THE EFFECT OF THE FLAVONOIDS QUERCETIN AND GENISTEIN ON THE ANTIOXIDANT ENZYMES Cu, Zn SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE REDUCTASE IN MALE SPRAGUE-DAWLEY RATS

by

ANNETTE CAIRNS GOVERNO

(Under the Direction of Joan G. Fischer)

ABSTRACT

Quercetin (QC) and genistein (GS) are phytochemicals found in fruits and vegetables. These compounds may exert protective effects by altering antioxidant enzyme activities. The objective of the study was to examine the effects of QC and GS supplementation on the activities of the antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GSHPx), and Cu, Zn superoxide dismutase (SOD) in liver, and SOD activity in red blood cells (RBC), as well as the Ferric Reducing Antioxidant Potential (FRAP). Male, weanling Sprague-Dawley rats (n=7-8 group) were fed quercetin at 0.3, 0.6 or 0.9g/100g of diet or genistein at 0.008, 0.012, or 0.02g/100g diet for 14d. GS supplementation significantly increased liver GSHPx activity compared to control ($p<0.01$). GS did not significantly alter activities of liver SOD and GR, or RBC SOD. QC did not significantly alter antioxidant enzyme activities in liver or RBC. Neither QC nor GS increased the antioxidant capacity of serum. In conclusion, low levels of GS significantly increased liver GSHPx activity, which may contribute to this isoflavone's protective effects.

INDEX WORDS: Flavonoids, Quercetin, Genistein, Copper Zinc Superoxide Dismutase, Glutathione Peroxidase, Glutathione Reductase

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CHAPTER 1

INTRODUCTION

High fruit and vegetable consumption has been associated with a decreased risk of cardiovascular diseases including ischemic heart disease, stroke and coronary heart disease (Steinmetz & Potter 1991, Joshipura et al. 2001). Epidemiological studies have also shown that consumption of higher levels of fruits and vegetables is consistently associated with a reduced risk of cancer at many sites (Steinmetz & Potter, 1991). High fruit and vegetable intake may also help control diabetes, obesity, high cholesterol and hypertension (World Cancer Research Fund (WCRF) & American Institute for Cancer Research (AICR), 1997). The exact mechanisms by which fruits and vegetables help in the protection against these diseases are not yet understood. There are many beneficial dietary components in fruits and vegetables including vitamins, minerals, fiber and phytochemicals that may be responsible for their health benefits.

Phytochemicals are non-nutrient components found in plants. Flavonoids are one of the many classes of phytochemicals. Quercetin is one of the most common flavonoids, and is present in foods such as apples, onions, tea and berries. Many studies have demonstrated quercetin's beneficial effects on tumor initiation and promotion in animals, DNA damage, protection against hepatic ischemia-reperfusion injury and gastric lesions, and growth of human cancer cells in vitro (Rice-Evans & Packer, 1998; Su et al. 2003; Martin et al. 1998). Quercetin is one of the most effective antioxidants of the flavonoids. It can function directly as an antioxidant, readily quenching free radicals, and indirectly by chelating transition metals (WCRF

& AICR, 1997). Genistein, an isoflavone, is associated with reduced cancer risk. While it is thought that some of genistein's beneficial effects result from its antiestrogenic actions, genistein also inhibits angiogenesis and promotes apoptosis (Stephens, 1997; Fotsis et al. 1993). Studies have likewise shown the benefits of genistein in protecting against oxidative damage (Suzuki et al 2002). Like quercetin, genistein acts directly as an antioxidant by quenching free radicals.

Quercetin and genistein's protection against disease may also include the ability to alter the activities of antioxidant enzymes. Reactive oxygen species (ROS) are formed from oxygen and are necessary in many biological systems. However, due to their high reactivity, high levels of ROS can cause damage to DNA, proteins and lipids, and interfere with cell function, normal signal transduction, cell proliferation and cell metabolism (Halliwell & Gutteridge, 1999; Chance et al. 1979; Schraufstatter, 1986). Three important antioxidant enzymes in the body include copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GSHPx), and glutathione reductase (GR). These antioxidant enzymes work together to defend against oxidative damage by several of the major ROS, including superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2). CuZnSOD removes $O_2^{\bullet-}$ by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (Marklund & Marklund, 1974). GSHPx is important in removing H_2O_2 produced by the dismutation of $O_2^{\bullet-}$ by CuZnSOD. GR, using NADPH as its substrate, is critical for recycling glutathione, making this substrate of GSHPx available for removal of H_2O_2 (Paglia & Valentine, 1967; Figure 1).

Previous studies examining the effects of flavonoids on antioxidant activity have had conflicting results, with some showing increased (Cai & Wei, 1996; Suzuki et al. 2001; Bok et al. 2002; Duarte et al. 2001; Fischer, 2001) and others showing decreased enzyme activity (Breinholt et al. 1999; Rohrdanz et al. 2003) with flavonoid supplementation (Tables 1 & 2).

Results depend on dose, length of study and organ studied. Due to inconsistent results, more studies are needed to identify the possible beneficial effects of genistein and quercetin in disease prevention, their effects on antioxidant enzymes, and the mechanisms involved.

Breinholt et al. (1999) studied the individual effects of six flavonoids, including quercetin and genistein, on antioxidant enzyme activity in rodents. They found that red blood cell (RBC) GSHPx and GR activities were inversely associated with increasing antioxidant potential of the flavonoid administered. The authors suggested that the flavonoids, due to their antioxidant properties, decreased the need for these enzymes (Breinholt et al. 1999). This study tested the hypothesis that the dietary administration of quercetin and genistein would decrease the activity of antioxidant enzymes in the liver and RBC but increase the overall antioxidant capacity of the serum in male Sprague-Dawley rats. The objectives of this study were 1) to test if quercetin and genistein would decrease the activity of the antioxidant enzymes CuZnSOD, GSHPx and GR in the liver, and CuZnSOD in the RBC and 2) to test if genistein and quercetin would increase the total antioxidant capacity of the serum despite causing a decrease in antioxidant enzyme activity.

The major finding of this study was that genistein, supplemented at levels achievable in the human diet, increased the activity of hepatic GSHPx. In addition, doses of quercetin at 0.3%-0.9% of the diet did not affect antioxidant enzyme activity in rats.

Genistein has been shown to be protective against chronic disease in animal models (Rice-Evans & Packer, 1998; Suzuki et al. 2002). Further, elevated GSHPx activity may be associated with increased protection against chronic diseases such as cancer and cardiovascular disease (Sun et al. 1990; Blankenberg et al. 2003). Increased GSHPx activity may be one of the mechanisms by which genistein protects against chronic disease.

CHAPTER 2

LITERATURE REVIEW

Free Radicals

Free radicals are "any species containing one or more unpaired electrons". Free radicals are highly reactive and many reactions involving these molecules result in the formation of new radical species (Nijveldt et al, 2001). Free radicals can also oxidize molecules in the body through the transfer of electrons from one atom to another (Pietta, 2000). Reactive species include nitric oxide, hydroxyl, superoxide, peroxyl, alkoxyl and sulphur radicals, as well as some non-radicals such as singlet oxygen, hypochlorous acid and peroxynitrite (Halliwell & Gutteridge, 1999). Transition metals such as iron and copper are essential for the synthesis and/or activity of many enzymes and other proteins involved in respiration, oxygen transport and nitric oxide (NO•) formation. However, when in their free state, they are potentially dangerous due to their ability to undergo one-electron transfers. This makes them powerful catalysts of free radical reactions, allowing for conversion of hydrogen peroxide (H_2O_2) to the highly reactive hydroxyl radical as well as other reactive radicals (Halliwell & Gutteridge, 1999).

Many, but not all, free radicals and related reactive species are oxygen-derived (Nijveldt et al, 2001). Reactive oxygen species (ROS) are any oxygen-derived species more reactive than the ground state oxygen molecule (Sun, 1990). ROS include the superoxide ($O_2^{\bullet-}$) and hydroxyl (OH•) radicals, as well as some non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2). ROS such as $O_2^{\bullet-}$, OH•, and H_2O_2 are necessary reactive species in biological systems. They are important in the process of phagocytosis when produced by phagocytes in order to kill

bacteria (de Groot & Rauen, 1998). Also, $O_2^{\bullet-}$ is naturally produced as a result of the electron transport chain, a necessary biological process for the production of adenosine triphosphate (ATP) (Young & Woodside, 2001).

Reactive Oxygen Species and Disease

Although ROS play important biological roles in the body, they are only useful in controlled amounts. Because of their high reactivity, high levels of ROS can cause damage to cells. ROS can damage all types of biomolecules including lipids, proteins, DNA, and carbohydrates. ROS cause lipid peroxidation, which is "the oxidative deterioration of polyunsaturated lipids" (Halliwell & Gutteridge, 1999) and results in impairment of important membrane functions (Thomas, 1994). Enzymes, receptors, and transport proteins are damaged by direct attack on proteins by ROS and by end products of lipid peroxidation. The hydroxyl radical directly attacks sugars, purines and pyrimidines, causing damage to DNA and interfering with DNA repair, replication and transcription (Thomas, 1994; Halliwell & Gutteridge, 1999). Ultimately, ROS can affect gene transcription, cell growth and proliferation, cell metabolism, and signal transduction (Yamasaki & Naus 1996; Sun, 1990; Halliwell & Gutteridge, 1999).

It is believed that excess ROS play a major role in initiating or furthering tissue injury in several major diseases including certain cancers, atherosclerosis, and inflammatory diseases (Sun, 1990; Young & Woodside, 2001; Halliwell & Gutteridge, 1999). Atherosclerosis is the leading cause of death in the U.S. and was responsible for 931,108 deaths in 2003 (American Heart Association, 2003). ROS may contribute to atherosclerosis, stroke, and myocardial infarction. This may occur through ROS oxidation of LDL, leading to the plaque build-up characteristic of atherosclerosis. ROS can weaken arterial plaques, resulting in ischemia when

these plaques detach from the vessel wall and block blood flow (Halpert et al. 1996). Cancer is the second leading cause of death in the U.S. and was responsible for approximately 556, 500 deaths in 2003 (American Cancer Society, 2003). ROS may contribute to all stages of carcinogenesis including tumor promotion, progression and metastasis through direct DNA damage, damage to lipids, proteins, and DNA repair enzymes, and through activation of chronic inflammation and interference with cell to cell communication (Yamasaki & Naus, 1996; Halliwell, 1982; Halliwell & Gutteridge, 1999). ROS have been implicated in many other chronic diseases including hypertension, diabetes, and respiratory, inflammatory, and neurodegenerative diseases (Halliwell & Gutteridge, 1999; Nakazono et al. 1991; Halliwell, 1982).

Superoxide Radical

Superoxide ($O_2^{\bullet-}$) is formed when a single electron is added to the ground-state oxygen molecule, leaving oxygen with one unpaired electron. Superoxide is produced in all aerobes depending upon the oxygen concentration of the environment. Some $O_2^{\bullet-}$ is produced by activated phagocytic cells, cytosolic enzymes, from binding of oxygen to hemoglobin and myoglobin (Haem proteins), and via auto-oxidation reactions. The major source of $O_2^{\bullet-}$ in the body is the electron transport chain (Young & Woodside, 2001; Halliwell & Gutteridge, 1999).

