutathione peroxidase was determined according to the method developed by Agergaard and Jenson (1982). Absorbance was monitored at 340 nm for 100 seconds after tertiary butyl hydroperoxide was added. The activity of glutathione peroxidase was determined by the change of absorbance and calculated as follows: mmole/l/s=2813*(A_{sample} - A_{blank}).
6.3.5.2. Ferric reducing/antioxidant ability in plasma

Ferric reducing/antioxidant ability (FRAP) in plasma was measured according to the methods of Benzie and Strain (1996) and Langley-Evans (2000). Antioxidant power of plasma was determined against a standard curve of ferrous sulfate (250, 500, 1000, 1500, and 2500 µM).

6.3.5.3. Vitamin C and uric acid analyses

The method developed by Rümelin et al. (1999) was used for the measurement for plasma ascorbic acid, total ascorbic acid, and uric acid by HPLC. Unknown concentrations of ascorbic acid, total ascorbic acid, and uric acid in plasma were calculated from standard curves. Dithiothreitol freshly made on a daily basis was used to reduce dehydroascorbic acid to ascorbic acid. The reaction mixture was kept in the dark at 25°C for 30 minutes before HPLC analysis.

6.3.5.4. Glutathione determination in blood

Total glutathione and oxidized glutathione concentrations in whole blood were measured by the methods developed by Griffith (1980). Unknown concentrations of glutathione in the samples were calculated from a standard curve created by using 200, 400, 600, and 800 nmol/ml of glutathione.

6.3.5.5. Malondialdehyde amounts in plasma and LDL

*In vitro* lipid resistance to oxidation was assessed by means of oxidizing LDL by incubation with cupric ion according to the method modified from Jialal and Grundy (1991) and Naito et al. (1993). The resulting malondialdehyde was measured by HPLC (Fukunaga et al., 1998). LDL protein was measured by Sigma kit 5656 in order to determine LDL amount after dialysis and to standardize LDL-MDA in samples. In this method, LDL was dialyzed in 10-micromolarEDTA phosphate buffer solution at 4°C for 24 hours. Two hundred microliters of 100 micromolarCuSO₄ and 600 µl distilled water were added to all tubes containing 200 µl dialyzed LDL. The mixture was incubated at 37°C for 24 hours. Seventy-five microliters of oxidized LDL mixture was mixed with 1 ml thiobarbituric acid (TBA) reagent (0.2% (w/v) thiobarbituric acid in 0.1 M sodium acetate buffer, pH 3.5) in a
1.5 ml polypropylene microtube. The TBA and LDL mixture was capped and heated in a water bath at 95°C for 60 minutes. After cooling with tap water, the reaction mixture was centrifuged at 10,000 x g at ambient temperature for 15 minutes. The five microliters of supernatant was injected into HPLC for MDA determination. The thiobarbituric acid-malondialdehyde adduct was monitored by the fluorescence detector with excitation at 515 nm and emission at 553 nm.

The method developed by Fukunaga et al. (1998) was used to measure in vivo MDA in plasma and LDL. In order to increase the sensitivity in the determination of in vivo LDL-MDA, 50 µl dialyzed LDL were mixed with 100 µl 0.2% TBA solution. The following steps for measuring in vivo LDL-MDA were the same as those described above in in vitro LDL-MDA determination by HPLC. The concentrations of MDA in LDL and plasma were calculated from the peak area, based on a calibration curve prepared from 1,1,2,2-tetraethoxypropane as standard.

6.3.5.6. Plasma isoflavone determination

The determination of plasma genistein and daidzein by HPLC, modified from methods of Wang and Murphy (1994) and Xu et al. (1994). Half ml plasma and 19 µl fluorescein (199.541 ng/µl, internal standard) were mixed with 20 ml of methanol and then the mixture was centrifuged at 5000 x g for 10 minutes at 10°C. Methanol supernatant was evaporated to closely complete dryness at 37°C. The hydrolyzate generated by adding sodium acetate buffer (pH 5.5) was incubated with 50 µl B-glucuronidase/sulfatase at 37°C overnight (16-20 hours). After incubation, the hydrolyzate was filtered with solid phase extraction cartridge (J&W Scientific, Folsum, CA) before proceeding with HPLC analysis. The filtrate was dried under nitrogen. The residue was re-dissolved in 170 µl of 80% acetonitrile. Then, 100 µl of extract was injected into HPLC for genistein and daidzein determination. The recovery rate of plasma isoflavone from all extraction steps was 97.4±5.9 % according to the amount of fluorescein determined by HPLC. Unknown concentrations of plasma genistein and daidzein were calculated from the standard curves of genistein and daidzein.

6.3.5.7. Plasma volume change
A hematocrit, analyzed by microcentrifugation, was used to account for plasma volume change ($\Delta PV(\%)$) after exercise. The following formula from Van Beaumont et al. (1973) was used to estimate plasma volume change based on the hematocrit before exercise ($Hct_1$) and after exercise ($Hct_2$).

$$\Delta PV(\%) = \frac{100}{(100-Hct_1)} \times \frac{100(Hct_2 - Hct_1)}{Hct_2}$$

The concentrations of plasma vitamin C, uric acid, isoflavones, and MDA were adjusted by plasma volume change before statistical analyses.

6.3.6. Statistical Analyses

The effect of supplementation (isoflavone vs. placebo), exercise (pre- vs. post-) and time (week 0 vs. week 4) were studied in a matched-pair repeated-measure (split-plot) mixed-model analysis of variance in two stages (Sall et al., 2001).

The first stage was used to determine (i) whether there is a change in pre-exercise response level over the four-week period of supplementation and (ii) whether there was a change in response level due to exercise (pre vs. post). The linear model used for the first-stage analysis used matched pairs of subjects (whole-plot) factor. The interaction between matched pairs and supplementation was the between-subjects (whole-plot) error term. Exercise (pre- vs. post-) and time (week 0 vs. week 4) were within-subject (split-plot) factors. The three two-way interactions and the three-way interaction were included in the first-stage model. A linear contrast was used to make determination (i) above.

