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An In Vivo Study of The Antioxidant Potentials of Plant Food Concentrates

by

Vincent Edward Ziccarelli 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

In

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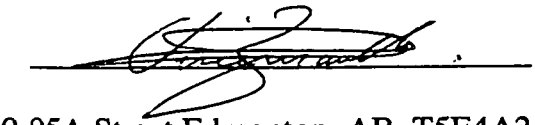

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **An In Vivo Study of The Antioxidant Potentials of Plant Food Concentrates** submitted by Vincent Edward Ziccarelli in partial fulfillment of the requirements for the degree of Master of Science in Nutrition and Metabolism.

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ABSTRACT

"Green factors" concentrate (GF) and germinated wheat powder (WP) are rich sources of antioxidants, however, their in vivo antioxidant effects are unknown. The present longitudinal study investigated the antioxidant and lipidemic responses in 15 male hypercholesterolemic subjects to the supplemental intakes of GF, and to determine if the combined intake of GF plus WP would increase these effects. Erythrocyte superoxide dismutase (SOD), whole blood glutathione peroxidase (GPX), and plasma concentrations of zinc, copper, lipids and thiobarbituric acid-reactive substances (TBARS) were determined. The SOD activity was significantly elevated with intake of GF while total and LDL-cholesterol concentrations in the plasma were significantly decreased. The latter responses remained unchanged when the supplemental intake was enriched with WP supplement. These results indicate that, in the presence of hypercholesterolemia, the antioxidant and cholesterol status can be appreciably improved with the supplemental intake of GF, and that the addition of WP has no additive effects.

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LIST OF ABBREVIATIONS

BMI	Body mass index
CHeFF	Center for Healthy Functional Foods
CVD	Cardiovascular disease
GF	"Green factors" concentrate
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized Glutathione
WP	Germinated wheat powder
HDL	High density lipoprotein
LDL	Low density lipoprotein
MDA	Malondialdehyde
SOD	Superoxide dismutase
TBARS	Thiobarbituric-acid reactive substances
TC	Total cholesterol
TG	Triglycerides
UV	Ultraviolet
VIS	Visible
VLDL	Very low density lipoprotein

CHAPTER 1. INTRODUCTION

1.0 FREE RADICALS VERSUS DISEASE

A free radical is defined as any chemical species with an unpaired electron. In humans, free radicals are biochemical and physiological by products of aerobic metabolism, phagocytosis, transition metals, and the biosynthesis of endoperoxides (Table 1.1). In addition, environmental exposure to oxidizing pollutants, cigarette smoke and electromagnetic radiation generate free radicals. Some of these reactive compounds include the superoxide anion, hydroxyl radical, thiyl radical, peroxyl radical, alkoxyl radical, and nitric oxide (Halliwell 1994). Refer to Table 1.2 for a list of some common free radicals. In low concentrations, reactive oxygen species play a part in normal physiological processes such as intracellular signaling, and cell differentiation. However, in high concentrations, free radicals may increase oxidative stress (Mates et al. 1999).

The reactivity of a free radical varies and therefore the extent of potential damage to the body may be affected. For instance, the hydroxyl radical, an extremely reactive oxygen centered radical generated from ionizing radiation, is considered to be one of the most potent. Whereas, nitric oxide, synthesized by phagocytes, endothelial cells and brain cells, is less reactive and therefore less harmful to the body. In general, most free radicals are very unstable and will react with almost any biological compound, thereby creating a series of chain reactions. Consequently, many cellular

disturbances may result with excess exposure to oxidants and include oxidative DNA damage, protein and lipoprotein peroxidation, which have been implicated in degenerative disease states (Table 1.3, Maxwell 1995) such as cancer, diabetes, cardiovascular disease (CVD), rheumatoid arthritis, and age related macular degeneration (Betteridge 2000).

Table 1.1: Sources of oxidative stress (Maxwell 1995)

Source	Mechanism
Mitochondrial electron transport	Leakage of superoxide as a result of inadequate reduction of oxygen
Transition metal ions	Iron and copper give rise to hydroxyl radical formation
Inflammation	Activated phagocytes produce free radicals
Enzymes, e.g. xanthine oxidase	Superoxide release due to reperfusion of ischaemic tissues
Drug metabolism, e.g. paraquat acetaminophen	Free radical metabolites produced during metabolism
Cigarette smoke	Gas phase with concentrated free radicals
Radiation	ultraviolet light, x-rays

Table 1.2: Examples of free radicals (Morton et al. 2000)

Name	Chemical formula	Comments
Superoxide	$O_2^{-\bullet}$	Oxygen-centred radical with limited reactivity
Hydroxyl	$HO^{\bullet}$	Oxygen-centered radical with high reactivity
Peroxyl, alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Oxygen-centered by product of organic peroxides
Oxides of nitrogen	$NO^{\bullet}$, $NO_2^{\bullet}$	Oxidative by products of L-arginine, and cigarette smoke
Peroxynitrite	$ONOO^-$	Formed from the reaction of $O_2^{-\bullet}$ and $NO^{\bullet}$, damages biomolecules

Evidence from clinical studies suggests that free radicals play a strong role in the pathogenesis of heart disease. It has been reported that an increase in myocardial oxidative stress may cause adverse cardiomyopathic changes which may lead to depressed contractile function and ultimately heart failure (Singal et al. 1998). As well, current research indicates that oxidized low density lipoprotein (LDL)

cholesterol may be one of the initiating factors in the atherogenic process. Oxidized LDL cholesterol activates macrophage foam cell generation, stimulates platelet adhesion, and impairs the fibrinolytic and anticoagulant capacities of the endothelium (Figure 1.1). These events may increase the risk for developing arterial plaque, which may set the stage for myocardial infarction (Holvoet and Collen 1998, Heller et al. 1998). It has been found that body mass index (BMI) is significantly correlated to the oxidation of non-HDL (non-high density lipoprotein) lipoproteins, suggesting that obese individuals are particularly prone to lipid peroxidation, which may account for the increased risk of CVD with obesity (Van Gaal et al. 1998).

In addition to coronary events, other pathophysiological conditions are associated with oxidative stress. For instance, oxidative DNA damage may be important in the etiology of many cancers. DNA damage may result from free radical attack of cellular lipid components that generates reactive metabolites that may couple to DNA bases. The resulting DNA lesions are considered to be genotoxic and mutagenic (Poulsen et al. 1998, Marnett 2000). As well, nitric oxide metabolites have been found to adversely effect lung tissue. The reaction of the superoxide anion with nitric oxide generates the peroxynitrite radical, which in turn damages lung parenchyma (Suga et al. 1998). Further, the release of iron from the renal cortical mitochondria has been reported to increase oxidative stress on the kidneys, which has been associated with acute renal failure (Baliga et al. 1997). In addition, prolonged exposure to hyperglycemia leads to advanced glycosylation end products, thereby

increasing the oxidative load of people with diabetes, which has been implicated in the development of diabetic complications (Maxwell 1995).