Superoxide is recognized as a major factor in oxygen toxicity. Superoxide may depress energy metabolism in the Krebs cycle, damage proteins involved in signal transduction, and damage enzymes necessary for DNA synthesis. Other superoxide-dependent damage to biomolecules include enzyme inactivation, DNA damage and lipid peroxidation. Superoxide is also able to form other dangerous ROS. The spontaneous dismutation reaction involving $O_2^{\bullet-}$

and $HO_2^{\bullet}$, the protonated form of $O_2^{\bullet-}$, forms H_2O_2 (Aikens & Dix, 1991; Halliwell & Gutteridge, 1999):

$$HO_2\bullet + O_2^{\bullet-} \longrightarrow H_2O_2 + O_2$$

Once H_2O_2 is formed, the highly reactive hydroxyl radical (OH•) can then be formed in the presence of transition metal ions such as iron via the Haber-Weiss reaction (Young & Woodside, 2001):

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$$

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

The protonated superoxide radical can cause peroxidation of peroxisomes and lipoproteins, and can stimulate peroxidation by reacting with pre-formed lipid hydroperoxides (Halliwell & Gutteridge, 1999).

Hydroxyl Radical

The hydroxyl radical is generated by several reactions in the body including the Fenton reaction, in which Fe^{2+} reacts with H_2O_2, and in the reaction of UV radiation with H_2O_2. The hydroxyl radical can be generated from ozone, H_2O_2, hypochlorous acid reacting with $O_2^{\bullet-}$, and may be generated during ethanol metabolism. Hydroxyl radicals are highly reactive and react with the molecules in their immediate vicinity. The hydroxyl radical is produced from exposure

to high-energy radiation, and is responsible for much of the damage done to cellular DNA, proteins and lipids by ionizing radiation. The hydroxyl radical attacks sugars, purines or pyrimidines, generating many end products and causing DNA damage (Thomas, 1994). Finally, OH• can readily initiate lipid peroxidation (Barber & Thomas 1978; Halliwell & Gutteridge, 1999):

$$\mathbf{-CH_2- + OH\bullet \longrightarrow -\dot{C}H- + H_2O}$$

Hydrogen Peroxide

Hydrogen peroxide is a non-radical, a weak oxidizing and reducing agent, and is generally poorly reactive. Despite its relatively poor reactivity, H_2O_2 can be cytotoxic and can cause direct cell damage (Halliwell & Gutteridge, 1999). Several enzymes in the body can generate H_2O_2 including peroxisomal enzymes associated with fatty acid metabolism and cytoplasmic enzymes involved in the oxidation of cell metabolites (Thomas, 1994). Any biological system that generates $O_2^{\bullet-}$ will also produce H_2O_2 by $O_2^{\bullet-}$ dismutation. H_2O_2 inactivates enzymes, oxidizes certain keto-acids and degrades certain haem proteins, releasing iron ions. H_2O_2 leads to DNA damage and strand breakage of DNA, which could lead to mutations or cell death. However, much of the damage from H_2O_2 in cells is likely due to the conversion of H_2O_2 to OH• by transition-metal ions or ultraviolet light (Halliwell & Gutteridge, 1999; Young & Woodside, 2001):

$$\mathbf{H_2O_2 + Fe^{2+}\ (Cu^{+}) \longrightarrow OH\bullet + OH- + Fe^{3+}(Cu^{2+})}$$

$$\mathbf{H_2O_2 \xrightarrow{UV} OH\bullet + OH\bullet}$$

Oxidative Stress

Oxidative stress refers to a serious imbalance between ROS or free radicals and antioxidant defenses. An antioxidant is "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Young et al. 2000). Antioxidants prevent tissue damage by free radicals by scavenging free radicals, preventing their formation, or by promoting their decomposition (Young et al. 2000). The body has a natural antioxidant defense system to protect it from cellular damage by ROS and free radicals (Mates et al. 1999). This antioxidant system includes enzymes that catalyze reactions that convert radicals to more stable, less reactive species. It also includes chain-breaking antioxidants that can donate or receive an electron to or from a radical, transition metal binding proteins, and plasma antioxidants such as uric acid and albumin (Young & Woodside, 2001).

Oxidative stress results from any of four situations: 1) Excess free radical exposure from the environment, 2) insufficient dietary antioxidant intake, 3) disturbance in biochemical systems that generate ROS, or 4) failure in internal protective antioxidant mechanisms such as antioxidant enzyme systems (Halliwell & Gutteridge, 1999). When cells experience mild oxidative stress, the body up-regulates the antioxidant defense system and is often able to restore oxidant/antioxidant balance, thus preventing damage to the cells. Cells are able to alter gene expression to elevate antioxidant defenses or decrease transcription of certain genes in order to restore oxidant/antioxidant balance. When the amount of free radical exposure in the body is not balanced by antioxidant defense systems, damage will result. Oxidative stress can result from inadequate antioxidants, including dietary or internal antioxidants such as enzymes, or from increased production of ROS from exposure to high oxygen concentrations, excessive activation

of natural ROS-producing systems, or by the presence of toxins that produce ROS (Halliwell & Gutteridge, 1999).

Oxidative stress is measured by assessing oxidative damage to molecules such as DNA, lipids and proteins. Several types of measurement of oxidative DNA damage are used. Measuring steady-state damage, damage found in DNA isolated from aerobic cells, reflects the balance between damage to DNA and the activity of DNA repair enzymes. Increased steady-state oxidative DNA damage has been reported in some human cancerous tumors and often occurs during oxidative stress (Musarrat et al. 1996; Halliwell & Gutteridge, 1999). Peroxidation of membrane lipids, lipoproteins or fatty acids can be determined by measuring the net balance between peroxidation and the removal of peroxidation products, or by assessing the overall rate of peroxidation in vivo. Protein damage can be determined by measuring damage to specific amino acid residues or by measuring the balance between oxidative protein damage and the repair or removal of damaged proteins (Halliwell & Gutteridge, 1999).

Antioxidant Defenses

In general, organisms have only enough antioxidant defenses to cope with their normal exposure to oxygen (Halliwell & Gutteridge, 1999). Because many radicals and oxygen-derived species are damaging to cells, aerobes have developed antioxidant defenses in order to survive oxygen exposure. Antioxidant defenses can be induced by exposure to ROS, free radicals, and cellular signal molecules. Certain antioxidant defenses such as enzymes catalytically remove free radicals and other reactive species. Other antioxidants scavenge ROS or minimize the availability of pro-oxidants such as metal ions. The level of antioxidant defense differs between tissues and cell types in a given tissue. For example, extracellular fluids have different

protective antioxidant mechanisms, including different enzymes, than the intracellular environment (Sun, 1990).

Some antioxidants, such as enzymes, are found naturally in the body, while other antioxidants, such as vitamins and phytochemicals, can be obtained in the diet. Three important antioxidant enzymes in the body include copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GSHPx), and glutathione reductase (GR). Cells can tolerate mild oxidative stress, in part due to the up-regulation of antioxidant enzymes. It is well understood that these antioxidant enzymes work together in defending against oxidative damage. For instance, GR is critical for recycling GSH and making this substrate of GSHPx available for removal of H_2O_2. GSHPx is important in removing H_2O_2 produced by the dismutation of $O_2^{\bullet-}$ by CuZnSOD, and overexpression of the CuZnSOD gene has been shown to induce GSHPx activity (Sun, 1990; Figure 1).

There are assays in which total antioxidant activity can be tested. One of the first of these assays was the total (peroxyl) radical trapping antioxidant parameter (TRAP). This assay measures the peroxidation of lipids in body fluids on exposure to azo initiators. TRAP can also be used to compare the antioxidant activities of different molecules (Halliwell & Gutteridge, 1999). Another method is the ferric reducing antioxidant potential assay, or FRAP. This assay measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} at low pH (Benzie & Strain, 1996).

Copper, Zinc Superoxide Dismutase

Cu, Zn superoxide dismutase (CuZnSOD) is an unusually stable protein that contains two protein subunits, each with an active site containing one copper and one zinc ion. It is found in virtually all eukaryotic cells, and is located primarily in the cytosol, but is also present in

lysosomes, nuclei and the space between inner and outer mitochondrial membranes. CuZnSOD is found in high concentrations in the liver of animals (Halliwell & Gutteridge, 1999). CuZnSOD removes $O_2^{\bullet-}$ by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (Marklund & Marklund, 1974):

$$\mathbf{O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{CuZnSOD} H_2O_2 + O_2} \text{ (ground state)}$$

The biological role of CuZnSOD is to scavenge $O_2^{\bullet-}$ and it is essential in defending against $O_2^{\bullet-}$ damage. Gradual exposure of rats to increasing oxygen levels has shown that the ability of rats to adapt to and survive oxygen toxicity is correlated with an increase in CuZnSOD activity (Frank, 1985).

Manganese superoxide dismutase (MnSOD) is a protein that contains manganese at its active site. MnSOD is not as stable as CuZnSOD and is widespread in bacteria, plants and animals. In most animal tissues, MnSOD is almost entirely located in the mitochondria (Fridovich, 1995). The relative activities of MnSOD and CuZnSOD depend on the tissue and species. Increases in MnSOD and CuZnSOD activities have been seen in copper ion and manganese deficiencies, respectively. Cells with no mitochondria, such as erythrocytes, do not contain MnSOD. MnSOD catalyzes virtually the same reaction as CuZnSOD by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (Marklund & Marklund, 1974) as follows:

$$\mathbf{Mn^{3+} + O_2^{\bullet-} \xrightleftharpoons{MnSOD} [Mn^{3+} - O_2^{\bullet-}] \xrightleftharpoons{MnSOD} Mn^{2+} + O_2}$$

$$\mathbf{Mn^{2+} + O_2^{\bullet-} \xrightleftharpoons{MnSOD} [Mn^{2+} - O_2^{\bullet-}] + 2H+ \longrightarrow Mn^{3+} + H_2O_2}$$

Glutathione Peroxidase

Glutathione peroxidase (GSHPx) is made up of four protein subunits, each containing an atom of selenium (Se). GSHPx is found primarily in the cytosol of cells, but is also found in the matrix of mitochondria and in the cell nucleus. It is widely distributed in animal tissues and is in highest concentrations in the liver, kidney and adrenals. Its substrate is reduced glutathione (GSH), which acts as a hydrogen donor. GSHPx removes H_2O_2 by catalyzing its reduction to H_2O, and the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) (Paglia & Valentine, 1974; Chance et al. 1979):

$$\mathbf{H_2O_2 + 2GSH \xrightarrow{GSHPx} GSSG + 2H_2O}$$

GSHPx is a key enzyme of the antioxidant defense system under normal conditions and during oxidative stress (Remacle et al. 1992). GSHPx acts in cooperation with the enzyme catalase to remove H_2O_2 in vivo. GSHPx removes H_2O_2 produced in erythrocytes and by mitochondria, endoplasmic reticulum, or enzymes such as CuZnSOD. Activity of GPx depends upon the availability of GSH. Activities of GSHPx increase with exposure to high oxygen concentrations.

Catalase consists of four protein subunits, each with a ferric haem group at its active site. Catalase is found primarily in peroxisomes, with very little catalase being located in mitochondria. Most aerobic cells contain catalase activity. The highest concentrations in human tissues are in liver, erythrocytes and kidneys (Marklund et al. 1982). Catalase directly catalyzes decomposition of H_2O_2 to ground-state oxygen (Mates et al. 1999):

$$\mathbf{2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2}$$

The normal low levels of H_2O_2 in erythrocytes are mainly taken care of by GPx. However, if the concentration of H_2O_2 is increased, catalase becomes more important in removing H_2O_2. Catalase has a high affinity for H_2O_2, and can achieve a very high rate of H_2O_2 destruction when levels of H_2O_2 are high (Halliwell & Gutteridge, 1999).