The second-stage was used to determine (i) the main effects of supplementation (isoflavone vs. placebo) and (ii) the interactions between supplementation and time (week 0 vs. week 4). The linear model used for the second-stage analysis was a simplification of the first-stage model achieved by analyzing the post-exercise minus pre-exercise difference. As in the first-stage model, matched pairs of subjects were used as blocks, supplementation was the between-subjects factor, time as the within-subjects factor, and the interaction between supplementation and time was included. Tukey’s HSD procedure was used for multiple comparison (Sall et al., 2001).
Results were reported as means±SE; statistical significance was determined at P<0.05. All analyses were performed by JMP IN 4 statistical software package (SAS Institute Inc., Cary, NC).

6.4. RESULTS

6.4.1. Nutrient intake and plasma isoflavones

Two dietary records were collected from each subject before the first and last session of the submaximal exercise. Dietary intake of each subject was analyzed by Nutritionist V. The results showed that the subjects in the HGI group consumed significantly less macro- and micro-nutrients in final week of the study than before the first submaximal exercise (Table 6.2). However, nutrient intake of subjects in placebo group did not show the same trend as that in HGI group.

After the four-week HGI supplementation, plasma genistein and daidzein concentrations were 499 and 415 ng/ml, respectively (n=15, SE=100 and 98), and they were significantly increased to 633 and 539 ng/ml, respectively (n=15, SE=146 and 144), after 30 minutes of submaximal exercise (P=0.04 and P=0.05).

6.4.2. Exercise intensities of initial and final submaximal exercises

The intensities of the first and second submaximal exercise were significantly different (80.0 and 81.4% VO₂pk, ml/min/kg, respectively (n=30, SE=0.82 and 0.79)) (P=0.03). The variation in exercise intensity among participants was due to individual exercise capability on the cycle ergometer and/or his unwillingness to put more effort to increase exercise intensity. Furthermore, low fitness level in some physically inactive participants might be the reason they could not reach the goal exercise intensity.

6.4.3. Exercise effect on biomarkers of oxidative stress

Submaximal exercise significantly changed hematocrit mean value from 46.1% to 47.2% (P=0.002), consequently resulting in 4.3% plasma volume decrease after exercise. Plasma MDA, isoflavones, uric acid, and ascorbic acid were adjusted according to this
plasma volume shift before the statistical analyses. The effects of 30 minutes of 80%VO$_2$pk exercise on erythrocyte antioxidant enzymes, non-enzymatic antioxidants, and indices of lipid peroxidation had been fully discussed in chapter 5, thus, they are only briefly mentioned here. As to products of lipid peroxidation, plasma MDA was significantly increased by exercise (P<0.0001) (Table 6.3; Fig 6.2 and 6.3), however, neither in vitro LDL-MDA nor in vivo LDL-MDA was influenced by the submaximal exercise (Table 6.4 and 6.5; Fig 6.4 and 6.5). Exercise significantly decreased blood TGSH (P<0.0001) (Table 6.6; Fig 6.6 and 6.7) and GSH (P<0.0001) (Table 6.7; Fig 6.8 and 6.9) and increased blood GSSG (P=0.0066) (Table 6.8; Fig 6.10). The ratio of GSH/TGSH was significantly decreased following exercise (P<0.0001) (Table 6.9; Fig 6.11). Vitamin C was not influenced by the exercise protocol (Table 6.10, 6.11, and 6.12; Fig 6.12-14). Uric acid, the product of purine metabolism, was significantly elevated by submaximal exercise (P=0.0097) (Table 6.13; Fig 6.15). FRAP value, an index of total antioxidant power of plasma, was significantly enhanced by exercise (P<0.0001) (Table 6.14; Fig 6.16 and 6.17). Exercise also significantly enhanced SOD activity in red blood cells (P<0.0001) (Table 6.15; Fig 6.18 and 6.19), however, it decreased GPx (P=0.0006) (Table 6.16; Fig 6.20 and 6.21).

6.4.4. The effect of isoflavone supplementation

6.4.4.1. Products of lipid peroxidation

Products of lipid peroxidation were measured in plasma and LDL. Pre-exercise plasma MDA was significantly decreased by four-week HGI supplementation (P<0.05) (Table 6.3; Fig 6.2 and 6.3). However, in vivo and in vitro LDL-MDA was not affected (Table 6.4 and 6.5; Fig 6.4 and 6.5). Although HGI supplementation resulted in a decrease of in vivo pre-exercise plasma MDA, it did not prevent the increase of MDA due to exercise.

6.4.4.2. Glutathione homeostasis in whole blood

Four weeks of HGI supplementation resulted in significant increase of pre-exercise TGSH (P=0.01) (Table 6.6; Fig 6.6 and 6.7). GSH concentration was not affected. Pre-exercise GSSG in both groups was increased (P<0.0001) (Table 6.8; Fig 6.10). The ratios of
pre-exercise GSH/TGSH in both groups were decreased with time (P<0.001) (Table 6.9; Fig 6.11).

Glutathione homeostasis was significantly influenced by exercise (Table 6.6, 6.7, 6.8, and 6.9; Fig 6.6-11). HGI supplementation led to significantly decreases of TGSH and GSH in the blood by exercise than those before the supplementation. However, HGI supplementation did not affect any change in GSSG concentration and GSH/TGSH ratio due to exercise.

6.4.4.3. Plasma vitamin C, uric acid, and FRAP value

The concentrations of pre-exercise ascorbic acid and total ascorbic acid in plasma were significantly decreased with time (P<0.005 and P<0.01, respectively) (Table 6.10 and 6.11; Fig 6.12 and 6.13). These decreases in vitamin C may be due to decreased intake of vitamin C from food during 4 weeks of study. The ratios of pre-exercise AA/TAA in both groups were not influenced by supplementation and time (Table 6.12; Fig 6.14).

There was no effect of HGI on pre-exercise uric acid concentration in plasma (Table 6.13; Fig 6.15). Although the pre-exercise FRAP value, total antioxidant power in plasma, increased after 4-week HGI supplementation, increased FRAP value in placebo was also noticed (P<0.002) (Table 6.14; Fig 6.16 and 6.17). The percentages of FRAP increase were 14.0% and 10.6% in HGI and placebo groups, respectively.