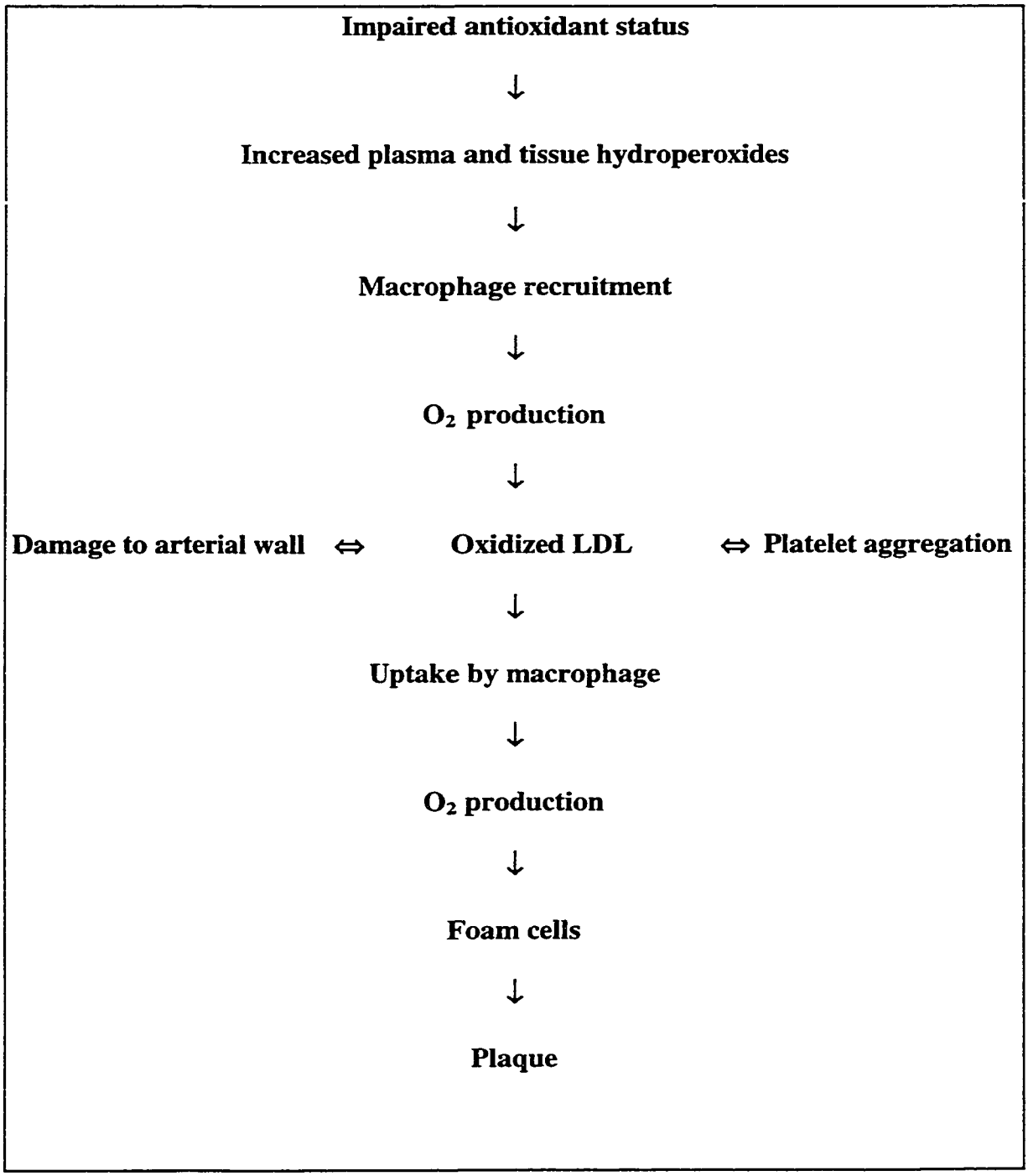

Figure 1.1: Flow diagram of the possible free radical attacks contributing to plaque formation (modified after Basu and Dickerson 1996)

Table 1.3: Diseases associated with oxidative stress (Maxwell 1995)

Cancer
Ischaemia-reperfusion injury
Drug toxicity
Inflammation:
rheumatoid arthritis, pancreatitis, and inflammatory bowel
Hematological disease
Neurological disease:
trauma, stroke, and Parkinson's disorder
Critical care medicine:
sepsis, hypoxia, trauma, and organ hypoperfusion
Ocular disease:
senile macular degeneration, cataract, and retrolental fibroplasia
Atherosclerosis

1.1 ANTIOXIDANT THEORY

Oxidative stress occurs in the body when the number of free radical species produced is greater than the number of available antioxidants. In the human body, antioxidant mechanisms exist to help maintain an internal balance of pro-oxidants to antioxidants, thereby minimizing oxidative stress. Antioxidants are defined as any substance or compound that prevents oxidation. Antioxidant sources in the body include the endogenous antioxidants such as selenium dependent glutathione peroxidase, copper-zinc dependent superoxide dismutase, catalase, uric acid and albumin (Betteridge 2000, Halliwell 1994).

1.2 ENDOGENOUS ANTIOXIDANTS

Given the extent of free radicals found in the human body and the potential for tissue damage, natural antioxidant defenses have evolved to counteract free radical attack. These defenses can be categorized into enzymatic and non-enzymatic functions (Betteridge 2000, Halliwell 1994).

1.2.1 ENZYMATIC ANTIOXIDANTS

SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) is an enzymatic antioxidant. Three forms of SOD exist in humans. These include extracellular SOD (EC-SOD), cytosolic Cu/Zn dependent SOD, and mitochondrial SOD (Mn-SOD) (Betteridge 2000, Mates et al. 1999). All forms of SOD act to quench the highly unstable superoxide anion (O_2^-) to the less reactive hydrogen peroxide radical (H_2O_2) and oxygen (O_2):

$$2O_2^- + 2H \rightarrow H_2O_2 + O_2$$

As well, SOD quenches single charged anions such as fluoride and azides. Certain disease states have been associated with SOD activity. For example, SOD levels tend to be higher in hepatitis patients, muscular dystrophy patients, and in patients with various forms of leukaemia. Hence, elevated SOD levels may be used as biomarkers for specific conditions (Gonzales et al. 1984, Kubsta et al. 1985, Stern et al. 1982). Whereas in cataractous lens, rheumatoid arthritis and immunodeficiencies, SOD activities have been found to be low (Fecondo and Augusteyn 1983, Yousseff 1983, Umeki et al. 1987). Thus, monitoring for potentially low levels of SOD in individuals that are prone to deficiency, may allow for preventative measures to reduce tissue damage.