Glutathione Reductase

Glutathione Reductase (GR) catalyzes the reaction in which GSSG is recycled back to GSH, thereby maintaining a high ratio of GSH to GSSG in normal cells (Xia et al. 1985; Carlberg & Mannervik, 1974):

$$\mathbf{GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+}$$

Glutathione's importance in antioxidation includes its role as a cofactor for GSHPx, its involvement in ascorbic acid metabolism, and its prevention of protein –SH groups from oxidizing and cross-linking. GSH also chelates copper ions and decreases their ability to release radicals into solution (Sun, 1990; Halliwell & Gutteridge, 1999). Actions of GR depend upon availability of NADPH, mainly derived from the pentose phosphate pathway.

Plant Foods and Disease Risk

For years, researchers have studied the effects of diet on the development and prevention of disease. The National Cancer Institute estimates that one in three cancer deaths are diet related and eight of ten cancers have a nutrition or diet component (American Cancer Society, 2004). For example, high-energy diets, diets high in alcohol and certain foods such as refined sugars

and red meat, may contribute to an increased risk for cancer (American Cancer Society, 2004). Consumption of a diet high in saturated fat and cholesterol may contribute to heart disease (American Heart Association, 2003). Likewise, deficiencies of certain microconstituents of foods such as antioxidants, vitamins, minerals and bioactive compounds have also been associated with increased cancer risk and heart disease (American Cancer Society, 2004; American Heart Association, 2003).

In contrast, diets low in energy and high in certain foods and food components such as fruits, vegetables, whole grains, fiber, vitamins and minerals appear to be protective against cancer (WCRF & AICR, 1997). Epidemiological, cohort and case-control studies have shown that consumption of higher levels of fruits and vegetables (5 servings or more) is associated consistently with a reduced risk of cancer at many sites (Steinmetz & Potter, 1991; Block et al. 1992). A review by Steinmetz and Potter (1991) found certain vegetables, in particular carrots and green leafy and cruciferous vegetables, were associated with a decreased risk of certain cancers including lung, esophageal, laryngeal, oral, pharyngeal and stomach. High fruit and vegetable consumption has also been associated with a decreased risk of cardiovascular diseases including ischemic heart disease, stroke, and coronary heart disease (Joshipura et al. 2001). Further, high fruit and vegetable intake may help control diabetes, obesity, high serum cholesterol levels and hypertension (WCRF & AICR, 1997). The American Heart Association, the American Cancer Society, and the American Institute for Cancer Research recommend at least five to ten servings of fruits and vegetables a day to reduce the risk of cardiovascular disease and certain cancers (Van Duyn & Pivonka, 2000; American Heart Association, 2003; American Cancer Society, 2003). The exact mechanisms by which fruits and vegetables help in the protection against these diseases are not yet known. However, the effects of fruits and

vegetables on cancer prevention may include inhibiting tumor promotion, protecting against oxidative DNA damage, blocking carcinogens from DNA, and facilitating carcinogen metabolism (Steinmetz & Potter, 1991). Diets high in fruits and vegetables can increase the antioxidant capacity of the serum and protect against lipid peroxidation (Miller et al. 1998).

Many dietary components in fruits and vegetables have been studied for possible protective effects against diseases including vitamins, minerals, fiber, and phytochemicals (Steinmetz & Potter, 1991). Phytochemicals are non-nutrient components found in plants. Specific effects of phytochemicals commonly found in fruits and vegetables include enhancement of immune function, reduction of serum cholesterol levels, detoxification of carcinogens, and protection against lipid peroxidation and cellular DNA damage (Van Duyn & Pivonka, 2000; Nijveldt et al. 2001).

Phytochemicals and Flavonoids

Phytochemicals have been associated with the prevention and/or treatment of at least four of the leading causes of death in the U.S.: cardiovascular disease, cancer, diabetes and hypertension (Bloch & Thompson, 1995). Among these are carotenoids, including lycopene, a powerful antioxidant that may help in prostate cancer prevention (Miller et al. 2002) and lutein and zeaxanthin, found in spinach, kale, and turnip greens, which may reduce the risk of lung cancer and age-related macular degeneration (American Cancer Society, 2003; Landrum et al. 2001). Sulfides, found in garlic and onions, may help prevent tumor promotion (Sakamoto et al. 1997), inhibit the growth of bacteria, help lower blood pressure, and strengthen the immune system (American Cancer Society, 2003). Anthocyanins are the components that give red wine, blueberries and red cabbages their intense color. Epidemiological studies have shown that

anthocyanin intake is associated with improvement of visual functions and a lower risk of cardiovascular disease. There is also evidence that anthocyanins may help in cancer prevention (Hou et al. 2003).

Flavonoids are one of the major classes of phytochemicals. The basic structure of flavonoids includes a 3-carbon ring between two benzene rings. Flavonoids are naturally occurring phenolic compounds found ubiquitously in fruits and vegetables (Van Duyn, et al. 2000). Flavonoids are able to scavenge free radicals and chelate iron (de Groot & Raun, 1998). The antioxidant and the copper-initiated pro-oxidant activities of flavonoids depend upon the number of hydroxyl substitutions in its backbone structure. In general, the more hydroxyl group substitutions, the stronger the radical scavenging activity of a flavonoid. The 3', 4' –dihydroxy substitution at ring B and a keto structure (4-keto, 3-OH) at ring C are responsible for the chelation of metal ions (de Groot, 1997). The degree of hydroxylation and the relative positions of hydroxyl groups are key in determining antioxidant ability of flavonoids (Halliwell & Gutteridge, 1999).

Positive effects of flavonoids include anti-inflammatory, antiallergic, antihemorrhagic and anticarcinogenic activities (Nijveldt et al. 2001; de Groot & Rauen, 1998). For example, flavonoids suppress hyperproliferation of colonic epithelial cells, colon tumor incidence, chemically induced tumors, and the development of squamous cell carcinoma and acute leukemias (Rice-Evans & Packer, 1998).

The positive effects of flavonoids are thought to result primarily from two properties: their antioxidant activity and their effects on certain enzymes (de Groot & Rauen, 1998). Flavonoids are able to quench singlet oxygen and scavenge hydroxyl radical and superoxide anion (de Groot & Rauen, 1998; Rice-Evans & Packer, 1998). Flavonoids undergo reactions with ROS in which

the flavonoids are oxidized, resulting in a more stable, less-reactive radical (Nijveldt et al. 2001). The ability of flavonoids to create a more stable oxygen radical contributes to their potency to act as antioxidants (de Groot & Rauen, 1998) and suppress lipid peroxidation (da Silva et al. 1998; Kerry & Abby, 1998).

Flavonoids affect the catalytic activities of many enzymes. Some flavonoids induce the expression of cytochrome P450 enzymes, but may inhibit or stimulate their metabolic activity (Hodek et al. 2002). Flavonoids inhibit lipoxygenase and cyclooxygenase enzymes, enzymes involved in lipid and lipoprotein metabolism, and enzyme systems associated with cell activation processes (Reed et al. 2002; Hodek et al. 2002; Middleton et al. 1998). Flavonoids also have significant effects on antioxidant enzyme systems (Breinholt et al. 1999). Flavonoids' effects on antioxidant enzymes are not well understood, and results of studies in this area vary, with studies showing both up-regulation and inhibition of antioxidant enzyme activity by flavonoids (Aherne & O'Brien, 1999; Breinholt et al. 1999; De et al. 2000).

Antioxidant vs. Prooxidant Potential

Also to be considered is that antioxidants, despite their protective effects, have the ability to act as pro-oxidants under certain circumstances, and could therefore lead to oxidative damage (Young et al. 2000). The ability of certain antioxidants to react with metal ions in chelation also enables them to act as pro-oxidants (Yang et al. 2001). Antioxidants reduce metal ions, resulting in free radicals (Halliwell & Gutteridge, 1999). For example, ascorbate, an antioxidant, reduces iron and results in the formation of OH•, as shown by the following reaction:

$$Fe^{3+} + \text{ascorbate} \longrightarrow Fe^{2+} + \text{ascorbate}$$

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

These pro-oxidant effects can be seen with many reducing agents in the presence of transition metal ions. The determining factor of pro-oxidation by antioxidants is often the availability of catalytic transition metal ions (Halliwell & Gutteridge, 1999). High levels of iron, copper, or other transition metals in the presence of antioxidants can lead to oxidative damage (Young et al. 2000). Because these reactions can occur in vivo, they are important to consider when supplementing antioxidants or altering antioxidant enzyme levels.

Quercetin

Flavonols are one of the 13 subclasses of flavonoids that are categorized according to their structure. Quercetin is a well-studied flavonol and one of the most abundant flavonoids found in plants (Figure 2). Quercetin is found in high concentrations in onions, broccoli, lettuce, tomatoes, berries, grapes, apple skins, and tea (Elliot et al. 1992; Knight, 1999). Many believe quercetin to be protective against certain diseases. Quercetin inhibits tumor initiation and promotion in animals and DNA damage in HepG2 cells (Rice-Evans & Packer, 1998). Quercetin has been shown to inhibit the growth of cells from various human cancers in vitro, protect against gastric lesions and hepatic ischemia-reperfusion injury (Rice-Evans & Packer, 1998; Martin et al. 1998; Su et al. 2003).

Many of the positive effects of quercetin in chronic disease prevention may be due to its antioxidant activity (Halliwell & Gutteridge, 1999; Rice-Evans & Packer, 1998). Quercetin acts

directly as an antioxidant by scavenging free radicals. It acts indirectly by chelating iron and copper, and by inhibiting H_2O_2 and transition metal-induced lipid peroxidation (da Silva et al. 1998). For example, quercetin has been shown to remove iron from iron-loaded rat hepatocyte cultures and protect against iron-induced lipid peroxidation and hemolysis (Morel et al. 1993; Ferrali et al. 1997). Quercetin's structure contributes to it being one of the most effective antioxidants of the flavonoids. Wang et al. (1999) studied the structural properties of quercetin in relation to its antioxidant activities and found two structural characteristics of quercetin to be crucial for protection against H_2O_2-induced oxidative stress 1) the 3',4'-hydroxyl groups in the B ring and 2) a 2,3-double bond in conjugation with a 4-oxo-group in the C ring. These structural components of quercetin are important determinants of quercetin's antioxidant capacity due to the reaction of the hydroxyl groups with free radicals.

Quercetin may also exert protection by altering the activity of certain enzyme systems. Quercetin induces activities of the phase II enzymes quinone reductase and glutathione-S-transferase, each of which promotes the metabolic deactivation of carcinogens (Halliwell & Gutteridge, 1999; Penn et al. 2003). The body's defense system for protecting cells from damage includes antioxidant enzymes. Numerous animal studies have examined the effects of quercetin on the antioxidant enzymes CuZnSOD, GSHPx, GR and CAT (Table 1). These studies have found conflicting results, however, showing that quercetin increases, decreases or has no effect on the activities of these antioxidant enzymes.