Neither isoflavone supplementation nor exercise had any effect on AA, TAA, and AA/TAA ratio (Table 6.10, 6.11, and 6.12; Fig 6.12-14). Plasma uric acid was increased by exercise; however, HGI supplementation did not result in any change (Table 6.13; Fig 6.15). Post-exercise FRAP value representing total antioxidant power in plasma was enhanced most likely due to elevated uric acid concentration. However, isoflavone did not significantly contribute to the increased FRAP value after exercise.

6.4.4.4. Erythrocyte antioxidant enzyme activities

Pre-exercise erythrocyte SOD activity was significantly increased after four weeks of HGI supplementation (P=0.0006), however, isoflavones did not have any apparent effect on
the increase of SOD activity due to exercise. (Table 6.15; Fig 6.18 and 6.19). Neither pre-exercise activity of CAT nor GPx was affected by HGI supplementation, however, decreased activities of GPx due to exercise were maintained at pre-exercise levels after 4 weeks of supplementation (Table 6.16, and 6.17; Fig 6.120-21).

6.5. DISCUSSION

A considerable body of research focused on the beneficial effects of soy protein on human health. It has been suggested that the amount of soy protein intake may influence the low incidence rate of cardiovascular diseases in Asians (Adlercreutz et al., 1993). Soy protein contains phytonutrients that may play a key role in the prevention of and treatment of diseases. These are compounds such as isoflavones, saponins, fiber, and the amino acid composition of the protein. Isoflavones, especially, have drawn a good deal of attention because of their antioxidant activity and anticarcinogenic potential. The results from most published studies that investigated the antioxidative activity of isoflavones, especially genistein, were based on in vitro models and animal models (Bakhit and Potter, 1995; Kerry and Abbey, 1998; Trieu et al., 1999). The current study is the first one to investigate in vivo antioxidative activity of isoflavones from high-genistin extract and their effect on oxidative stress initiated by intensive exercise. Isoflavones diminished pre-exercise plasma MDA; however, they did not prevent the increase of plasma MDA due to exercise. With regard to antioxidant enzymes, HGI increased pre-exercise SOD activity; however, they did not diminish the increase of activity due to exercise. Although isoflavone supplementation did not modulate GPx and CAT activities, they maintained the decreased GPx activities due to exercise. Isoflavones did not provide antioxidative protection against oxidative stress initiated by intensive exercise; however, they exerted their antioxidant activity in humans under normal physiological conditions.

Isoflavones have been shown to act as antioxidants in an in vitro model (Bakhit and Potter, 1995; Kapiotis et al., 1997; Kerry and Abbey, 1998). However, their in vivo antioxidative activity counteracting oxidative stress initiated by any means was not fully explored in humans. It has been recognized and evidenced that exercise can dramatically increase ROS production (Alessio, 1993; Davies et al., 1982; Ji, 1995b), leading to oxidative
stress in the body. In the current study, oxidative stress induced by 30 minutes of 80% VO$_2$pk exercise has been discussed in Chapter 5. Briefly, exercise increased SOD activity and decreased GPx activity in red cells. As to glutathione homeostasis, exercise decreased blood GSH, TGSH, and GSH/TGSH ratio, and increased blood GSSG. Uric acid concentration in plasma was increased by exercise; however, vitamin C concentration remained the same as before exercise. Total antioxidant power in plasma determined by the FRAP method was significantly elevated, and most of the increased FRAP value was due to increased uric acid after exercise. Following the changes of antioxidant defenses challenged by exercise, in vivo product of lipid peroxidation (MDA) in plasma was significantly increased, while in vivo and in vitro LDL-MDA was not changed. Therefore, the oxidative stress initiated by exercise is a good approach to explore the mechanism by which isoflavones may exert their functionality in the body such as antioxidant.

The growing concerns for general health, chronic disease prevention, and aging have fueled consumer interest in phytonutrients because of their curative, preventative, and nutritive value (Guhr and Lachance, 1997). Phytonutrients have been demonstrated to have functionalities in antioxidation, enzyme modulation, cell proliferation, and apoptosis (Balentine et al., 1999). Brandi (1997) reported that soy isoflavones might play the major beneficial role of soy in lowering the incidence of diseases. Therefore, increased soy isoflavone consumption may contribute to low incidence of cardiovascular diseases and cancers.

There are many varieties of isoflavones extracted from soy protein, especially daidzein and genistein. These two isoflavones and their corresponding glycosylated forms are the major isoflavones in soy. Most isoflavones in soy products and blood circulation are present in the conjugated forms. A dramatic rise in plasma isoflavone concentrations was observed after soy protein supplementation to a typical Western diet, reaching 901±245 nmol/L for genistein and 498±102 nmol/L for daidzein (Gooderham et al., 1996). Adlercreutz et al. (1993) also observed that the average genistein concentration in plasma in Japanese men is 86.9 ng/ml, 44-fold as in Finnish men. In agreement with Gooderham’
study, 150 mg isoflavones led to a dramatic increase in plasma genistein and daidzein in this study.

Lichtenstein (1998) reported that a variety of factors influence the bioavailability of soybean isoflavones. Xu et al. (1994) and Zhang et al. (1999) suggested that gut microflora play an important role in the bioavailability of isoflavones in humans. Moreover, plasma genistein concentrations vary even though the same amount of genistein was consumed in humans. Large variation of genistein and daidzein concentrations in plasma in the present study also indicated that factors in foods or intestinal environment probably influence the isoflavone bioavailability. Apparently, the bioavailability of isoflavones in some humans may be relatively low because of the varied extent of the degradation of conjugated isoflavones into aglycone forms for absorption by gut microflora.

In another study from our laboratory (Chapter 3), exhaustive exercise doubled genistein concentrations in the liver, muscle, and plasma of rats fed high-genistin diet for 4 weeks. The results of the present study were in agreement with our rat study that 30 minutes of 80%VO₂pk exercise significantly elevated plasma daidzein and genistein concentrations. However, future work is needed to determine where genistein and daidzein are stored and how they are redistributed in the body during exercise. Since Chang et al. (2000) noticed that genistein is stored in other tissues other than the liver, such as the mammary gland, prostate, testes, ovaries, thyroid gland, and brain, it could be hypothesized that isoflavones might be moved from these tissues into the circulation during exercise.