GLUTATHIONE PEROXIDASE

Glutathione peroxidase (GPX) contains selenium, present as a selenocysteine residue, which is essential for its enzyme activity. GPX enzymes are found in most tissues, such as the testes, liver and kidneys, and are responsible for catalyzing the reduction of highly reactive lipid or other organic hydroperoxides. Consequently, in the presence of glutahione (GSH), GPX further reduces the hydrogen peroxide radical generated from SOD reactions, to produce H_2O and oxidized glutathione (GSSG). GPX has therefore been considered one of the most important antioxidant mechanisms (Mates et al. 1999) :

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

In addition to reducing hydroperoxides, GPX helps to regenerate ascorbic acid from its oxidized derivative, dehydroascorbic acid (Maxwell 1995).

Low levels of GPX activity have been found in people with alcoholism, cancer, cardiovascular disease (CVD), Crohn's disease and cystic fibrosis (Girre 1990, Salonen 1984, Therond 1988, Perona 1990, Rannem 1992). However, debate still exists as to whether deficiency in GPX gives rise to these diseases, or if the disease state itself compromises GPX status, or both scenarios.

In addition to GPX, catalases are found in the peroxisomes of many tissues and also aid in the catalytic removal of hydrogen peroxide from tissues.

1.2.2 NON ENZYMATIC ENDOGENOUS ANTIOXIDANTS

Transport or storage proteins such as transferrin, lactoferrin, and ceruloplasmin, act as important extracellular preventative antioxidants in that they bind metals such as copper and iron, thereby inhibiting free metals from promoting oxidative damage. As well, the catabolic waste products, uric acid and bilirubin, have been found to act as low molecular weight scavenging antioxidants (Halliwell 1994).

Overall evidence points to the fact that there exists endogenous antioxidant mechanisms, whereby the oxidants generated in the body could be potentially counteracted. However, if these mechanisms become overwhelmed, due to increased free radical load and or impaired antioxidant functions, other antioxidant sources may become increasingly more important in maintaining antioxidative homeostasis (Jacob and Burri 1996, Lampe 1999).

1.3 EXOGENOUS (CHAIN-BREAKING) ANTIOXIDANTS

Exogenous antioxidants, provided exclusively from dietary sources, such as vitamin C, tocopherols, carotenoids, isoflavones and phenolic compounds, complement the body's endogenous antioxidants in countering free radical attack (Betteridge 2000, Halliwell 1994, Carr and Frei 1999, Hessler et al. 1983). These small-molecule antioxidants are present in the body both at the intra and extracellular levels, and can

reach up to the millimolar range, as seen with vitamins E and C (Frei 1999). Dietary antioxidants, are called 'scavenging antioxidants' because of their ability to quench free radical chain reactions, and in turn become oxidized to products with very low reactivity. As a result, the antioxidant radical is unable to react with biological molecules, hence reducing tissue damage. The sacrificed (oxidized) antioxidant may then be reduced back to its original state, by some other reducing agent, so as to regain its redox function (Maxwell 1995).

1.3.1 PHYTOCHEMICALS

Consuming a diet rich in fruit and vegetables will provide, in addition to vitamins and minerals, many phytochemicals. These substances include phenolic compounds, terpenoids, phytates, phytosterols, saponins, indoles, lignans, protease inhibitors and isoflavonoids (Craig 1997, Dragsted 1999, Ziegler 1991). Although many phytochemicals have been recently identified, more remain to be found (ADA reports 1995). According to epidemiological evidence, phytochemicals may be contributing to the protective effects of fruit and vegetables against many diseases (Craig 1999). These non nutritive plant substances may work to counteract disease via antioxidant effects, stimulation of detoxification enzymes, decreased cell proliferation, and altered estrogen metabolism (Anderson and Smith 1999, Aldercreutz, Dragsted 1999).

1.3.2 ISOFLAVONOIDS

Isoflavonoids, unique to foods such as soy beans, chic peas, red clover and alfalfa, are a subclass of flavonoids, and their structural features include a flavone nucleus composed of 2 benzene rings which are linked through a heterocyclic pyrane ring. Genistein and daidzein are the predominant isoflavones (Messina 1999). There are roughly 1-3 mg of isoflavones/g soy protein. Therefore, a serving of soy food would provide approximately 25-40 mg of isoflavonoids (Messina 1999). The latter may act as antioxidants to reduce the peroxidation of lipoproteins. The rate of very low density lipoprotein (VLDL), LDL and HDL oxidation in CVD patients has been reported to be markedly reduced with soy protein (Kanazawa et al. 1995). In vitro studies have indicated that genistein and daidzein may be transported in LDLs and thus act in similar fashion to vitamin E in reducing in vivo membrane lipid oxidation (Anderson et al. 1999). Further, genistein has also been shown to protect against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced oxidative stress, and to significantly reduce the activity of glutathione peroxidase, catalase, glutathione reductase, and superoxide dismutase (Breinholt et al. 1999). The antioxidant properties of genistein may therefore stimulate a feedback mechanism on antioxidant enzymes, due to improved oxidative status in animals (Breinholt et al. 1999).

In addition to antioxidant properties, isoflavones reduce blood lipids. In an in vivo study, isoflavones significantly lowered both LDL and VLDL cholesterol by 30-40% in males and females, decreased total cholesterol to HDL cholesterol ratio in females

by 50% and in males by 20%, and raised HDL cholesterol in females by 15% (Anthony et al. 1996). Changes in hormonal metabolism may account for the hypocholesterolemic effects of isoflavonoids. Data from animal studies suggest that feeding soy protein to laboratory animals consistently increases plasma thyroxine levels (Forsythe 1995). It has been postulated that soy protein affects metabolism in a similar fashion to hyperthyroidism. Whereby, HMG CoA reductase activity, LDL receptor activity, and bile acid excretion increase and plasma lipids decrease. As well, soy protein has been associated with reducing the insulin to glucagon ratio, possibly preceeding the decrease in plasma cholesterol. The component in soy, responsible for these hormonal changes, still remains to be elucidated (Potter 1995).

1.3.3 PHENOLIC COMPOUNDS

Other polyphenolic compounds include flavonols, flavones, catechins, anthocyanidins, flavanones, and ferulic acid (Table 1.4). On a daily basis, human intake of total flavonoids is approximately in the hundreds of milligrams(Hollman and Katan 1999). Tea and red wine contain concentrated levels of these compounds, thus possibly accounting for the potential health benefits associated with the intake of these beverages (Bourne et al. 2000).