Several studies have shown protection against hepatic ischemia-reperfusion injury and gastric lesions in rats associated with the ability of quercetin to up-regulate SOD and GSHPx (Su et al. 2003; Kahrama et al. 2003). Bok et al. (2002) studied the supplementation of 1 g/kg diet of quercetin for six weeks to high cholesterol-fed rats. Quercetin supplementation increased

hepatic SOD and GSHPx activities. Duarte et al. (2001) treated spontaneously hypertensive and normotensive Wistar Kyoto rats with 10 mg quercetin/kg body weight for five weeks. Hepatic GSHPx activity was significantly decreased in spontaneously hypertensive rats when compared to Wistar Kyoto rats. However, spontaneously hypertensive rats showed significantly increased hepatic GSHPx activity after quercetin supplementation.

In contrast, Breinholt et al. (1999) studied the effects of two weeks gavage administration of 0.1g/kg body weight of various flavonoids on antioxidant enzyme activities in female rats. They found quercetin significantly decreased the activities of red blood cell (RBC) GR and GSHPx in female rats. These decreases in enzymatic activities tended to be more pronounced with increasing antioxidant potential of the different flavonoids tested. The authors hypothesize the antioxidant enzymes in RBC were down regulated by quercetin in response to an improved antioxidant status of the RBC due to the presence of quercetin. Rohrdanz et al. (2003) found quercetin, at a concentration of 5-100 umol, decreased MnSOD, GSHPx and CuZnSOD mRNA expression levels in rat hepatoma H411E cells. Despite this decrease in antioxidant enzyme mRNA expression, only a mild oxidative stress was induced and pretreatment of cells with quercetin protected against oxidative stress from H_2O_2 exposure.

Still, other studies have shown quercetin to have no effect on antioxidant enzyme activities. Coldiron et al. (2002) studied the effects of 14 days intraperitoneal administration of quercetin and coenzyme Q(10) at 10 mg/kg b.w. on oxidative stress in normal and diabetic rats. Hepatic SOD and GSHPx levels were decreased, while renal GSHPx levels were increased in diabetic rats compared to non-diabetic rats. However, administration of quercetin, coenzyme Q(10), or a combination of the two antioxidants did not affect the altered enzyme activity levels in diabetic rats. Galvez et al (1995) tested eight flavonoids, including quercetin, for their

antiperoxidative activities against lipid peroxidation induced in rat liver cell membranes. Quercetin protected against lipid peroxidation, but failed to influence GSHPx activity. Similar results were found in a study by Aherne and O'Brien (1999) in which preincubation of colon cancer (Caco-2) and liver cancer (Hep G2) cells with 200 umol/L quercetin significantly protected cells against H_2O_2-induced DNA damage, but failed to significantly affect catalase or SOD activity.

Genistein

Genistein, an isoflavone, is found naturally in soybeans, whole grain cereals, seeds, berries and nuts (Smythies, 1998). Genistein acts directly as an antioxidant, and its structure is thought to be at least partly responsible for its antioxidant properties (Figure 3). The hydroxyl group at position 4' is the active center to scavenge peroxyl radicals, and was shown to be crucial in protecting against superoxide anion and H_2O_2 formation (Zhang et al. 2003; Wei et al. 1995). Likewise, the location of ring B at the 3-position of the heterocyclic ring greatly affects the radical scavenging capacity of genistein (Pietta, 1999). Genistein may be beneficial in protecting against a number of diseases. It has been shown to reduce cancer incidence and severity in animals, inhibit the proliferation of colon or breast cancer cell lines, and inhibit proliferation of prostate cancer cells in vitro (Rice-Evans & Packer, 1998; Suzuki et al. 2002). Genistein inhibits angiogenesis and promotes apoptosis and cell differentiation, all of which are possible mechanisms of genistein's anticancer activity (Fotsis et al. 1993; Stephens et al. 1997; Polkowski et al. 2000). Genistein has been shown to inhibit platelet aggregation and improve monocyte and endothelial function in a macrophage cell line, effects that may help protect against coronary artery disease (Gottstein et al. 2003).

Due to its antioxidant properties, genistein suppresses H_2O_2 production (Wei et al. 1993; Wei et al. 1995; Peterson, 1995), scavenges H_2O_2 and $O_2^{\bullet-}$, and prevents hemolysis of red blood cells by H_2O_2 (Gyorgy et al. 1964; Pratt et al. 1981). Genistein has also been shown to inhibit low-density lipoprotein (LDL) oxidation, as well as protect endothelial cells from damage by oxidized LDL (Kapiotis et al. 1997; Win et al. 2002). Genistein inhibits DNA strand breaks induced by H_2O_2 and inhibits iron-induced lipid peroxidation in vitro (Win et al. 2002; Hou et al. 2003; Halliwell & Gutteridge, 1999).

As with quercetin, part of genistein's antioxidant activity may relate to its ability to affect antioxidant enzyme systems. Results of studies in this area vary, with some showing genistein to increase, decrease, or have no effect on antioxidant enzymes (Table 2). Cai and Wei (1996) found that 30 days dietary administration of 250 ppm genistein to Sencar mice significantly increased SOD and GSHPx activities in the skin and increased GR activity in the skin & small intestine, while 50 ppm increased CAT activity in the liver, kidney, and small intestine. Suzuki et al. (2002) studied the effects of 100 umol/L genistein on human prostate cells (LNCAP & PC-3). Gene expression of CuZnSOD, MnSOD and catalase (CAT) were not significantly affected by genistein. However, genistein significantly induced gene expression of GSHPx in a dose dependent manner. Proliferation of prostate cancer cells was also inhibited in a dose-dependent manner after genistein treatment. Other studies have shown genistein supplementation to suppress human prostate tumor proliferation, inhibit growth of prostate tumors and tumor angiogenesis in mice (Setchell et al. 2001; Knowles, et al. 2000; Mitchell et al. 2000). Increasing GSHPx gene expression or activity may partly explain genistein's role in protecting against cell damage.

Breinholt et al. (1999) studied the effects of various flavonoids, including genistein, on antioxidant enzyme activity of rats exposed to 2-amino-1-methyl-6-phenylimidazo [4,5-B] pyridine (PhIP) to induce oxidative stress. Genistein significantly decreased the activities of GR, GSHPx and SOD in female rats after two weeks gavage administration of 0.1 g genistein/kg body weight/day. Despite this decrease in antioxidant enzyme activity by genistein, there was protection against PhiP-induced oxidative stress. Decreases in antioxidant enzyme activities caused by flavonoids correlated with increasing antioxidant potential of the flavonoid administered. Due to this finding, the authors suggested that the antioxidant properties of quercetin caused down regulation of these enzymes. Kameoka et al (1999) studied the administration of 100 umol/L genistein to human intestinal cancer cells (Caco-2) for 48 hours. No effects on CuZnSOD or CAT activities due to genistein administration were seen.

Inconsistencies in the results of studies using quercetin or genistein may be due to the animal model, tissue or cell line studied, flavonoids administered, the doses of flavonoids, other antioxidants present, and length of exposure to the flavonoid. More studies are needed to identify the effects of flavonoids on antioxidant enzymes in the body and to determine their possible mechanisms of action in cell protection.

Absorption, Bioavailability and Intake of Quercetin and Genistein

Understanding the absorption and bioavailability of flavonoids is important in studying their effects in vivo, particularly when considering results of in vitro and animal studies. Bioavailability of quercetin and genistein to the target organ is an important factor in the protective effects of these flavonoids in vivo. The bioavailability of flavonoids is influenced by their chemical properties, conjugation in the intestines, intestinal absorption, and enzymes

available for metabolism (Yang et al. 2001). Quercetin is generally found in foods in its glycosylated form. It has been thought that for passive diffusion of flavonoids to occur, the glycosylated forms must be converted to the aglycone by glycosidases (Yang et al. 2001). However, human studies of quercetin have shown that some glycosylated forms can be directly absorbed in the intestines (Morand et al. 2000).

Studies involving humans have shown bioavailability values of 52% for quercetin glycosides from onions, 24% for pure quercetin, and 17% for rutin (Yang et al. 2001; Olthof et al. 2000). The remaining 48% of glycosides not absorbed in the small intestine are metabolized by the colonic microflora into quercetin aglycone and phenolic acids, which can then be absorbed from the colon (Olthof et al. 2000). Whether the aglycone form or the quercetin glycosides are better absorbed remains unclear. Studies have found both the aglycone form (Meng et al. 2004) and the quercetin glycosides (Morand et al. 2000; Hollman, 1996) to be better absorbed.

The relative absorption and bioavailability of quercetin glycosides are affected by their sugar moiety (Olthof, 2000). De Vries et al. (2001) found that the comparative absorption of quercetin in humans differs between different foods and beverages and pure quercetin, as measured by plasma concentrations. The aglycone form of genistein is absorbed via passive diffusion from the intestines. All isoflavones are rapidly and efficiently absorbed from the intestinal tract (Setchell, 2001). While there are differences between the metabolism of aglycone and β-glycosides of genistein, their fates are similar. In humans fed genistein, Setchell et al. (2001) found that the mean time to reach peak plasma concentrations for the aglycone form was significantly shorter than for the β-glycoside, suggesting more rapid absorption of the aglycone. However, the authors also found the bioavailability to be greater in humans when ingested as the

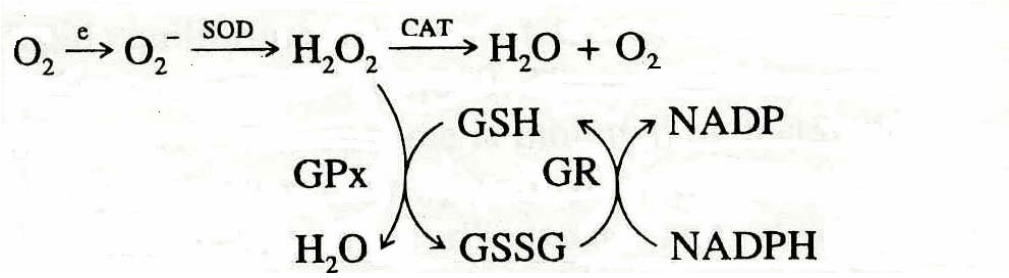
β-glycoside rather than the aglycone form (determined by the plasma appearance and disappearance concentrations). The authors suggest that the glycoside moiety acts as a protecting group to prevent biodegradation of the isoflavone structure in the blood, thereby causing the glycosides to be more bioavailable to the tissues.

Fritz et al (1998) studied the effects of genistein on mammary tumors of rats. Twenty-one days dietary administration of 50 mg/kg diet and 250 mg/kg diet of genistein resulted in dose dependent protection against mammary tumors in female Sprague-Dawley rats. This study also demonstrated the bioavailability of genistein in rats by studying the serum concentrations of genistein after supplementation. Serum concentrations in rats fed 25 and 250 mg genistein/kg diet were 40 and 418 pmol/ml, respectively. Dalu et al. (1998) also found a dose response relationship of serum concentrations of rats fed 25, 100, 250, and 1,000 mg genistein/kg diet (252, 307, 1,094, and 2,712 pmol/ml, respectively). These serum genistein concentrations in these studies are comparable with those found in Asian men on a traditional diet high in soy (276 pmol/ml; Aldercreutz et al. 1993).

Quercetin accounts for the majority of flavonol intake, and average flavonol intake of adults in the United States is approximately 20-25 mg/d (Manach et al. 2004). The typical Asian diet includes 25-40 mg/d of isoflavones including genistein, while the typical American diet contains just a few mg/d of isoflavones (Manach et al. 2004).