Isoflavones have a potential to reduce the risk for cardiovascular disease by inhibiting LDL oxidation because isoflavones have multiple hydroxyl groups which act as effective antioxidants by donating hydrogen atoms from their phenolic hydroxyl groups to peroxyradicals (Tikkanen et al., 1998; Wang, 2000). Genistein with the highest antioxidant characteristic among isoflavones has been shown to prevent LDL oxidation initiated by oxidizing agents (cupric ions or azo-induced oxidants) in vitro (Bakhit and Potter, 1995; Kapiotis et al., 1997; Kerry and Abbey, 1998) in a concentration-dependent manner. LDL
from the serum of rats fed a genistein-rich soy protein diet for 3 weeks showed a decrease in LDL oxidation (Anderson et al., 1998); and lag time of LDL oxidation mediated by copper ion was significantly increased in humans fed soy bars (Tikkanen et al., 1998). The results from animal and human studies indicated that isoflavone supplementation into food can boost antioxidant capacity. However, it has to be mentioned that most of the main isoflavonoids of soy, genistein and daidzein, are conjugated in the liver and then released into the circulation. Furthermore, only minute amounts of unmodified isoflavone, corresponding to approximately one isoflavone molecule/500 LDL molecules, were found in LDL (Tikkanen et al. 1998; Yamakoshi et al., 2000). In contrast to the reduction of *in vivo* pre-exercise plasma MDA after 4-week HGI supplementation, pre-exercise LDL-MDA amounts measured *in vivo* and *in vitro* were unaffected. This result was also supported by data from LDL migration on gel electrophoresis (data not shown). It could not be understood why isoflavone administration in the our study did not have antioxidative protection on LDL. It has been suggested that plasma is a more suitable medium than LDL in which to study lipid peroxidation (Kristiina et al., 1997). This is because plasma contains the full spectrum of lipoproteins. Consequently, in this study, LDL-MDA was not affected by isoflavone supplementation, however *in vivo* pre-exercise plasma MDA was significantly decreased after 4-week HGI supplementation. Such results are in agreement with a previous study performed in our laboratory on rats fed a high-genistin isoflavone extract (Chapter 4). It can be speculated that isoflavones may not influence the extent of LDL lipid peroxidation under normal physiological conditions because they are not incorporated in the LDL particle. However, there is the possibility that isoflavones provide antioxidative protection to other lipoproteins and free fatty acids besides LDL.

*In vivo* plasma MDA was decreased by 4-week HGI supplementation in our subjects under normal physiological conditions; and it was increased by submaximal exercise. However, isoflavones in the circulation could not inhibit the increase of *in vivo* plasma MDA after the second submaximal exercise. These results indicated that isoflavones might not be a potent antioxidant in the circulation because predominant forms of isoflavones are conjugated. Mitchell et al. (1998) have reported that the isoflavones were relatively poor
hydrogen donors compared with the other estrogenic-like compounds such as kaempferol and coumestrol; thus, they suggested that the biological activity of genistein is more likely linked to its estrogenic property. Furthermore, the circulating forms of isoflavones are predominantly glucuronidated on the 7 position of A ring, thus removing a hydroxyl group which is needed to execute their antioxidant activity (Mitchell et al., 1998), likely resulting in a decreased antioxidant activity. Kapiotis et al. (1997) also observed that genistin which loses the hydroxyl group from the same position as glucuronidated genistein was less active in inhibiting LDL oxidation *in vitro* (Kapiotis et al., 1997). Therefore, our results of *in vivo* plasma MDA suggest that isoflavone supplementation could enhance antioxidant defense against free radical attack by some means under normal physiological conditions; isoflavones in the current supplemented dose and for a short period of 4 weeks may not have sufficient antioxidative capability to diminish the oxidative stress initiated by intensive exercise.

GSH is the most abundant non-protein thiol source in the cell and serves multiple functions in protecting tissues from oxidative damage and keeping the intracellular environment in the reduced state (Meister and Anderson, 1983). GSH reduces hydrogen- and organic-peroxides via a reaction catalyzed by GPx; and it serves as a scavenger of hydroxyl radical and singlet oxygen (Niki et al., 1985). Furthermore, studies have demonstrated that increased blood oxidized glutathione (GSSG) and reduced GSH/TGSH ratio are indices of oxidative stress (Stephen et al., 1995; Erhola et al., 1997; McIntyre et al., 1997). Increased total GSH concentration and elevated expression of gamma-glutamylcysteine synthetase mRNA, the rate-limiting enzyme for GSH synthesis, was observed when 1 micromolar genistein was incubated with vascular smooth muscle cells of rats (Mizutani et al., 2000). Furthermore, Appelt and Reick (1999) observed that 810 PPM soy isoflavones fed to rats led to increased GSH and decreased GSSG concentrations in plasma. In a rat study conducted in our laboratory (Chapter 4), HGI supplementation increased the ratio of GSH/TGSH, indicating that HGI supplementation partially protects the body against oxidative insults. In agreement with Mizutani et al. (2000), whole blood TGSH increased after a 4-week isoflavone supplementation. Increased pre-exercise GSSG in the HGI and placebo group unexpectedly observed after 4-week treatment was not understood.
Isoflavones led to greater decreases of GSH and TGSH between pre- and post-exercise, however, they did not result in any significant change in difference of GSSG between pre- and post-exercise. Greater decreases of post-exercise blood TGSH and GSH in the HGI group suggested that isoflavones in the circulation might promote the mobilization of GSH from blood to other tissues. Further research will be necessary to explore whether isoflavones may accelerate the mobilization of glutathione from the circulation into tissues. Furthermore, increased GSSG by submaximal exercise, which was not prevented by isoflavone supplementation, indicated that isoflavones in the circulation are not effective as an antioxidant and do not prevent oxidative stress on the glutathione system.