Table 1.4: Dietary sources of polyphenolic acids (Morton et al. 2000)

Source	Compounds
Red wine and grapes	Cinnamic acid derivatives, flavonoids
Tea	Gallic and caffeic acid, complex polyphenols
Apricots	Caffeic acid (as chlorogenic acid)
Cherries	Caffeic and gallic acids
Apples and peaches	Caffeic, coumaric and ferulic acids
Raspberry	Ellagic, gallic and cinnamic acids
Apple cider	Caffeic acid
Citrus fruit	Cinnamic acids
Olives, olive oil	Dihydroxyphenylethanol, oleuropeine
Alfalfa, cabbage, spinach	Cinnamic acids, mainly caffeic acid
Wheat	Cinnamic acid derivatives, minor amounts of coumaric and caffeic acids

However, in vivo antioxidant effectiveness of plant-derived phenolic compounds may depend on their bioavailability (Duthie and Crozier 2000). Plant phenolic metabolites are present in plasma and body tissues, and display appropriate pharmacokinetic properties to elicit a physiological response (Duthie and Crozier 2000, Terao1999, Bourne et al. 2000). As antioxidants, phenolic compounds may act to break free radical chain reactions, and quench reactive oxygen and nitrogen species, thereby

inhibiting oxidation of lipids and other biological molecules (Morton et al. 2000). The hydrogen-donating abilities as well as the metal-chelating properties of polyphenols may help explain their antioxidant potentials (Pannala et al. 1998). Moreover, similar to other scavenging antioxidants, oxidized polyphenols are relatively stable and thus less likely to initiate a chain reaction. Nevertheless, under certain circumstances, such as the presence of excess phenolic compounds, and/or transition metals, the potential for polyphenols to act as prooxidants is possible (Bravo 1998).

Although many in vitro studies indicate that plant phenolic compounds act as antioxidants, in vivo studies are still limited. This is especially the case with hydroxycinnamates (simple phenolic acids such as ferulic acid) which are usually present in plant foods at much higher concentrations, versus other flavonoids. Nevertheless, it has been reported that ferulic acid prevents LDL peroxidation suggesting possible peroxyl radical scavenging effects (Pannala et al. 1998). Ferulic acid may also possess stronger abilities to quench peroxynitrite radicals as opposed to other hydroxycinnamates, possibly indicating greater antioxidant properties. Indeed, in vivo studies are required to further explore the relative importance of phenolic compounds and their reducing properties, and their overall contribution to antioxidant defences.

1.3.4 CAROTENOIDS

More than 600 carotenoids naturally exist and include β-carotene, lycopene, α-carotene, lutein, canthaxanthin, and zeaxanthin. Carotenoids are lipophilic antioxidants and therefore stored in the LDL of the plasma. Supplementation with β-carotene increases the presence of β-carotene in the plasma LDL, and reduces cell-mediated oxidation (Dugas et al. 1999). Intensity of oxidative inhibition may be directly associated with the carotenoid concentration in the plasma. In addition, it has been speculated that different carotenoid compounds may have preferential scavenging properties, such that β-carotene may quench thiyl radicals more readily whereas canthaxanthin may selectively quench peroxy radicals. Thus, a mixed intake of carotenoids may offer better antioxidant defences against a multitude of free radicals versus a single carotenoid (Carpenter et al. 1997, Kontush et al. 2000). Recently, the lack of other carotenoids may account for the neutral or negative results seen in intervention trials with supplemental β-carotene in isolation (Steinberg and Chait 1998, Hininger et al. 1997). As well, β-carotene has demonstrated to be prooxidative in certain circumstances (Hininger et al. 1997). Instead, lutein, α-carotene, α- and β-cryptoxanthin and lycopene show antioxidant potentials that may be working to reduce both risk for lung cancer and cardiovascular events (Ziegler 1991, Hininger et al. 1997, Agarwal and Rao 1998).

1.3.5 VITAMIN AND MINERAL ANTIOXIDANT FACTORS

Nutrient antioxidants are also found in plant foods and include ascorbic acid, tocopherols, tocotrienols, zinc, and selenium. These vitamins and minerals have also been reported to be associated with the prevention of many degenerative diseases. However, unlike the phytochemical antioxidants, the nutrient antioxidants have been well studied and their roles are more clearly understood, in terms of their antioxidant potentials (Morton et al. 2000).

1.3.6 VITAMIN C (ASCORBIC ACID)

Vitamin C is a water soluble antioxidant vitamin that consists of two compounds, its reduced form L-ascorbic acid, and its oxidized form L-dehydroascorbic acid (Basu and Schorah 1982). The antioxidant potential of vitamin C may thus lower the risk for numerous diseases (Solzbach et al. 1997). Current research indicates that ascorbic acid significantly lowers the blood levels of isoprostanes, improves endothelium-dependent vasodilation in vascular smooth muscle, and prevents smoke-induced leukocyte aggregation, thereby reducing CVD risk (Solzbach et al. 1997, Reilly et al. 1996, Heitzer et al. 1999). As well, ischaemic heart disease patients have been found to have low levels of plasma ascorbic acid, and upon supplementation this was reversed. It is speculated that during the acute stages of myocardial infarction, many aqueous phase free radicals may be rapidly consuming vitamin C, accounting for its compromised status (Herbaczynska-Cedro et al. 1995). Also, it has been suggested

that maintenance of adequate plasma ascorbic acid is required to inhibit oxidative damage to the eye lens, thereby possibly reducing age-related cataract risk (Van der Pols 1999). Further, plasma levels of vitamin C were found to be inversely associated with gastric cancer mortality in Japan (Tsubono et al. 1999).

As an antioxidant, vitamin C functions in many ways to reduce oxidative stress. Ascorbic acid quenches many aqueous phase free radicals, such as nitrogen dioxide, superoxide anion, and singlet oxygen. Thus, ascorbic acid directly reduces oxidative stress in biological fluids, and acts as the first line of antioxidative defence in the plasma. Further, vitamin C helps to indirectly decrease cell membrane lipid peroxidation by recycling the α-tocopherol (a lipophilic antioxidant) by reducing its α-tocopheroxyl radical within the lipid bilayer. In addition, it has been suggested that ascorbic acid may be working to scavenge superoxide radicals in a similar fashion to superoxide dismutase (Herbaczynska-Cedro et al. 1995). Alternatively, in vitro studies have found that vitamin C may also react with free metal ions thereby forming the reactive alkoxyl and hydroxyl radicals, subsequently increasing oxidative stress. However, it is still unclear if this occurs in vivo (Carr and Frei 1999). Therefore, more in vivo studies are required to determine if ascorbic acid may become prooxidative in physiological conditions.

1.3.7 VITAMIN E (TOCOPHEROLS AND TOCOTRIENOLS)

Vitamin E is fat soluble and consists of both the tocopherols and the tocotrienols. Vitamin E deficiency has been characterized by lipid free radical chain reactions resulting in pervasive tissue damage. The predominant fat soluble antioxidant found in LDL is α-tocopherol, whereby LDL consists of a mean value of 6 mol vitamin E/mol. α-tocopherol thus becomes a potentially important lipophilic reducing agent. Other fat soluble antioxidants such as β-carotene, lycopene, and γ-tocopherol are also present in the LDL fraction, however, each of these antioxidants are only one-tenth that of α-tocopherol (Esterbauer et al. 1991, Princen et al. 1995).