Figures

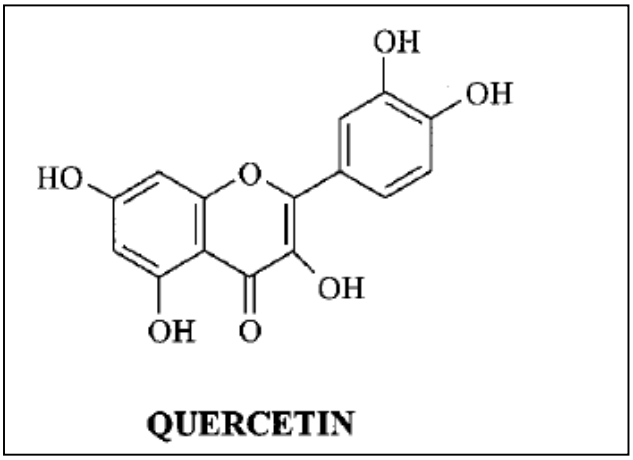
Figure 1 Interaction of the antioxidant enzymes CuZnSOD, GSHPx, GR, and Catalase



SOD = Superoxide dismutase; GPx = Glutathione Peroxidase; GR = Glutathione Reductase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; e = electron, $O_2^{\bullet -}$ = superoxide radical; H_2O_2 = hydrogen peroxide; O_2 = oxygen; H_2O = water

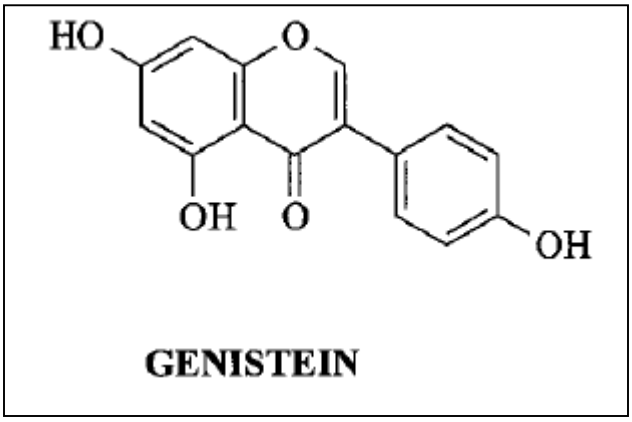
Adapted from Sun, 1990

Figure 2 Structure of Quercetin



Breinholt et al. 1999

Figure 3 Structure of Genistein



Breinholt et al. 1999

Tables

Table 1 Studies on the effects of quercetin on antioxidant enzymes

Author	Subject	Length of Study	Route of Administration	Dose	Stress induced	Results
Aherne & O'Brien, 1999	Caco-2 and Hep 2 cells	24 hours	---------	0-200 umol/L	H_2O_2(50microM)-induced DNA damage (30min)	No effect on SOD. Decreased H_2O_2-induced DNA damage
Breinholt et al. 1999	Female rats	2 weeks	Gavage administration	0.1 g/kg body weight	PhiP-induced(250ug) oxidative stress	Decreased GR and GSHPx activities in RBC
Nagata et al. 1999	Cultured rat hepatocytes (BL-9)		--------		H2O2-induced oxidative damage	Increased GSHPx activity in Se+ but not Se- media
De et al. 2000	Swiss albino mice	31 days	Oral	2% of diet	20-methyl cholanthrene induced cervical neoplasia	Increased hepatic GSHPx, SOD activity. No effect on CAT
Fischer & Fischer, 2000	Rats		Oral	Low dose :1% diet High dose: 2% diet	None	2% increased colon mucosa GR & hepatic CuZnSOD activities
Duarte et al. 2001	Hypertensive Wistar Kyoto rats	5 weeks	Oral	10 mg kg body weight	Hypertension	Increased hepatic GSHPx activity
Kahraman & Inal, 2002	Rats	(4h UV) 3, 6, 9 days	Pretreatment Intraperitoneally	50 mg/kg body weight	UVA light	Increased GSHPx, GR, & SOD activities diminished with UVA exposure

Table 1 continued

Author	Subject	Length of Study	Route of Administration	Dose	Stress Induced	Results
Bok et al. 2002	Rats	6 weeks	Oral	1 g/kg diet	High-cholesterol fed (10g/kg)	Increased hepatic SOD & GSHPx activities
Mikulcik et al. 2002	Rats		Oral	Low dose: 0.2% diet High dose: 1% diet	None	High dose increased colon mucosa GR activity
Coldiron et al. 2002	Diabetic Sprague-Dawley rats	14 days	Intraperitoneally	10 mg/kg body weight	30-day streptozotocin-induced DM	No effect on decreased hepatic SOD and GSHPx or increased renal GSHPx activities
Kahraman & Inal 2003	Rats	--------	Pretreatment intraperitoneally 60 min prior to ischemia	50 mg/kg body weight	Induced renal ischemia (45 min)/reperfusion (60 min) injury	Increased SOD, CAT activities
Molina et al. 2003	Mouse liver	15 days	Pretreatment with quercetin	25,50,75 mg/kg body weight	Ethanol-induced lipid peroxidation	Increased GSHPx, SOD, GR, CAT activities
Rohrdanz et al. 2003	Rat hepatoma H4IIE cells	24 hours	-----	5-100 umol/L	H2O2-induced oxidative stress	5, 10, 50 & 100 microM decreased GSHPx, CuZnSOD mRNA expression

Table 2 Studies on the effects of genistein on antioxidant enzymes

Author	Subject	Length of Study	Route of Administration	Dose	Stress Induced	Results
Breinholt et al. 1999	Female rats	2 weeks	Gavage administration	0.1 g/kg body weight	PhiP-induced oxidative stress	Decreased RBC GSHPx, SOD, GR & CAT activities
Cai & Wei, 1996	Sencar mice	30 days	Oral	50, 250 ppm	None	Increased SOD & GSHPx activity in skin (250ppm); increased GR activity in skin & small intestine (250ppm); increased CAT activity in small intestine (50ppm), liver & kidney (250ppm)
Kameoka et al. 1999	Human intestinal Caco-2	48 hours	-------	100 umol/L	None	No effect on CAT or CuZnSOD
Suzuki et al. 2002	Human prostate cells (LNCAP & PC-3)	92 hours	-------	100 umol/L	None	Increased GSHPx gene expression and activity; No effect on CuZn- or MnSOD or CAT gene expression or activity
Appelt & Reicks, 1999	Female Sprague-Dawley Rats	2 weeks	Oral	0.03, 0.4, and 0.81 mg/g diet isoflavones	None	Increased liver GSHPx and GR activities in group fed 0.81mg/g diet

CHAPTER 3

THE EFFECTS OF THE FLAVONOIDS QUERCETIN AND GENISTEIN ON THE ANTIOXIDANT ENZYMES COPPER ZINC SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE REDUCTASE IN MALE SPRAGUE-DAWLEY RATS[1]

[1] Governo, A.C., Penn, D.M., Power, J.D., Fischer, J.G. To be submitted to *The Journal of Nutrition*.

Abstract

Reactive oxygen species (ROS) from internal metabolism and from the environment contribute to disease development and progression by causing damage to macromolecules (Sun, 1990). The body's defense against ROS includes natural antioxidant enzymes synthesized in the body, as well as antioxidants that must be obtained from the diet. Flavonoids are non-nutrient food components found in fruits and vegetables, and are thought to contribute to the protective roles of fruits and vegetables against disease. The antioxidant properties of flavonoids may prevent lipid peroxidation and other cell damage caused by ROS (Rice-Evans & Packer, 1998).

Two antioxidant flavonoids, genistein and quercetin, not only act as antioxidants but are thought to affect the activities of antioxidant enzymes in the body. However, their effects on antioxidant enzymes are not well understood. Fifty-six male Sprague-Dawley rats were fed low, medium, and high doses of genistein (0.0008%, 0.0012%, or 0.002% of the diet, respectively) or quercetin (0.3%, 0.6%, 0.9% of the diet, respectively) for fourteen days. The effects of genistein and quercetin on the antioxidant enzyme activities of copper zinc superoxide dismutase (CuZnSOD) in the liver and RBC, hepatic glutathione peroxidase (GSHPx), and hepatic glutathione reductase (GR) were measured. It was proposed that supplementation with quercetin and genistein, due to their antioxidant properties, would cause a decrease in antioxidant enzyme activities, but increase the overall antioxidant capacity of the serum. Quercetin supplementation did not significantly affect CuZnSOD, GSHPx or GR activity in the liver, or CuZnSOD activity in RBC. Genistein supplementation did not significantly affect CuZnSOD or GR activities. The low dose of genistein, however, significantly increased hepatic GSHPx activity. Total antioxidant capacity of the serum, as measured by the FRAP assay, was not significantly affected

by quercetin or genistein. Genistein's ability to increase GSHPx activity may be one of the mechanisms by which it protects against chronic disease.

Introduction

Phytochemicals are non-nutrient components found in plants. Phytochemicals have been associated with the prevention and/or treatment of at least four of the leading causes of death in the U.S.: cardiovascular disease, cancer, diabetes and hypertension (Bloch & Thompson, 1995). Specific protective functions of phytochemicals commonly found in fruits and vegetables include enhancement of immune function, reduction of serum cholesterol levels, detoxification of carcinogens, protection against lipid peroxidation and cellular DNA damage, and improvement in antioxidant protection (Van Duyn et al, 2000; Bloch & Thompson, 1995; Nijveldt et al. 2001; Kerry & Abbey, 1998).

Flavonoids are one of the many classes of phytochemicals and are subclassified into flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavones. Quercetin is one of the most common flavonols found in foods (Manach et al. 2004). Quercetin inhibits tumor initiation and promotion in animals and DNA damage in HepG2 cells. Quercetin also inhibits the growth of cells from various human cancers in vitro, and protects against hepatic ischemia-reperfusion injury and gastric lesions (Rice-Evans & Packer, 1998; Su et al. 2003; Martin et al. 1998). Quercetin is one of the most effective antioxidants of the flavonoids. It acts directly as an antioxidant, but also acts indirectly by chelating iron, thus blocking iron-induced free radical production (de Groot & Rauen, 1998; Rice-Evans & Packer, 1998; da Silva et al. 1998). Quercetin reduces lipid peroxidation and oxidative DNA damage in vitro and in vivo (Morel et al. 1993; Ferrali et al. 1997).

Genistein, an isoflavone, is another phytochemical with antioxidant properties. Genistein also inhibits angiogenesis and promotes apoptosis (Suzuki et al. 2002). These protective effects, along with genistein's antioxidant, anti-thrombogenic and anti-atherogenic characteristics may help protect against diseases such as cancer and coronary artery disease (Gottstein et al. 2003).

Along with quercetin and genistein's ability to act directly as antioxidants, mechanisms by which they protect against disease may include the ability to affect the activity of antioxidant enzymes. Antioxidant enzymes are important defenses in surviving exposure to oxygen. Reactive oxygen species (ROS) are formed from oxygen and are necessary in many biological systems. However, due to their high reactivity, high levels can cause damage to DNA, proteins and lipids, and interfere with cell function, normal signal transduction, cell proliferation and cell metabolism (Halliwell & Gutteridge, 1999). Excess ROS play an important role in initiating or furthering tissue injury in several major human diseases including certain cancers, atherosclerosis, many inflammatory diseases, and possibly hypertension, diabetes, and respiratory and neurodegenerative diseases. Antioxidant enzymes protect against ROS by catalytically removing reactive species, reducing their availability, and by scavenging ROS (Halliwell & Gutteridge, 1999).