Vitamin C, also one of the water-soluble antioxidant, was not influenced by isoflavone administration. Uric acid, the product of purine metabolism and a strong antioxidant, was also not affected. The pre-exercise FRAP value was significantly higher in the HGI group. Benzie and Strain (1996) suggested that uric acid and ascorbic acid contributed 60 and 15% of FRAP value, respectively. Therefore, the pre-exercise isoflavone antioxidant contribution to FRAP value may be not as significant as uric acid and ascorbic acid. The increase of pre-exercise FRAP values can be partially attributed to the dramatic increase of isoflavone concentrations from dietary isoflavone supplementation.

The significantly enhanced differences of antioxidant power in plasma (FRAP) between pre- and post-exercise in both groups after 4 week treatment demonstrated that isoflavones may not be directly involved in antioxidation in the circulation. Four-week isoflavone administration resulted in dramatic increases of plasma genistein and daidzein and exercise further elevated their plasma levels higher than those before exercise. However, FRAP value after the second exercise did not reflect an effect of the increase of plasma isoflavones. Our results suggested that an increased FRAP value after the second exercise might be attributed to other antioxidants such as vitamin E whose increase in plasma after exercise has been observed (Pincemail et al., 1988; Vasankari et al., 1997) and uric acid.
In addition to the effects of isoflavones on non-enzymatic antioxidants, it has been noticed that genistein could modulate antioxidant enzyme activities in tissues and red blood cells. Dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased the activities of antioxidant enzymes in skin and small intestine of SENCAR mice (Cai and Wei, 1996). In contrast, Breinholt et al. (1999) observed that dietary genistein administration at 0.1 g/kg body weight for 2 weeks consistently inhibited the activity of GR, CAT, SOD, and GPx in RBC of female rats, while there was no effect of genistein on liver antioxidant enzymes. Furthermore, GPx and CAT enzyme activities in erythrocyte were not affected by dietary HGI supplementation in a rat study conducted in our lab (Chapter 3), while SOD activity was decreased. Hsu et al (2001) found that 150 mg/d isoflavone supplementation for 6 months did not result in any difference of erythrocyte antioxidant enzyme activities of postmenopausal women. Our results showed that isoflavone supplementation increased pre-exercise SOD activity in red blood cells, but had no effect on CAT and GPx. The effect of isoflavones on antioxidant enzymes may have tissue- and species-specific characteristic. Furthermore, the varied doses administered to animals may have led to discrepant results in the different studies. There was no reasonable theory to simultaneously explain increased or decreased enzyme activities. The most appropriate explanation for the current study was that isoflavones, through unknown mechanisms, enhanced pre-exercise SOD activity that was not up-regulated by oxidative stress because GPx activity, decreased by the oxidative stress initiated by exercise, maintained normal level. In addition, pre-exercise plasma MDA in vivo was decreased by HGI supplementation. Therefore, isoflavone administration for 4 weeks enhanced total antioxidant capability in red blood cells through the increased SOD activity.

In the present study, increased erythrocyte SOD activity after exercise reflected the increased ROS production. Isoflavones may not directly participate in the reduction of superoxide, therefore, enhanced superoxide generation due to exercise will still lead to the increase of SOD activity in red blood cells. Regarding GPx activity in red blood cells, our results agreed with our previous exercised rat study (Chapter 3) in which enzyme activity was decreased due to exercise, and maintained as normal values by isoflavone
supplementation. Leeuwenburgh and Ji (1995) reported that severe GSH depletion resulted in a significant down-regulation of liver GPx. The reduction of blood GSH, thus, might inhibit erythrocyte GPx activity during and/or after exercise. Isoflavones increased blood TGSH before exercise. This increased TGSH amount could provide sufficient substrate for GPx reaction, and further maintain normal level of GPx activity.

6.6. CONCLUSIONS

Even though isoflavones are considered to be effective antioxidants, an assumption predominantly based on in vitro studies, very little is known about their actual in vivo capacity to function as antioxidants. Furthermore, most studies conducted previously used aglycone isoflavones, other than conjugated isoflavones which are the major compounds existing in the circulation and tissues. Therefore, the interpretation of in vivo results via in vitro data must be taken with precaution. Our study is the first one to test the effect of dietary isoflavone supplementation as a possible defense mechanism against oxidative stress resulting from intensive exercise in healthy young men. We investigated how isoflavones exert their antioxidative activity in vivo, and whether isoflavones are directly involved in the antioxidant defense against exercise induced oxidative stress.

Isoflavone supplementation at 150 mg/d led to a dramatic increase of plasma genistein and daidzein concentrations; and 80% VO$_2$pk exercise for 30 minutes further enhanced concentrations of genistein and daidzein, which might have been mobilized from other tissues during exercise. Four-week isoflavone administration under normal physiological conditions led to decreased plasma MDA through their influence on blood TGSH and SOD activities, but not directly through their antioxidative capability to reduce free radicals or lipid peroxides. Isoflavones may increase blood TGSH by stimulating gamma-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis. The mechanism by which isoflavones activated SOD activity in red blood cells was not understood.

In vivo and in vitro LDL-MDA both before and after exercise was not influenced by isoflavone supplementation, probably reflecting minimum isoflavone presence in the LDL particle. The increase of isoflavones after submaximal exercise did not inhibit the increase of
plasma MDA due to exercise. However, isoflavone supplementation restored decreased GPx activity to normal level, partially elevating antioxidant defense capability. The reason why isoflavones could modulate enzyme activity was not understood. Furthermore, mobilization of GSH by isoflavones might augment total antioxidant power in active tissues, such as skeletal muscle.

Our results demonstrated that isoflavones have antioxidant activity in vivo under normal physiological conditions in healthy young men. Isoflavones also normalized the decrease of antioxidant enzyme activities due to exercise. However, isoflavones may not be potent enough to decrease all the parameters of oxidative damage due to exercise.

6.7. REFERENCES


Table 6.1. Characteristics of subjects.

<table>
<thead>
<tr>
<th></th>
<th>HGI</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>Age, years</td>
<td>15</td>
<td>20.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>15</td>
<td>76.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;pk, ml/min/kg</td>
<td>15</td>
<td>40.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Means in the same column without the same letter were different, using t-test, P<0.05.*
Table 6.2. Dietary intake before two bouts of submaximal exercise.