According to epidemiological evidence, intake of vitamin E is inversely associated with risk for CVD (Rimm et al. 1996). It has been reported that men with higher dietary consumption of vitamin E and longer duration of intake (including supplementation) display the strongest protective effects against coronary risk factors (Keaney et al.1999). In vitro studies indicate a dose dependent response to α-tocopherol and individual variation, in relation to LDL oxidation (Princen et al. 1991, Esterbauer et al. 1995). Animal studies also show that vitamin E strongly attenuates copper-induced oxidation of β-VLDL (Stewart-Lee et al. 1994). It has been hypothesized that the reduced oxidation of LDL, and VLDL by vitamin E, possibly inhibits a cascade of events promoting necrotic foam cell development, and ultimately atherosclerotic lesion development (Keaney et al. 1999). As well, vitamin E has been found to enhance nitric oxide bioactivity, reduce proliferation of smooth muscle cells,

and inhibit the stimulation of protein kinase C, which may also account for its inverse association with CVD (Keaney et al. 1999). In addition to lowering CVD risk, Vitamin E was also found to significantly reduce the renal content of malondialdehyde, and SOD activity, suggesting potential protective effects against nephrotoxicity in rats (Abdel-Naim et al. 1999). Also, higher levels of plasma tocopherols was inversely associated with risk for age-related nuclear cataract in humans (Lyle et al. 1999).

Recently, it has been reported that the tocotrienols, natural analogues of tocopherol, exert hypocholesterolemic effects, anti-tumor, and anti-thrombotic properties. Moreover, tocotrienols were found to significantly improve total antioxidant status, and SOD activity in hypertensive rats (Newaz and Nawal 1999). Thus, other forms of vitamin E may also act as effective fat soluble antioxidants and therefore need to be further explored in relation to their redox potentials (Theriault et al. 1999). Nevertheless, more in vivo studies of vitamin E are required to investigate dose effects, and mechanism of action before conclusions can be made.

1.3.8 TRACE ELEMENTS

In addition to the antioxidant vitamins, trace minerals have also demonstrated antioxidant potentials. Zinc, an important mineral essential for both humans and animals, is found in all human tissues. Zinc plays an important structural role in metalloenzymes, thereby influencing metabolic processes such as protein metabolism

(Sandstead 1985, Wada et al. 1985). Increased oxidative status in animals, as a result of zinc deficiency, provided the first clues indicating that zinc may be involved in the antioxidative process. In theory, zinc can indirectly exert antioxidant properties (DiSilvestro 2000). For instance, long term intake of zinc has been shown to induce the metallothioneins, metal-binding proteins, which in turn exert antioxidant effects in many conditions, such as ethanol toxicity and radiation exposure (Maret 2000, Powell 2000). As well, zinc has been found to compete with metals, such as copper and iron, for site specific binding to a low molecular weight cellular component, thereby inhibiting formation of hydroxyl radicals (Powell 2000). Some other proposed antioxidant mechanisms for zinc include its structural presence in SOD, its ability to spare vitamin E, and its membrane stabilizing properties (Bunk et al. 1989, Bray and Bettger 1990, Davis et al. 1998, DiSilvestro 2000). In light of these findings, future studies are needed to determine if long term zinc supplementation can improve antioxidant status in zinc deficient conditions (ie. people with diabetes) and if vitamin C may work in synergy with zinc (DiSilvestro 2000).

Selenium, an essential trace mineral used by the body for protein synthesis, is also an integral part of the GPX enzyme. Deficiencies in selenium may therefore reduce the GPX activity in the body, consequently affecting antioxidant status (Stadtman 1987). The biochemical functions of selenium, similar to that of vitamin E, include the reduction of fatty acid peroxyl radicals. The related functional properties of selenium and vitamin E therefore attributes a sparing effect of each nutrient on the other. It has been found that plasma selenium is inversely associated with cancer incidence

(Salonen 1986, Clark and Combs 1986). As well, dietary deficiencies of selenium have been linked to other degenerative states such as heart disease, arthritis, pancreatitis, and AIDS (May and Pollock 1998, Bowrey et al. 1999). For instance, chronic pancreatitis disease has been associated with increased oxidative stress, and decreased glutathione and selenium status, which may contribute to pancreatic complications. Alternatively, the supplementation of selenium, may reduce the pain, the frequency of acute exacerbations, and the need for surgery, in chronic pancreatitis patients (McCloy 1998, Bowrey et al. 1999).

1.4 ANTIOXIDANTS IN ISOLATION-CONTRADICTORY RESULTS

Although many studies have consistently demonstrated that antioxidant rich fruit and vegetables reduce the onset of chronic disease, supplementations of synthetic antioxidant nutrients, in isolation, have shown inconsistent results. For instance, intake of a diet high in beta-carotene is inversely associated with lung cancer incidence and coronary events, however, supplemental intake of high dose beta-carotene, by asbestos workers and smokers, was found to increase the onset of lung cancer and mortality due to CVD (Vainio 2000). In order to explain this paradox, in vivo and in vitro studies suggest that the very high level of free radicals, in the lungs of smokers, initiates oxidized metabolites of beta-carotene, thereby promoting carcinogenesis.(Wang and Russel 1999). Further, in smokers who also drink alcohol on a regular basis, supplemental beta-carotene increases the risk for hepatotoxicity,

and pulmonary cancer. Ethanol prevents the metabolic conversion of beta-carotene to retinol, which may also account for these adverse effects (Leo and Lieber 1999).

The potential deleterious functions of supplementation in smokers is not only limited to beta-carotene. High intake of vitamin E may also cause problems. High dose intake of vitamin E in smokers was found to increase red blood cell peroxidation in vitro, and reduce plasma levels of vitamin C, which may consequently increase platelet activating factor in the plasma (Handelman 1997). In light of these findings, supplementation with high levels of vitamin E or beta-carotene is cautioned, particularly in smokers and those who regularly consume alcohol, until future studies prove otherwise (Albanes 1999).

In addition, current evidence indicates that supplemental intake of ascorbic acid may promote the onset of atherosclerosis. Although dietary intake of foods rich in vitamin C has been found to reduce arterial wall thickness, supplementation of vitamin C increases the arterial wall thickness in men and women, and the effects are dose dependent. At the lowest dose (up to 500mg/day), arterial wall thickness was 1.2-2.1 times greater, whereas at the highest dose (up to 3355mg/day) wall thickness was 2.7 times greater than those that did not consume supplements (Anonymous 2000). As well, ascorbic acid supplementation may be contraindicated in individuals prone to high iron stores. In theory, an excess of iron in the blood may give rise to non-transferrin-bound iron, which may induce lipid peroxidation and reduce antioxidant status. Hence, high doses of vitamin C is discouraged, so as to limit the absorption of

non-heme iron in these individuals. Further, in vitro studies demonstrate that ascorbic acid, in the presence of transition metals such as iron, may become prooxidative (Gerster 1999).

1.5 ANTIOXIDANT SYNERGY

It is well established that antioxidants work in a synergistic fashion. For instance, glutathione regenerates dehydroascorbic acid to ascorbic acid, and ascorbic acid is required to reduce the tocopherol radical to its antioxidant form (Fuchs 1998). The discouraging results attained with supplemental antioxidants in isolation may therefore be partly explained, due to the lack of other associated antioxidant factors found in vegetables (Bohlke et al. 1999).