Three important antioxidant enzymes in the body include copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GSHPx), and glutathione reductase (GR). These antioxidant enzymes work together in defending against oxidative damage by several of the major ROS, including superoxide ($O_2^{\bullet-}$) and hydroxyl (OH•) radicals, and hydrogen peroxide (H_2O_2). CuZnSOD removes $O_2^{\bullet-}$ by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (Marklund & Marklund, 1974). GSHPx is important in removing H_2O_2 produced by the

dismutation of $O_2^{\bullet-}$ by CuZnSOD. GR is critical for recycling GSH, a substrate of GSHPx, and making it available for removal of H_2O_2 (Paglia & Valentine, 1967).

The effects of quercetin and genistein on antioxidant enzyme activity have been examined previously. However, results of previous studies vary according to the flavonoid dose and the tissue studied (Breinholt et al. 1999; Aherne & O'Brien, 1999; De et al. 2000; Cai & Wei, 1996). Many studies using high doses of flavonoids have shown increases in enzyme activity. In contrast, Breinholt et al. (1999) showed that decreased antioxidant enzyme activity was inversely associated with increasing antioxidant potential of the flavonoid administered at relatively low levels of supplementation. The authors concluded that the flavonoids might decrease the need for antioxidant enzyme activity due to the antioxidant properties of the flavonoids (Breinholt et al. 1999). Due to inconsistent results, more studies are needed to clarify the effects of quercetin and genistein on antioxidant enzyme activity.

I tested the hypothesis that the dietary administration of quercetin and genistein, due to their antioxidant properties, would decrease the activity of antioxidant enzymes in the liver and RBC but increase the overall antioxidant capacity of the serum in male Sprague-Dawley rats. The objectives of this study were 1) to test whether quercetin and genistein would decrease the activity of the antioxidant enzymes CuZnSOD, GSHPx and GR in the liver, and CuZnSOD in the RBC and 2) to test whether genistein and quercetin would increase the total antioxidant capacity of the serum despite causing a decrease in antioxidant enzyme activity. In addition, most studies evaluating the effects of quercetin and genistein on antioxidant enzymes, used levels of quercetin and genistein that were well above normal intake levels for humans, with some doses being cytotoxic (Rohrdanz et al. 2003). In the current study, lower doses of genistein and quercetin were used to determine if the previously reported effects could be found

at lower levels of intakes. The low and medium doses of genistein selected were levels attainable in the human diet (Appelt & Reicks, 1999). Previous studies using rat models have found increased hepatic antioxidant enzyme activity with 2% quercetin and altered antioxidant enzyme activity in the colon with 1% and 2% quercetin supplementation (Fischer & Fisher, 2000; Fischer et al. 2002). However, no effect on enzyme activity was seen with 0.2% quercetin. The present study examined the effects of quercetin at doses between 0.2% and 1% of the diet to attempt to determine the levels at which quercetin is able to affect antioxidant enzyme activity.

Methods

Experimental design

Male, weanling Sprague-Dawley rats (n=56; initial weight 70-90 g; Harlan, Indianapolis, IN) were housed individually in stainless steel wire-bottomed cages in a temperature (21±1ºC), humidity and light (12 hour light:dark cycle), controlled environment. The decision to use this gender and age of rat was to test the hypothesis in animals were similar to those used in past flavonoid feeding studies (Cai & Wei, 1996; Appelt & Reicks, 1997). Animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee. Rats were acclimated for 24 hours and randomly assigned to one of seven different treatment groups (n=8) with similar mean group body weights (mean group weights: 70-74 g). A semi-purified, modified AIN-93 diet (Table 3, American Institute of Nutrition, 1993) was fed with zero, low, medium and high doses of either quercetin (Sigma Chemical Company, St. Louis MO, USA) or genistein (Toronto Research Chemicals, ON, Canada) for 14 days. The levels of quercetin used were 0, 0.3% (0.3 g/100 g diet), 0.6% (0.6 g/100g diet), or 0.9% (0.9 g/100g diet). The levels of genistein used were 0, 0.0008% (0.8 mg/100g diet), 0.0012% (1.2 mg/100g diet), or 0.002% (2

mg/100g diet); Rats had free access to diet and water throughout the study. Body weight was measured weekly and food intake measured for three-day periods each week of the study. This data has been reported previously (Penn, 2003). Fresh food was added daily and diet was stored at -20ºC until use.

Tissue Collection and Preparation

The animals were fasted overnight prior to sacrifice and tissue collection. Rats were anesthetized with a 3:2:1 (v/v/v) ratio of ketamine:acepromazine:xylazine (0.8 ml/kg body weight). Blood was obtained via heart puncture into non-heparinized syringes.

Whole blood was centrifuged for 20 minutes (4º C, 2000g; model J2-HS, JS-7.5 rotor, Beckman). Serum was removed and red blood cells were washed three times with ice-cold saline and centrifuged between each wash to remove saline. An equal volume of water was added to the cells prior to freezing. The red blood cells and serum were frozen at –80º C until analysis. The liver was removed following whole body perfusion. An incision was made from the abdomen to the chest area, and the skin was folded back from the rib cage in order to make two cuts through the ribs. A canula was placed through the apex of the left ventricle of the heart and inserted into the aorta. Ice-cold heparinized saline was pumped through the animal for about ten minutes to remove any residual blood. The livers were excised, rinsed with ice-cold saline, blotted dry and weighed. Livers were frozen in liquid nitrogen and then stored at -80º C until they were homogenized and separated for analysis.

One-gram sections of liver were homogenized at 4º C in 4 ml phosphate buffer (pH 7.0; 0.05 mol/L potassium phosphate) using a hand-held (Omni International) homogenizer and stored at -80º C for two weeks until further centrifugation. To obtain cytosol, the liver

homogenate was centrifuged (model J2-HS, Beckman) for 20 minutes at 4º C and 10,000 X G. The supernatant was saved and transferred to a polycarbonyl centrifuge tube and then centrifuged again in a LE-80K Optima Ultracentrifuge (Beckman) for 1.16 hours, at 4º C, 100,000 X G. Cytosol was collected and frozen at –80º C until analyzed.

Tissue Analysis

CuZnSOD activity of liver cytosol and RBC were determined with a spectrophotometer (DU 650, Beckman) using the method of Marklund and Marklund (1974) with pyrogallol as the substrate. To determine CuZnSOD activity, a ethanol/chloroform mixture (5:3) was added to undiluted liver homogenate to inactivate MnSOD before centrifugation for 30 min at 7200 RPM as described by Johnson and Murphy (1988). CuZnSOD activity was measured in triplicate. Supernatant was diluted to achieve linearity of reaction. For liver, the reaction mixture included 0.01 ml sample, 0.03 ml pyrogallol (4 mmol/L) and 0.96 ml SOD buffer (pH 8.2) which contained N-Tris [hydroxymethyl] methyl- 3-aminopropane-sulfonic acid (TAPS), and diethylenetriamine pentaacetic acid (DTPA, 1mmol/L). For RBC, the reaction mixture included 0.05 ml sample, 0.92 ml SOD buffer and 0.03 ml pyrogallol. CuZnSOD inhibits the oxidation of pyrogallol, which autoxidizes rapidly. Superoxide dismutase activity was determined by measurement of the inhibition of pyrogallol autoxidation by CuZnSOD at absorbance of 420 nm and was run for 4 minutes with readings every 60 seconds. DTPA prevents interference from Fe^{2+}, Cu^{2+} and Mn^{2+}. One unit of CuZnSOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%.

GSHPx activity of liver cytosol was measured spectrophotometrically with *t*-butyl hydroperoxide (TBH; 0.3 mmol/L) as the substrate according to the method of Paglia and

Valentine (1967). Liver supernatant was diluted by adding 0.1 ml supernatant to 0.75 ml potassium phosphate buffer (0.05 mol/L) with EDTA (0.005 mol/L, pH 7.4). Supernatant was diluted to achieve linearity in the reaction. Reaction mixture included 0.1 ml diluted sample, 0.1 ml TBH and 0.8 ml of a mixture that contained potassium phosphate buffer, NADPH (2 mmol/L), GSH (10 mmol/L), GSSGR (10 IU/ml), and NaN_3 (10 mmol/L). Samples were run in duplicate for 4 minutes, with readings every 60 seconds. GSHPx activity was determined by change in absorbance at 340 nm. One unit of GSHPx activity is defined as one umol of NADPH oxidized per minute, and is expressed as units/mg protein.

GR activity of liver cytosol was measured with a spectrophotometer according to the method of Xia et al. (1985) with glutathione (0.5 mmol/L) as the substrate. In the presence of GR, hydrogen is transferred from NADPH to GSSG. GR activity was determined by measuring the rate of oxidation of NADPH in the presence of GSSG. Liver supernatant was diluted by adding 0.1 ml supernatant to 0.4 ml potassium phosphate buffer. The reaction cuvette contained 0.8 ml NADPH (0.25 mmol/L), 0.1 ml GSSG (5 mmol/L), and 0.1 ml diluted sample (50 mmol/L, pH 7.0). The reaction was started by the addition of GSSG. Samples were run in duplicate at absorbance 340 nm for 4 minutes, with readings taken every 60 seconds. One unit of GR activity is defined as the amount of GR to oxidize 1 umol NADPH/minute.

The FRAP assay was used to measure the antioxidant potential of the serum (Benzie, 1996). Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion reduction at low pH causes a colored ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex to form. This complex forms an intense blue color with absorption maximum at 593 nm. Excess Fe^{3+} is used and the rate-limiting factor of Fe^{2+}-TPTZ (and therefore color) formation is the reducing ability of the sample. Undiluted serum samples were used, and the change in absorbance was measured spectrophotometrically at 593 nm.

FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (Benzie & Strain, 1996).

The sample protein concentrations of liver and RBC cytosol were determined according to the method of Lowry et al. (1951). Data for CuZnSOD, GSHPx and GR assays were expressed as units/mg protein.

Statistical Analysis

Treatment means, standard error of the mean, analysis of variance (ANOVA), and least significant difference tests were determined using the statistical package SAS (version 6.10, SAS Institute, Cary, NC). Differences among treatment groups were considered significant if $p < 0.05$. A power analysis had suggested that n=8 per group is sufficient to show significance when change in enzyme activity is at least 35% from control.

Results

Food Intake, Body Weight and Organ Weights

This data was previously reported by Penn (2003). Food intake was decreased about 14% by the experimental diets during the first week of study. However, there were no significant differences in body weight among treatment groups at the end of two weeks of study (Table 4). The only significant elevation in liver to body weight ratio was found in rats fed 0.3% quercetin at the 14 day time point (p= 0.02) (Table 5). It is not clear why the liver weights were increased in the majority of the animals within this treatment group.

Effects of Genistein

No significant changes in CuZnSOD or GR activities were observed in RBC or liver of rats fed low, medium or high doses of genistein (0.0008%, 0.0012%, 0.002%, respectively) for 14 days. However, genistein supplementation significantly increased GSHPx activity in the liver of rats ($p < 0.01$; Table 6; Figure 4). Post-hoc tests showed that only the lowest level of genistein supplementation (0.0008%) significantly increased activity over control.

Effects of Quercetin

No significant changes in CuZnSOD or GSHPx activities were observed in the RBC or liver of rats after 14 days dietary administration of low, medium or high doses of quercetin (Table 7). The activity of GR was decreased 28-40% in the liver of rats fed medium and high doses of quercetin, but this change was also not significant.