<table>
<thead>
<tr>
<th></th>
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<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Week 4</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Calorie, Kcal/d</td>
<td>15</td>
<td>2633&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein, g/d</td>
<td>15</td>
<td>110.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHO, g/d</td>
<td>15</td>
<td>348.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fat, g/d</td>
<td>15</td>
<td>91.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fiber, g/d</td>
<td>15</td>
<td>20.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin A, RE</td>
<td>15</td>
<td>1314&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin C, mg</td>
<td>15</td>
<td>169.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E, mg</td>
<td>15</td>
<td>8.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>ab</sup>Means in the same group with different letters were significantly different between exercise 1 and 2, analyzed by a paired t-test, P<0.05.
Table 6.3. Plasma MDA in subjects before and after supplementation\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>1.02*</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>0.88</td>
</tr>
</tbody>
</table>

\(^{ab}\)Means of difference with different letters were significantly different, P<0.05.

*HGI supplementation significantly decreased MDA, P<0.05.

\(^1\)Concentration was expressed as micromolar.
Figure 6.2. Plasma MDA. Means with different letters were significantly different, $P<0.05$. 
Figure 6.3. The difference of plasma MDA between pre- and post-exercise. Means with different letters were significantly different, P<0.05.
Table 6.4. LDL-MDA in subjects before and after supplementation\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th></th>
<th>Week 4</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Diff</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>0.078</td>
<td>0.011</td>
<td>0.066</td>
<td>0.009</td>
<td>-0.012</td>
<td>0.071</td>
<td>0.008</td>
<td>0.075</td>
</tr>
<tr>
<td>Placebo</td>
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<td>0.082</td>
<td>0.008</td>
<td>0.079</td>
<td>0.008</td>
<td>-0.003</td>
<td>0.069</td>
<td>0.008</td>
<td>0.074</td>
</tr>
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\(^1\)Concentration was expressed as µmol/g protein.
Figure 6.4. LDL malondialdehyde in subjects before and after supplementation.
Table 6.5. *In vitro* LDL-MDA from subjects before and after supplementation\(^1\).

<table>
<thead>
<tr>
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<th>Week 0</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>HGI</td>
<td>53.2</td>
<td>3.5</td>
<td>44.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Placebo</td>
<td>54.8</td>
<td>3.4</td>
<td>51.8</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^1\)Concentration was expressed as microgram/ml.
Figure 6.5. *In vitro* LDL-MDA in subjects before and after supplementation.
Table 6.6. Blood TGSH in subjects before and after supplementation\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th></th>
<th>Week 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>1120*</td>
<td>49</td>
<td>1084</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>1146</td>
<td>59</td>
<td>988</td>
</tr>
</tbody>
</table>

*HGI supplementation increased blood TGSH, P=0.01.

\(^{ab}\)Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.

\(^1\)Concentration was expressed as nmol/ml.
Figure 6.6. Total glutathione concentration in whole blood. Means with different letters were significantly different, P<0.05.
Figure 6.7. The difference of total glutathione between pre- and post-exercise. Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.
Table 6.7. Blood GSH in subjects before and after supplementation\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th></th>
<th>Week 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>HGI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>15</td>
<td>864</td>
<td>49</td>
<td>706</td>
<td>54</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>854(^*)</td>
<td>64</td>
<td>650</td>
</tr>
</tbody>
</table>

*Time led to GSH decrease, \(P<0.0001\).

\(^{ab}\)Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), \(P<0.05\).

\(^1\)Concentration was expressed as nmol/ml.
Figure 6.8. 2 Glutathione concentration in whole blood. Means with different letters were significantly different, P<0.05.
Figure 6.9. The difference of glutathione in whole blood between pre- and post-exercise. Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.
Table 6.8. Blood GSSG in subjects before and after supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 4</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>128*</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>146*</td>
</tr>
</tbody>
</table>

*Time significantly led to the increase of pre-exercise GSSG in both groups, P<0.0001.

Concentration was expressed as nmol/ml.
Figure 6.10. Oxidized glutathione in whole blood. Means with different letters were significantly different, P<0.05.
Table 6.9. Blood GSH/TGSH ratio in subjects before and after supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>0.77*</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>0.74*</td>
</tr>
</tbody>
</table>

*Time significantly led to the decrease of pre-exercise ratio in both groups, P<0.001.
Figure 6.11. GSH/TGSH ratio in whole blood. Means with different letters were significantly different, P<0.05.
Table 6.10. Plasma ascorbic acid in subjects before and after supplementation\textsuperscript{1}.

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th></th>
<th>Week 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Diff</td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>6.85*</td>
<td>0.68</td>
<td>6.87</td>
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<tr>
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<td>15</td>
<td>6.26*</td>
<td>0.57</td>
<td>6.21</td>
</tr>
</tbody>
</table>

*Time significantly led to the decrease of pre-exercise AA in both groups, P<0.005.

\textsuperscript{1}Concentration was expressed as microgram/ml.
Figure 6.12. Plasma ascorbic acid in subjects before and after supplementation. Means in the same diet with different letters were significantly different, P<0.05.
Table 6.11. Plasma total ascorbic acid in subjects before and after supplementation\(^1\).

<table>
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<tr>
<th></th>
<th>Week 0</th>
<th></th>
<th>Week 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>HGI</td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.88*</td>
<td>0.80</td>
<td>6.81</td>
</tr>
<tr>
<td>Placebo</td>
<td>15</td>
<td>6.22*</td>
<td>0.57</td>
<td>6.33</td>
</tr>
</tbody>
</table>

\(^*\)Time significantly led to the decrease of pre-exercise TAA in both groups, \(P<0.01\).

\(^1\)Concentration was expressed as microgram/ml.
Figure 6.13. Plasma total ascorbic acid in subjects before and after supplementation. Means in the same diet with different letters were significantly different, P<0.05.
Table 6.12. Plasma AA/TAA ratio in subjects before and after supplementation.