In the highly oxidative environment of the lungs of smokers, β-carotene may become highly unstable. Ascorbic acid and vitamin E may both be required to protect β-carotene from becoming oxidized, and in turn β-carotene can exert chemopreventive properties. The vitamin C status in smokers is already compromised, as a result of plasma ascorbate depletion, thereby increasing the potential to oxidize β-carotene. Hence, upon high dose supplementation with β-carotene in smokers, the more likely that β-carotene can become procarcinogenic.

Alternatively, in vitro studies indicate that supplemental ascorbic acid maintains β-carotene in its antioxidative state (Wang and Russel 1999). As well, the tocopherols

have been found to stabilize carotenoids. An in vitro study found that the oxidation of β-carotene by smoke is reduced by up to 70 % with supplemental α-tocopherol versus no tocopherols. These findings are in line with a recent intervention study conducted in China, whereby combined supplemental intake of vitamin E, selenium and β-carotene decreased cancer mortality by 13% (Wang and Russel 1991).

In relation to cutaneous photoprotection, the use of combined antioxidant therapies has also been suggested to be better than singular antioxidants, in quenching reactive oxygen and nitrogen species and reducing skin photodamage. This includes the combined supplementation of vitamins C,E and β-carotene (Fuchs 1998).

In addition to the vitamin antioxidants, a synergistic reducing effect has been reported with phenolic compounds. Flavonoids prevent α-tocopherol, present in LDL, from being oxidized, and they also help to regenerate the α-tocopherol radical back to its reduced state, thereby sparing vitamin E (Bravo 1998, Carbonneau et al. 1997). Further, coumaric and caffeic acids were both found to act in synergy with ascorbic acid, which may partly explain the co-existence of these dietary factors in plant foods (Morton et al. 2000). As well, the combination of catechin with ascorbic acid was found to have an additive effect of their antioxidant capacities in vitro (Saucier and Waterhouse 1999).

There is also an interaction between trace elements, that may indirectly influence antioxidant status by altering endogenous antioxidant enzyme activity. For instance,

the adequate dietary intake of both Cu and Zn, may be required to attain optimal activity of Cu/Zn SOD and total SOD, in tissues such as the liver and the heart. As well, ceruloplasmin activity was reported to be at its maximum with moderately high dietary intakes of Cu, Zn and Fe. Further, low intake of dietary Zn may reduce the intestinal absorption of vitamin E, thereby decreasing liver vitamin E status (Roughead et al. 1999).

It is important to consider the potential synergetic roles that many antioxidant factors may play, in reducing oxidative stress. With this in mind, future studies that will assess for the combined effects of antioxidant nutrients are warranted, before conclusions can be made.

1.6 CONCLUSION AND OBJECTIVES OF THE PRESENT STUDY

There appears to be a substantial amount of evidence suggesting that the increased consumption of antioxidant rich fruit and vegetables, contributes many health benefits (Fraser 1999, Ziegler 1991). For instance, it has been found that high intake of vegetables decreases the risk for developing cancer by up to 50 %, or greater (Steinmetz 1996). Although many compounds found in plant foods, such as vitamins C, E and carotenoids, have demonstrated antioxidant properties that may account for these protective qualities, many pieces to the puzzle are still missing (Ziegler 1991). This is supported by the inconclusive results observed with isolated supplements (Hininger et al. 1997, Greenberg et al. 1994, Bloch and Thomson 1995). Hence,

recommendations to increase a singular antioxidant via supplementation, for improved antioxidant status, would be misguided. Rather, the increased intake of whole fruit and vegetables, containing a myriad of antioxidant compounds, working in synergy to prevent degenerative diseases, is highly encouraged (Bloch and Thomson 1995, Willet 1999 , Ziegler 1991).

Nevertheless, chronic disease states such as cancer, and CVD are of growing concern in affluent societies, and their incidence may be reduced if the general public were to make dietary and lifestyle changes, such as include more vegetables in the diet (Nutrition Recommendations 1990). Unfortunately, the general public is not consuming adequate quantities of fruit and vegetables. A 1991 survey demonstrated that North Americans consume on average 3.4 servings/day of fruit and vegetables, which is far from achieving the suggested 5-10 servings/day (Steinmetz 1996, Patterson 1990). In addition, only 23 % of American adults were reported to include 5 or more servings of fruit and vegetables in their daily diet (Steinmetz 1996, Patterson et al 1990). It has been suggested that the general public may be unaware of the likely benefits of plant foods, or may not be willing to include them, or are unable to have more of them due to economic factors. Thus, accounting for the reported low dietary intake of these foods (Patterson et al. 1990).

In response to the typically low intake of fruit and vegetables, the increased importance of these foods to attenuate disease pathologies, and the increased consumer demand, establishments such as the Center for Healthy Functional Foods

(CHeFF) have been developed. These centers are formulating convenience type nutraceutical and functional food products that incorporate fruit and vegetable extracts, that are compatible with the North American diet (Zammer 1995). As a result, these convenience foods, with added plant extracts, may help individuals to increase their intake of fruit and vegetables. For instance, gun-puffed vegetable snacks have been developed whereby naturally occuring carotenoids, and flavonoids have been added via fruit and vegetable concentrates. A serving of this snack food contains the amount of carotenoids equivalent to 1/3 serving of raw carrots (Zammer 1995). Many vegetable food concentrates have also been developed in the health food industry that are available over the counter in both capsule and powder forms. Some of these common products include the brand names such as Simons Super Mix, Enriching Greens, and Nu-Greens. Although in vitro studies demonstrate that food extracts have antioxidant capacities, in vivo studies are still lacking (Plumb et al. 1995). Therefore, clinical studies are warranted to evaluate for the antioxidant potentials as well as the safety of these food items, thereby justifying exploration in this area.

The present study was undertaken essentially to investigate the in vivo antioxidant potentials of "Green Factors" concentrate (GF) and germinated wheat powder (WP), in a select group of healthy subjects. GF is one of the commercially available supplements popularly used as a substitute for fruit and vegetables (see chapter 2). GF consists primarily of antioxidant rich plant food concentrates such as alfalfa, barley grass, wheat grass, carrot juice, bilberry extract, grapeseed extract and soy

protein extract. WP is a cereal sprout with increased nutrient value, with particular reference to antioxidants (see chapter 2).