FRAP Assay

Dietary administration of low, medium, or high doses of quercetin or genistein to rats for 14 days did not affect the ferric reducing antioxidant potential (FRAP) of the serum (**Table 8**).

Discussion

This study was conducted to test whether quercetin or genistein supplementation would decrease the activities of antioxidant enzymes in rats, but increase the total antioxidant capacity of the serum. The level of genistein used approaches levels attainable in the human diet. The low (0.8 mg/100g diet) and medium (1.2 mg/100g diet) doses of genistein can be obtained by eating a diet high in soy products, as seen in Asian populations (Appelt & Reicks, 1999). The

high dose of genistein is roughly twice that seen in Asian populations (Fritz at al. 1998). The dietary concentration of quercetin, 0.3-0.9 g/100g diet, which was used because of previous studies showing high levels reduce tumor development, could not reasonably be obtained in the diet.

The major finding of this study was that dietary administration of genistein at a level attainable in the human diet for 14 days significantly increased hepatic GSHPx activity in rats. The relatively short-term supplementation of genistein may not reflect increased enzyme levels possible with long term supplementation, but these results are consistent with other studies in which similar levels of genistein supplementation and effects on GSHPx were studied. Cai et al. (1996) investigated the effect of GSHPx activity in Sencar mice fed 250 ppm genistein for 30 days. Genistein supplementation significantly increased GSHPx activity in the skin, but not small intestine, liver or kidney. The genistein concentration used in this study was approximately equal to that in a diet containing 20% soybean, which is achievable in human diets (Cai et al. 1996), but is much higher than levels used in the current study. Fritz et al. (1998) demonstrated the comparative bioavailability of genistein in rats and humans. The serum genistein concentrations of female Sprague-Dawley rats fed 25 and 250 mg genistein/kg diet were 40 and 418 pmol/ml, respectively. Dalu et al. (1998) also found a dose response relationship in rats fed 25, 100, 250, and 1,000 g genistein/kg diet and serum concentration levels of genistein (252, 307, 1,094, and 2,712 pmol/ml, respectively). These serum genistein concentrations are comparable with those found in Asian men on a traditional diet high in soy (276 pmol/ml; Aldercreutz et al. 1993). Suzuki et al. (2002) observed a 2-fold increase in GSHPx-1 gene expression levels in human prostate cells (LNCAP & PC-3) after 92 hours treatment with 100 umol/L genistein. Proliferation of prostate cancer cells was inhibited in a

dose-dependent manner after genistein treatment. The ability to up-regulate GSHPx gene expression may be a mechanism by which genistein is able to protect against cancer. Finally, Appelt and Reicks (1999) fed low, medium or high doses of isoflavones (30, 400 or 810 mg/kg diet, respectively) to female Sprague-Dawley rats for 2 weeks. Hepatic GSHPx was significantly increased in rats fed the highest isoflavone dose, which was 40 times higher than the highest genistein dose in the current study.

An increase in GSHPx may be beneficial in protection against cancer, cardiovascular disease or other diseases of aging. A review by Sun (1990) of studies looking at antioxidant enzyme activities in tumor cells showed that activities of MnSOD, CuZnSOD and CAT are often decreased in tumor cells. GSHPx and GR activities have been highly variable in these studies. The decreased enzyme activity in tumors found in these studies does not reveal whether reduced enzyme activity is a cause or is one of the consequences of cancer. This question has important implications in the treatment of cancer through manipulating antioxidant enzyme activities. As explained by Sun (1990), if inactivation of the genes for certain antioxidant enzymes is one of the causes of carcinogenesis, we should be able to prevent the disease to some extent by the addition of these enzymes, or substances that increase their activity, in the early stages of carcinogenesis. On the other hand, if the decrease in antioxidant enzyme activity is a result of cell malignancy and is required for maintenance of the malignant state, then enhancement of these decreased enzymes could help in cancer treatment.

Benefits of enhanced GSHPx activity in cardiovascular risk reduction have been suggested in a number of studies. Blankenberg et al. (2003) studied the effects of GSHPx activity and cardiovascular events in patients with coronary artery disease. Six hundred and thirty six patients with suspected coronary artery disease were assessed for risk of cardiovascular

events associated with baseline erythrocyte GSHPx activity. The risk of future fatal and nonfatal cardiovascular events was significantly and inversely associated with increasing quartiles of GSHPx activity. This association was independent of smoking status, gender, inflammatory markers, and selenium status. Straif et al. (2000) related increased GSHPx activity to inhibition of 5-lipoxygenase activity, which strongly contributes to atherosclerotic susceptibility in mice. This function of GSHPx, along with its antioxidant functions, may help protect against cardiovascular disease. The authors concluded that low erythrocyte GSHPx activity in patients with coronary artery disease puts them at higher risk for cardiovascular events. Likewise, increasing GSHPx activity in those with cardiovascular risk factors may be beneficial in protection against cardiovascular events.

Finally, a review by Matsuo (1992) examined results of 13 rat studies in which age-related declines were observed in GSHPx activity levels in various rat tissues. Increasing GSHPx levels through genistein supplementation could possibly help slow or reverse the decline in GSHPx levels seen with age, helping to increase protection against ROS.

No significant changes in enzyme activity were observed for hepatic or RBC CuZnSOD in rats after 14 days dietary administration of low, medium or high doses of quercetin. This is similar to results of others who also found that quercetin had no effect on antioxidant enzyme activity (Galvez et al. 1995; Aherne & O'Brien, 1999). Many studies looking at the effects of quercetin supplementation on antioxidant enzyme activity have found results to be dose and tissue specific. Early studies on the benefits of flavonoids reported that supplementation with quercetin at 0.05-2% of the diet decreased tumor formation and aberrant crypt foci in rodents (Warren et al. 2003; Deschner et al. 1991). De et al. (2000) studied the effects of 31 days of quercetin supplementation at 2% of the diet in Swiss albino mice. Quercetin supplementation

increased hepatic GSHPx and SOD activity in normal mice and in mice with 20-methyl cholanthrene-induced cervical neoplasia. Quercetin was also found to arrest or reverse the progression of cervical neoplasia (De et al. 2000). The effects of 1-2% quercetin supplementation on antioxidant enzyme activity were also studied in our lab. Increases in hepatic CuZnSOD with 2% quercetin, as well as an increase in colon mucosa GR activity with 1% and 2% quercetin supplementation were seen in rats (Fischer and Fisher, 2000; Fischer et al. 2002). These studies also found no effect on GR activity in RBC, or hepatic or colon GSHPx activity at 1 and 2% of the diet. Fischer et al. (2002) found an increase in GR activity in the distal colon, but a decrease in GR activity in the proximal colon with 1% quercetin. However, no effect on enzyme activity was seen with 0.2% quercetin. The present study looked at the effects of quercetin at doses between 0.2% and 1% to attempt to determine the levels at which quercetin is able to affect antioxidant enzyme activity. Since concentrations between 0.3 and 0.9% did not significantly affect the activities of hepatic CuZnSOD, GR or GSHPx, or RBC CuZnSOD, it was concluded that doses of at least 1% quercetin are needed to see effects on these antioxidant enzyme activities. Because the effects seen were tissue specific, and results at 1% quercetin were primarily seen in the colon, even higher doses (2%) may be needed to see effects in organs other than the colon.

The current study tested the hypothesis that supplementation of quercetin and genistein would decrease the antioxidant enzyme activity of rats. Because quercetin and genistein act directly as antioxidants, it was hypothesized that the need for these enzymes, and therefore their activity, would be decreased. Breinholt et al. (1999) had previously reported that 0.1 g/kg b.w. quercetin decreased the activities of RBC GR and GSHPx, and that genistein decreased RBC

GSHPx, SOD, GR and CAT activities. My study did not confirm this, possibly because oxidative stress was not induced as it was in Breinholt's study.

Although some studies have shown no effect of quercetin on enzyme activity, others have shown that quercetin inhibited lipid peroxidation and decreased H_2O_2-induced DNA damage (Galvez et al. 1995; Aherne & O'Brien, 1999). For example, in a study by Rohrdanz et al. (2003) on rat hepatoma cells, GSHPx and CuZnSOD mRNA expression decreased with doses of 5, 10, 50 and 100 umol quercetin. Despite this decrease in antioxidant enzyme gene expression, cells showed only mild oxidative stress. Both studies suggest quercetin's positive effects on cell damage may be attributed to a mechanism other than increasing antioxidant enzyme activity. In contrast, Nagata et al. (1999) studied cultured rat hepatocytes and found that quercetin activated GSHPx in selenium (Se) supplemented media cells, and protected against H_2O_2 oxidative stress.

Dietary administration of either quercetin or genistein for 14 days did not affect the ferric reducing antioxidant potential (FRAP) of the serum in rats. Fischer et al. (2002) found that supplementation of 1% quercetin increased FRAP in plasma of rats. This suggests that doses of at least 1% quercetin may be needed to see increases in the overall antioxidant capacity of the plasma. Genistein levels higher than those used in this study may be needed to increase the serum concentrations enough to observe increased in antioxidant capacity. On the other hand, the positive effects of these flavonoids may not be due to an ability to increase the antioxidant capacity of the serum, but rather through another mechanism.

The results of this study and others suggest that dietary concentrations of quercetin must be much higher than levels commonly found in the human diet to affect antioxidant enzyme activity. While genistein did not impact CuZnSOD or GR, our results support findings by others

that genistein, at concentrations that can be obtained in the diet, may increase GSHPx activity. This may be another mechanism by which genistein protects against chronic disease.

Tables

Table 3 Composition of Experimental Diet[1,2]

INGREDIENT	g/kg diet
Casein	200
Cornstarch	529.5
Sucrose	100
Soybean Oil	70
Cellulose	50
AIN-93G Vitamin Mix	10
AIN-93G Mineral Mix	35
Quercetin	*
Genistein	*
L-Cystine	3
Choline Bitartrate	2.5

[1] AIN-93 diet (American Institute of Nutrition, 1993)
[2] Diet components, excluding flavonoids, were purchased from Harlan Teklad Diets (Madison, WI)
[*] Amount of flavonoid added; Quercetin: 0.3 g/100g diet, 0.6 g/100 g diet, 0.9 g/100g diet; Genistein: 0.8 mg/100g diet, 1.2 mg/100g diet, 2 mg/100g diet. Cornstarch dose made up the difference for quercetin and genistein in the experimental diets.

Table 4 Initial and final body weights of rats. Rats were fed low, medium, or high doses of quercetin or genistein[1,2,3,4]

Dietary Treatment	Initial Body Weight (g)	Final Body Weight (g)
Control	72±4	171±4
QC1	73±4	170±3
QC2	73±4	178±5
QC3	73±3	168±4
Control	72±3	171±4
GS1	71±3	164±4
GS2	73±2	168±3
GS3	71±2	167±3

[1]From Penn, 2003.
[2]Means ± SEM (n=8/group). There were no significant effects of diet on body weight.
[3]Abbreviations: QC1=0.3% quercetin, QC2=0.6% quercetin, QC3=0.9% quercetin, GS1=0.0008% genistein, GS2=0.012% genistein, GS3=0.02%genistein.
[4]Body weight was measured weekly.