<table>
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<tr>
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<th></th>
<th>Week 4</th>
<th></th>
<th></th>
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<tbody>
<tr>
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<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>HGI</td>
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<td>1.01</td>
<td>0.02</td>
<td>1.01</td>
<td>0.01</td>
<td>0.97</td>
<td>0.02</td>
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<tr>
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<td>1.01</td>
<td>0.01</td>
<td>0.98</td>
<td>0.02</td>
<td>-0.03</td>
<td>0.96</td>
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225
Figure 6.14. Plasma AA/TAA ratio in subjects before and after supplementation.
Table 6.13. Plasma uric acid in subjects before and after supplementation\(^1\).

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<tr>
<th></th>
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<th></th>
<th></th>
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<td>Post-ex</td>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Diff</td>
<td>Mean</td>
</tr>
<tr>
<td>HGI</td>
<td>15</td>
<td>54.8</td>
<td>3.2</td>
<td>58.3</td>
<td>3.1</td>
<td>3.5</td>
<td>54.6</td>
</tr>
<tr>
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<td>53.3</td>
<td>2.0</td>
<td>55.5</td>
<td>2.0</td>
<td>2.2</td>
<td>52.4</td>
</tr>
</tbody>
</table>

\(^1\)Concentration was expressed as microgram/ml.
Figure 6.15. Uric acid concentration in plasma.
Table 6.14. Plasma FRAP in subjects before and after supplementation.  

<table>
<thead>
<tr>
<th>n</th>
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<th>Mean</th>
<th>SE</th>
<th>Diff</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
<th>Diff</th>
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<td>12</td>
<td>15</td>
<td>10</td>
<td>87^-</td>
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<td>15</td>
<td>78^a</td>
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<td>886^-</td>
<td>6</td>
<td>8</td>
<td>72^a</td>
<td>7</td>
<td>1118</td>
</tr>
</tbody>
</table>

^-Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.

-^Time significantly led to the increase of pre-exercise FRAP in both groups, P<0.002.

^-Concentration was expressed as microgram/ml.
Figure 6.16. Plasma FRAP in subjects before and after supplementation. Means with different letters were significantly different, P<0.05.
Figure 6.17. The difference of plasma FRAP between pre- and post-exercise. Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.
Table 6.15. Erythrocyte SOD activity in subjects before and after supplementation\(^1\).

<table>
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<th></th>
<th>Week 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>HGI</td>
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<td>4.67*</td>
<td>0.20</td>
<td>5.34</td>
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<td>5.00</td>
<td>0.28</td>
<td>5.91</td>
</tr>
</tbody>
</table>

\(^*\)HGI supplementation increased week 4 pre-exercise activity, \(P=0.006\).

\(^1\)Activity was expressed as U/g Hb.
Figure 6.18. Superoxide dismutase activity in erythrocytes. Means with different letters were significantly different, P<0.05.
Figure 6.19. The difference of SOD activity in erythrocytes between pre- and post-exercise.
Table 6.16. Erythrocyte GPx activity in subjects before and after supplementation\textsuperscript{1}.

<table>
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<td>Mean SE</td>
<td>Diff Mean</td>
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<td>262 13</td>
<td>241 17</td>
<td>-21\textsuperscript{ab} 297</td>
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</table>

\textsuperscript{a,b}Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.

\textsuperscript{1}Activity was expressed as mmol/s/mg Hb.
Figure 6.20. Glutathione peroxidase activity in erythrocytes in subjects before and after supplementation.
Figure 6.21. The difference of glutathione peroxidase activity between pre- and post-exercise. Means with different letters were significantly different, using Tukey’s HSD procedure (Sall et al., 2001), P<0.05.
Table 6.17. Erythrocyte CAT activity in subjects before and after supplementation$^1$.

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<td>2.5</td>
<td>-1.6</td>
<td>51.3</td>
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$^1$Activity was expressed as U/mg Hb.
Figure 6.22. Catalase activity in erythrocytes in subjects before and after supplementation.
CHAPTER 7

CONCLUSIONS

The difference between Western and Asian diets has been suspected to result in the low incidence of diseases such as cardiovascular diseases and some cancers. Soy consumption by Asian peoples may contribute to this phenomenon because of its component phytonutrients such as isoflavones, saponins, and fiber. Isoflavones are considered to be antioxidants because of the multiple hydroxyl groups in their phenolic rings. Therefore, isoflavones may decelerate and/or prevent the development of diseases via their antioxidant activity. In addition, they are known to induce weak estrogenic and/or anti-estrogenic effects because of their structure similarity to estrogen whose activity has been demonstrated to decrease or retard the incidence of cardiovascular diseases and some of the hormone-related cancers.

Although isoflavones have been described as antioxidants due to their protective action against food spoilage by oxidation, and according to results predominantly based on \textit{in vitro} studies, very little is known about their actual \textit{in vivo} capability to function as antioxidants. Furthermore, most \textit{in vitro} studies conducted previously used aglycone isoflavones, rather than their conjugated isoflavones which are the major compounds existing in the circulation and tissues. Therefore, the interpolation of the antioxidative activity of isoflavones from \textit{in vitro} data to \textit{in vivo} models must be taken with precaution. Our first rat study (Chapter 3) and our human study (Chapter 6) are the first ones to test isoflavone defense against the oxidative challenge initiated by intensive exercise. These experiments were designed to investigate whether isoflavones exert their antioxidative activity \textit{in vivo} and whether they directly involved in the antioxidative defense against free radical attack. The second rat study (Chapter 4) is also the first one to investigate the dose effect of isoflavones on exerting antioxidant activity \textit{in vivo}. 

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Whether isoflavones have antioxidative activity in vivo is determined by their bioavailability in both animals and humans. The results of the second rat study (chapter 4) showed that there was a dose-dependent response to the bioavailability of isoflavones in rats. A diet containing 522 PPM high-genistin isoflavone extract (HGI), (250 PPM of genistein compounds, significantly accumulated genistein in the liver and blood, while 1045 PPM HGI (500 PPM total genistein) significantly accumulated genistein in muscle. These results suggested that the dose of administration to animals was important for bioavailability of isoflavones. Another variable which influences isoflavone bioavailability is the presence of gut microorganisms, which are needed to cleave the beta-linkage of the glycone isoflavone forms. Some humans and rats may not have microorganisms, resulting in none or low isoflavone accumulation in the body.