It was hypothesized that the intake of both GF and WP would increase antioxidant status, and reduce oxidative stress in vivo, and that these effects would be synergistic and additive. Using a group of healthy human subjects, this hypothesis was tested with the following objectives:

- To define responses of Cu/Zn SOD and GPX , to GF and WP intakes
- To assess the modifying effects of GF and WP intakes on the biochemical status of the antioxidant trace elements Cu and Zn
- To examine the correlations between trace element status and endogenous antioxidant enzyme activity
- To determine the effects of GF and WP intake on plasma concentrations of thiobarbituric acid reactive substances (TBARS)
- To evaluate the influence of GF and WP on the plasma lipid status

CHAPTER 2. GREEN FACTORS AND GERMINATED WHEAT POWDER

2.1 Green Factors.

" Green Factors" (GF) is a green food concentrate which is produced by Natural Factors Nutritional products (Burnaby, BC), and is widely available in North American health food stores as a supplement powder of fruit and vegetables. This product consists of a variety of freeze dried plant juice extracts, many of which are of grass origin. The latter includes barley and wheat grasses, comprising 40 % of the total mixture in "Green Factors."

The cereal grass content of GF is of young origin. These grasses, in their young stages, are generally rich in macronutrients and antioxidants (Seibold 1990). Table 2.1 shows the comparison between wheat grass and whole wheat flour, in terms of their content of a select group of nutrients (Seibold 1990). The nutrient density, with particular reference to protein, dietary fiber, niacin, folic acid, and antioxidants such as ascorbic acid, β-carotene, and chlorophyll, is appreciably higher in wheat grass than in whole wheat flour (Seibold 1990). On a dry weight basis, total dietary fiber is 3.7 fold greater in wheat grass than in whole wheat flour. As well, folic acid is 2.6 times higher in wheat grass than the folic acid content of wheat flour. The antioxidant content of the latter was not detected, in contrast, an appreciable amount of ascorbic acid, β-carotene, and chlorophyll was detected in wheat grass, ranging from 51 to 543 mg/100 gm (Seibold 1990).

In addition to the cereal grasses, "Green Factors" consists of antioxidant rich ingredients, such as soya, mango, beetroot, artichoke, pineapple, cranberry juice, grape seed, and carrot juice powder, accounting for approximately 25 % of the supplement product. The remaining composition of "Green Factors" is made of extracts of herbs such as Ginkgo Biloba, Rosehips, Milk thistle, and Siberian Ginseng. Most of these herbs are generally considered to be rich in antioxidant value (Sutter and Wang 1993). The overall composition of the "Green Factors" supplement is given in Table 2.2.

Table 2.1 Nutrient content of Wheat Grass and Whole Wheat Flour*

Component	Wheat Grass	Whole Wheat Flour
Protein (gm)	32.0	13.0
Carbohydrates (gm)	37.0	71.0
Total Dietary Fiber (gm)	37.0	10.0
β-carotene (I.U.)	23 136	0.0
Ascorbic Acid (mg)	51.0	0.0
Folic acid (mg)	100.0	38.0
Chlorophyll (mg)	543.0	0.0
Niacin (mg)	6.1	4.3

Modified from Seibold 1990

Values expressed per 100 grams dry weight

Table 2.2 Composition of "Green Factors" * (F.D.(freeze-dried); A.D.(air-dried))

Alfalfa, Barley and Wheat Grass Juice Powder (F..D.)	37.4
Pure Soya Lecithin (99% oil free, 96% Phosphatides)	17.5
Carrot Juice Powder (F.D.)	8.0
Hawaiian Spirulina Pacifica	5.0
Apple Pectin Powder	5.0
CGF Chlorella (Broken Cell Wall)	3.0
Rhamnosus acidophilus (non-dairy probiotics)	2.4
Peace River Bee Pollen Powder	2.4
Freeze-dried Mango juice powder	1.2
Black Currant Powder	0.06
Dandelion Root Extract (A.D.)	1.5
Beetroot Extract (A.D.)	1.4
Siberian Ginseng Extract (A.D.)	1.0
Pacific Kelp powder	1.0
Artichoke Extract (A.D.)	0.47
Soya protein Extract	0.2
Bilberry Extract (A.D.)	0.12
Pineapple Extract (A.D.)	0.06
Cranberry Juice Extract (A.D.)	0.06
Rosehip Extract (A.D.)	0.05
Lycopene (from tomato source)	0.04
Milk Thistle Extract (A.D.)	0.6
Ginkgo Biloba Extract (A.D.)	0.12
Grape Seed Extract (A.D.)	0.06

Values are expressed in grams per 100 g of "Green Factors"

The "Green Factors" product, obtained from Natural Factors Nutritional Products (Burnaby, B.C.), was sent to the General Laboratories Division of SGS (Vancouver, B.C.) for it's macro and micronutrient analysis. According to the analysis, the green food supplement is a significant source of micronutrients, particularly antioxidants such as β-carotene, ascorbic acid and vitamin E (Table 2.3). In addition, minerals such as Cu, Zn and Mg are also present in appreciable quantities in this supplement. These inorganic elements are constituents of superoxide dismutase (SOD), which catalyses the removal of superoxide radicals (O_2^-).

$$2\ O_2^- + 2\ H^+ \xrightarrow{Zn\ Cu\ Mg\ \ SOD} H_2O_2 + O_2$$

As well, the "Green Factors" supplement contains a substantial amount of dietary fiber (20 g / 100 g).

Table. 2.3 Nutrient composition of "Green Factors" (per 100 g)

Carbohydrates (g)	35.1
Fat (g)	24.8
Protein (g)	27.4
Total Dietary Fiber (g)	20.5
Energy (Kcal)	473.0
β-Carotene (IU)	1588.0
Vitamin C (mg)	364.7
Vitamin E (IU)	141.2
Copper (ug)	440.0
Magnesium (mg)	223.5
Selenium (ug)	not detected
Zinc (mg)	7.06

2.2 Germinated wheat.

Germinated wheat is a cereal sprout that has been found to contain considerably higher levels of antioxidant vitamins and phenolic compounds, in relation to non-sprouted cereal grains (Price 1988). The germination process that can influence nutrient values involves, modifications to temperature, culturing media, steeping (soaking), germination time, and humidity (Price 1988). After harvesting the germinated wheat, all sprouts were rinsed with distilled water, thereby removing the testas, and were then stored for 2 hours at - 45°C, prior to drying. Germinated wheat samples were freeze dried (REPP freeze drier) to a 4 % moisture content. A pressure of 0.31 milli bar and a temperature of -5°C were applied for the drying procedure.

Thereafter, the dried wheat sprouts were ground in a UD-Cyclone mill. The resulting wheat powder was placed into zipper freeze bags and stored at -30°C, until further analysis could be completed (Yang et al. 2000).