Table 5 Liver to body weight ratio of rats. Rats were fed low, medium, or high doses of quercetin or genistein[1,2,3]

Dietary Treatment	Liver weight/100g body weight
Control	3.51±0.10
QCL	4.21±0.31
QCM	3.39±0.07
QCH	3.83±0.17
Control	3.51±0.10
GSL	3.76±0.25
GSM	3.64±0.31
GSH	3.78±0.19

[1]From Penn, 2003.
[2]Means ± SEM (n=8/group)
[3]Abbreviations: QCL=0.3% quercetin, QCM=0.6% quercetin, QCH=0.9% quercetin, GSL=0.0008% genistein, GSM=0.0012% genistein, GSH=0.02% genistein.

Table 6 Mean Cu, Zn, superoxide dismutase, glutathione peroxidase, and glutathione reductase activities of rats treated with low, medium, or high doses of genistein for 14 days[1,2,3]

Dietary Treatment		
	U/mg Protein	
	LIVER	**RBC**
CuZnSOD		
Control	61.6±5	191±11
Low	65.5±14	191±30
Medium	53.0±9	201±7
High	58.3±5	158±17
ANOVA	NS	NS
	LIVER	
GSHPx		
Control	0.501±0.054[a]	
Low	0.874±0.113[b]	
Medium	0.588±0.065[a]	
High	0.642±0.026[a]	
ANOVA	$p<0.01$	
	LIVER	
GR		
Control	0.040±0.010	
Low	0.043±0.010	
Medium	0.033±0.005	
High	0.033±0.005	
ANOVA	NS	

[1]Means ± SEM (n=5-8 group). Means within a column with different superscript letters differ significantly ($p<0.05$).
[2]Low dose =0.0008% diet, medium=0.0012% diet, high=0.002% diet.
[3]Units of enzyme activities are defined as follows: CuZnSOD= the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%; GSHPx= one umol of NADPH oxidized per minute, and is expressed as units/mg protein; GR= the amount of GR to oxidize 1 umol NADPH/minute.

Table 7 Mean Cu, Zn, superoxide dismutase, glutathione peroxidase, and glutathione reductase activities of rats treated with low, medium, or high doses of quercetin for 14 days[1,2,3]

Dietary Treatment		
	U/mg Protein	
	LIVER	**RBC**
CuZnSOD		
Control	61.6±5	191±11
Low	61.6±10	166±26
Medium	61.6±6	188±27
High	65.9±12	179±16
ANOVA	NS	NS
	LIVER	
GSHPx		
Control	0.501±0.054	
Low	0.597±0.054	
Medium	0.537±0.026	
High	0.595±0.065	
ANOVA	NS	
	LIVER	
GR		
Control	0.040±0.010	
Low	0.040±0.008	
Medium	0.024±0.003	
High	0.029±0.005	
ANOVA	NS	

[1]Means ± SEM (n=6-8/group).
[2]Low dose =0.3% diet, medium=0.6% diet, high=0.9% diet.
[3]Units of enzyme activities are defined as follows: CuZnSOD= the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%; GSHPx= one umol of NADPH oxidized per minute, and is expressed as units/mg protein; GR= the amount of GR to oxidize 1 umol NADPH/minute.

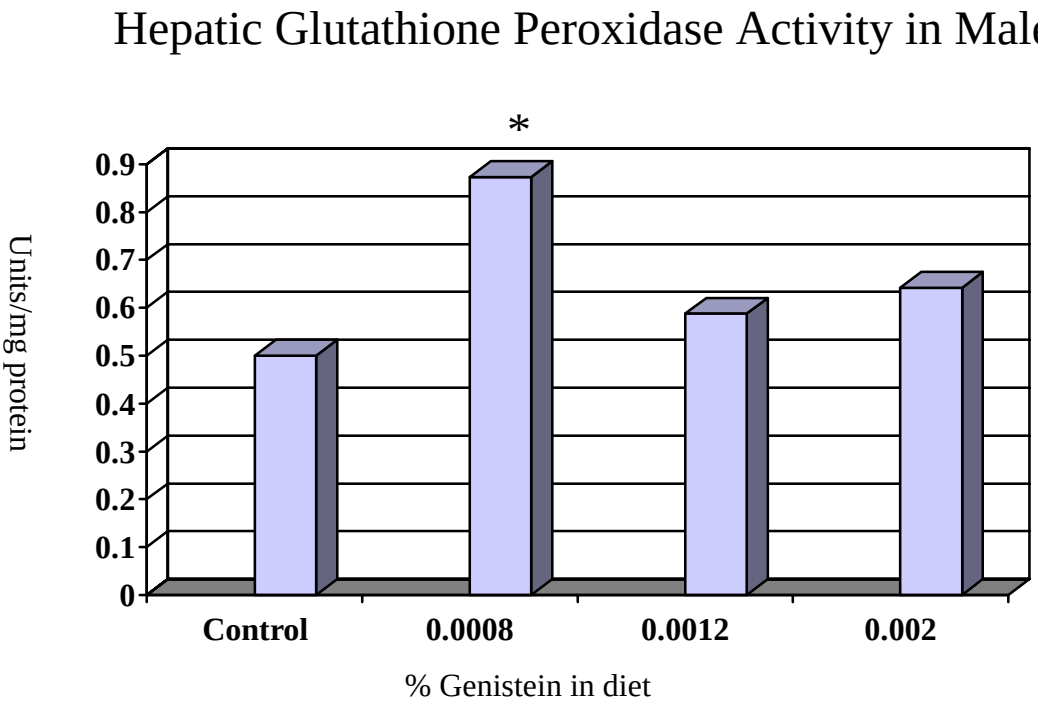
Table 8 Mean FRAP value of serum of rats treated with low, medium, or high doses of quercetin or genistein[1,2,3]

Dietary Treatment	ΔA_{593nm}
Control	424±24
GSL	456±47
GSM	406±31
GSH	469±62
ANOVA	NS
Control	424±24
QCL	386±13
QCM	413±36
QCH	412±22
ANOVA	NS

[1]Means ± SEM (n=7-8/group).
[2]FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration
[3]Abbreviations: QCL=0.3% quercetin, QCM=0.6% quercetin, QCH=.9% quercetin, GSL=0.0008% genistein, GSM=0.0012% genistein, GSH=0.0012% genistein; ΔA_{593nm}= change in absorbance at 593 nm.

Figures

Figure 4



Glutathione peroxidase activity within the liver of rats treated with varying doses of genistein. Means (n=8) within a column with different superscript letters differ significantly ($p<0.05$).

CHAPTER 4

SUMMARY

Major Findings and Implications

Low doses of genistein increased the activity of hepatic GSHPx in male Sprague-Dawley rats. These findings are consistent with other studies on the effects of genistein on GSHPx activity (Cai & Wei, 1996; Suzuki et al. 2001). Doses of genistein used in the current study were levels attainable in the human diet (Fritz et al. 1998) and were lower than doses used by Cai and Wei (1996). Increasing GSHPx activity may be one mechanism by which genistein protects against chronic disease. Blankenberg et al. (2003) showed that the risk of future fatal and nonfatal cardiovascular events was significantly inversely associated with increasing quartiles of GSHPx activity in six hundred and thirty six patients with suspected coronary artery disease. A review by Sun (1990) showed that activities of antioxidant enzymes are often decreased in tumor cells. Whether this reduced enzyme activity is a cause or is one of the consequences of cancer is not known. However, this question is important for the treatment of cancer. If decreased antioxidant enzyme activity is one of the causes of carcinogenesis, we should be able to prevent the disease to some extent by the addition of these enzymes, or substances that increase their activity, in the early stages of carcinogenesis. On the other hand, if decreased antioxidant enzyme activity is a result of cell malignancy and is required for maintenance of the tumor, then addition of these decreased enzymes could help in cancer treatment (Sun, 1990).

Quercetin supplementation between 0.3-0.9% of the diet did not affect antioxidant enzyme activity in the liver or RBC. Previous studies have demonstrated that while supplementation of quercetin at levels as low as 1% of the diet is sufficient to alter colon mucosa GR activity, at least 2% quercetin supplementation is needed to increase hepatic CuZnSOD activity in rats (Fischer & Fisher, 2001; Fischer et al. 2002). This suggests that effects of quercetin on antioxidant enzyme activity are dose and tissue specific. In combination with the studies of others, my data suggests that extremely high levels of quercetin well above that achievable in the diet are necessary to alter liver CuZnSOD, GSHPx, and GR, and RBC CuZnSOD activity.

Study Limitations

The isolated flavonoids quercetin and genistein were used in this study to test their effects on antioxidant enzyme activity. Because the action of flavonoids is likely affected by other nutrients, and because humans get flavonoids from whole foods, it would be ideal to use whole food extracts to study the effects of these flavonoids. However, little is known about the effects of quercetin and genistein on antioxidant enzyme activity. Therefore, investigating the effects of these flavonoids alone is appropriate in studying their role and determining the mechanisms by which they contribute to the protective effects of foods.

Another limitation is that purified quercetin and genistein were used. Quercetin and genistein are primarily found in foods in their glycolytic forms. However, the sugar moiety of the glycosides is cleaved in the intestines, leaving the aglycone form, which is what reaches the liver. Therefore, using the aglycone form is appropriate when relating findings to human intakes. Doses of quercetin in this study were well above levels found in the human diet.

However, levels used were based on studies in which quercetin was found to exert protective effects on tumor formation and aberrant crypt foci (Deschner et al. 1991; Warren et al. 2003).

The serum or organ levels of quercetin, genistein or their metabolites were not measured to determine the amount of flavonoid absorbed, and which metabolites reached the organs and may be responsible for any effects. Therefore, while genistein supplementation was found to increase hepatic GSHPx activity, it was not determined whether genistein itself or a metabolite is responsible for these effects. This is a critical limitation of all flavonoid research at this time (Manach et al. 2004).

No oxidative stress was introduced to the rats in this study in order to test whether flavonoids protect against oxidative stress through their effects on antioxidant enzymes. There was a shortage of RBC in order to determine effect on GSHPx and GR levels. The study was not able to determine if GSHPx levels were increased in RBC as well as in the liver.

Tissues were stored for up to one year before analysis. Multiple freeze-thaw cycles of tissues may alter protein or enzyme content. However, levels of enzymes found in the present study are comparable to levels seen in previous studies.

Additionally, as these studies are ultimately aimed at providing information useful for human health, the use of humans in studies of flavonoids is ideal. However, as the metabolism of nutrients has been shown to be similar in rats and humans, using rat models is useful, particularly in early studies, to determine the effects of flavonoids in various organs.

Future Research

In addition to measuring the antioxidant activity of tissues, future research should determine flavonoid levels in the serum and organs after flavonoid supplementation to determine

the amount of the flavonoid that reaches the tissue, and the flavonoid concentration needed to affect antioxidant enzyme activity. It is not clear which compounds are responsible for these effects. Therefore, future research should measure metabolite concentrations in tissues to help in determining the specific compounds responsible for effects on antioxidant enzyme activities or gene expression.

The ultimate goal of these experiments is to determine the effects of these flavonoids in humans. By measuring these compounds in the serum of animals relative to the dose of flavonoid supplementation, and comparing this to results found in humans consuming flavonoids, future research can bring us closer to determining levels necessary in humans in order to elicit responses in antioxidant enzyme activity.

Finally, while studies have shown genistein increases both GSHPx activity and gene expression, the mechanisms by which this occurs are not known (Cai & Wei, 1996; Suzuki et al. 2000). Future research should focus on determining the mechanisms by which genistein affects GSHPx activity.

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