The accumulated genistein was redistributed among different tissues after exercise. The liver, muscle, and plasma genistein amounts in the rats (Chapter 3) were enhanced after exhaustive exercise; and plasma genistein and daidzein concentrations in humans (Chapter 6) were increased after 30 minutes of 80%VO2pk exercise. The increased isoflavones in tissues were most probably mobilized from other tissues such as the brain, lung, and adrenal glands, leading to increased isoflavone concentrations in sites where it is most needed to defend against oxidative stress. Alternatively, reduced enterohepatic circulation due to diminished blood flow to the liver when may also decrease the excretion of isoflavones, resulting in increased isoflavone tissue levels.

It has been reported in the literature that isoflavones increase in vitro TGSH (GSH+GSSG) concentration and elevate the expression of gamma-glutamylcysteine synthetase mRNA, the rate-limiting enzyme for GSH synthesis, as well as increased GSH concentration and decreased GSSG concentration in the plasma. Similarly, HGI supplementation in rats (Chapter 4), under normal physiological conditions, increased the GSH/TGSH ratio. HGI supplementation in humans led to increased blood TGSH (Chapter 6). Isoflavones did not prevent the increase of blood GSSG after exercise, and led to a significant decrease of GSH and TGSH, indicating that circulating isoflavones may not be
directly involved in antioxidation during and immediately after exercise. Significant decrease of blood TGSH and GSH by exercise in the HGI group (Chapter 6) suggested that isoflavones in the circulation may promote the mobilization of GSH from blood to tissues involved in physical activity. Therefore, it can be concluded that isoflavones modulated glutathione homeostasis under normal physiological conditions. As to the effect of isoflavones on glutathione homeostasis under strenuous exercise, further work will be necessary to explore whether and how isoflavones accelerate the mobilization of glutathione from the circulation into the tissues.

The modulation of antioxidant enzymes by means of isoflavones in the tissues and blood of rats and mice have been reported in literature. However, conflicting results from these two animal studies showed possible tissue- and species-specificity of isoflavone effects on antioxidant enzymes. In our rat study (Chapter 3), erythrocyte SOD was decreased by HGI supplementation. However, erythrocyte SOD activity in humans was enhanced by 150 mg HGI supplementation (Chapter 6). It was not clear what mechanism led to such an effect. HGI supplementation did not modulate erythrocyte GPx activity under normal physiological conditions; however, this supplementation significantly prevented the decrease of erythrocyte GPx activity due to the exercise in both rats and humans (Chapter 3 and 6). The same phenomenon was observed in erythrocyte CAT of the rats (Chapter 3). It can only be speculated that HGI supplementation modulated erythrocyte antioxidant enzymes by means of isoflavone effect on glutathione homeostasis.

In agreement with previous in vitro studies, in vivo plasma MDA under normal physiological conditions was decreased in both humans and rats (Chapter 4 and 6). The results of the second rat study showed that the dose of HGI supplementation was associated with significant MDA reduction. However, HGI decreased neither the liver and muscle MDA in rats, nor LDL-MDA in humans. Although isoflavones demonstrate antioxidant activity in humans and rats under normal physiological conditions, they did not exhibit a measurable effect against oxidative challenge initiated by one bout of exercise in rats and humans. It could be concluded that isoflavones can act as antioxidants by increasing
erythrocyte SOD activity and blood TGSH in resting humans, as well as diminish some but not all parameters of oxidative stress initiated by exercise.

In summary, isoflavones provided antioxidant protection against oxidative stress under normal physiological conditions by means of their modulations of glutathione homeostasis and RBC antioxidant enzymes. However, isoflavones may not be potent enough to decrease all the parameters of oxidative damage due to exercise.
CHAPTER 8

FUTURE STUDIES

The consumption of soy protein is suggested as one of factors resulting in relatively low incidence of cardiovascular diseases in Asian population. A considerable body of research has been conducted in vitro and in vivo animal and human models. Soy protein has been claimed by the researchers and the FDA as a possible factor in the reduction of blood cholesterol in hypercholesteremic subjects. However, the exact mechanism in this scenario is still elusive. In addition to its hypocholesteremic effect, the antioxidant activity of soy isoflavones also have been investigated in vitro and in vivo animal and human models. However, it is premature to conclude that isoflavones act as strong antioxidants in vivo. Furthermore, the mechanism by which isoflavones execute their antioxidative functions in vivo is not completely explored. The results from in vitro studies showed that genistein inhibited oxidation induced by oxidants; and the results of animal and human studies showed that isoflavones could modulate antioxidant enzymes of tissues and decrease LDL susceptibility to oxidants. Our results showed that isoflavones may exert their antioxidant activity through their influence on glutathione homeostasis and antioxidant enzymes, especially when the body faces the oxidative stress initiated by exercise, indicating that they may not have direct antioxidative capability against free radicals in vivo. Thus, more questions were evoked by the results of these studies and are listed below.

Blood glutathione homeostasis is improved by isoflavone administration in rats and humans. The liver is the major site of synthesis of glutathione. However, our results did not directly investigate whether there was a correlation between the liver and blood glutathione concentrations. Furthermore, the mechanism by which glutathione synthesis is enhanced also needs to be explored. Is there a dose-response of isoflavone on glutathione synthesis in the liver? The hypothesis of this future study is that isoflavone administration will simultaneously increase the liver and blood glutathione in rats; there will be a positive correlation of glutathione concentrations between the liver and blood; and increased
glutathione amounts by isoflavone administration will be as a result of enhanced gamma-glutamylcysteine synthetase activity in the liver.

Isoflavones modulated antioxidant enzyme activities in red blood cells and other tissues of sedentary and exercised animals and humans. How isoflavones modulate enzyme activities must be elucidated. There are two phases of modulation of enzyme activities, direct modification of enzyme structure and increased synthesis of enzyme protein. The aforementioned question can be resolved by the quantitation of gene expression of antioxidant enzyme DNA in cells such as in the liver, skeletal muscle, and lymphocytes in animal model. The hypothesis of this study is that isoflavone administration in rats will increase mRNA amounts of antioxidant enzymes in the tissues, leading to increased enzyme activities.