Steeping wheat grains for 24 hrs in water at 16.5°C, and allowing to germinate for 7-8 days (in dark containers, at 98% relative humidity and 16.5°C) produced the most favorable sprouts, in terms of their antioxidant content (Yang et al. 2000). Vitamin C, β-carotene, and vitamin E content of wheat sprouts are thus increased by 5 fold, 3 fold, and 2 fold, respectively, after 7 days of germination. Ungerminated wheat grain, on the other hand, contains very little amounts of these antioxidant vitamins (Yang et al. 2000). The germination of wheat, also resulted in an elevated content of phenolic compounds, including ferulic acid and vanillic acid, of which a 50 % and a 200 % increase occured, respectively. These antioxidant phenolic compounds are considered to make up the largest group of antioxidants present in wheat sprouts (Yang et al. 2000). It has been suggested that during germination, an increase in cell wall biosynthesis of polyphenolics, and a reduction in wheat tannin content, may both account for the increased levels of phenolic acids found in germinated wheat (Maillard and Berset 1995, Cole et al. 1983). Table 2.4 gives the amounts of macronutrient, micronutrient, and phenolic acid contents of germinated wheat. Germinated wheat sprouts appear to be a potential dietary source of naturally occuring antioxidants. These include antioxidant vitamins, phenolic acids, and the inorganic elements that are involved in antioxidant enzyme activities, such as SOD.

Table 2.4 Nutrient content of Germinated Wheat*

Micronutrient (mg) :	Per 100 grams
Vitamin C	55.0
α-tocopherol	1.09
γ-tocopherol	0.15
β-carotene	3.11
Ferulic acid	93.24
Vanilic acid	1.29
Magnesium	155.0
Copper	0.55
Zinc	4.0
Selenium	<1.0
Macronutrient (g) :	
Carbohydrate	71.0
Protein	14.4
Fat	1.6
Calories (Kcal)	356
Soluble Fiber	17.2
Insoluble Fiber	0.5

Adapted from Yang et al, 2000.

* 7 day germination and 24 hour steeping

CHAPTER 3. MATERIALS AND METHODS

3.1 Participants

Strategies to attain participants involved newspaper advertisements, recruitment postings, and television announcements within the Edmonton area. 51 males, 30-68 years old, were initially screened, which resulted in 18 volunteers, who met inclusion/exclusion criteria (Table 3.1). Each subject provided informed consent for the study protocol. During the first week, subjects were instructed on the experimental details, and necessary information and materials were provided. The study protocol was approved by the Faculty of Agriculture, Forestry and Home Economics, Human Ethics Review Committee, at the University of Alberta.

The study was a total of 8 weeks in length consisting of four experimental periods (Table 3.3.). All subjects completed food frequency questionnaires (pre intervention and post intervention periods), and followed the same experimental protocol, thus, acting as their own controls. In addition, all participants were instructed to maintain their usual dietary intakes, with the exception of required supplementation, throughout the study. As well, in order to minimize experimental variation due to activity level, the subjects were asked to maintain their level of physical activity to pre intervention levels, for all experimental periods. No supplementation was taken during the first 2 weeks (control). This baseline period was followed by two weeks of taking green food concentrate. The latter was consumed twice daily with meals, as a

dietary supplement mixed with water (250 ml), whereby each dose level was 8.5 grams. During the next two weeks, the green food concentrate was administered in the same manner as previously described, however, in combination with germinated wheat powder, dose being 2.5 g twice daily. At the end of the supplemental period, there were two further weeks whereby study participants were kept under close supervision, so that they would continue to comply with the criteria required for the study. This period was called "washout period".

Table 3.1: Inclusion/exclusion criteria for participants to qualify for the present study

Inclusion Criteria:

- Individuals with borderline high to high plasma TC levels (> 5.2 mmol/L)
- males
- non - smokers
- low intake of alcohol (< 3 oz. Alcohol per week)
- individuals must reside within the greater Edmonton area, and be able to speak English

Exclusion Criteria:

- individuals that are exercising or dieting to lose weight, or persons who have lost a significant amount of body weight within 3 months prior to the initiation of the study
- persons with medical conditions that may alter blood lipid status such as thyroid disorder, liver problems (e.g. jaundice), diabetes, and gall bladder disease
- individuals that are taking or have taken hypolipidemic drugs within the last six months prior to start of study
- individuals that have an abnormal white blood cell, and or blood hemoglobin count
- individuals taking supplements such as vitamin, herbal or amino acid supplements (other than the supplements required for this study), that may influence blood lipid and or antioxidant status

Table 3.3: Experimental periods and their respective supplemental protocol for the present clinical trial.

EXPERIMENTAL PERIOD*	SUPPLEMENTATION PROTOCOL
• Baseline (control)	• Pre-supplemental period; subjects monitored to maintain required study criteria
• "GF" [1] supplementation alone	• Supplementation of Green factors (8.5g, twice daily); and supervision to ensure compliance to study protocol and acceptance to extract
• "GF" + WP[2] supplementation	• Supplementation of Green factors (8.5g, twice daily) plus germinated wheat powder (2.5g, twice daily); monitoring of the acceptance of the test supplements
• Post-supplementation	• Withdrawal of the supplements

* Each period is of 2 weeks duration; fasting blood samples were collected at the end of each period

[1] "GF", "Green factors" concentrate

[2] WP, germinated wheat powder

3.2 Blood Collections:

Following an overnight 12 hour fast, subjects were instructed to report to Dynacare Kasper Medical Laboratories, Southwest Edmonton, between the hours of 8:00 am and 9:30 am, in groups of 3 to 4 on a given day. In addition to blood withdrawals, interviews were regularly conducted with each participant, so as to monitor for the product acceptance, compliance and for any potential adverse effects, associated with the intake of the dietary supplements. Fasting heparinized (5.0 ml), and EDTA blood samples (7.0ml) were collected at baseline, post GF supplementation, post GF plus WP intake, and post washout period. The collected blood samples were transported on ice in a biohazard container from the Medical Laboratory (Kasper) to the University of Alberta, nutritional sciences biohazard area, where they remained on ice unless otherwise indicated. Thereafter, plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4 ° C. Within one hour of collection, the separated EDTA plasma was divided into aliquots and stored at -70 ° C, until laboratory analysis. The separated heparinized fresh plasma and erythrocytes were immediately used to determine antioxidant enzyme activities. All parameters measured in the study protocol were conducted in duplicate.

3.3 ANALYTICAL METHODS AND MATERIALS

3.3.1 Determination of Dietary Intake

Estimates of energy and macronutrient intake, including total lipids and total dietary fiber, were obtained by using a comprehensive, semiquantitative food-frequency questionnaire (FFQ) validated previously for the determination of individual dietary intakes (Bright-See E et al. 1994). Subjects were instructed to fill out a FFQ for baseline period and intervention periods (ie. GF alone and in combination with WP). Prior to the distribution of the FFQ's, details about the questionnaires (ie. standard portion sizes) were explained to the participants and inquiries regarding their use were responded to by a research coordinator. Standard portion sizes (ie. small, medium, or large) were assigned to each food category. Amounts of each food item were specified according to standard measures (i.e. 125, 250, or 375ml). To facilitate the estimation of portion sizes, food models and sample containers were presented to the study participants. Upon the collection of completed FFQ's, the research coordinator was available to respond to any questions or concerns regarding the questionnaires and their completion. The FFQ's were analyzed by a computerized nutrition software program (version 6.0, THE FOOD PROCESSOR; ESHA Research, Salem, Or). Final data analysis of the FFQ's for intervention periods included the supplemental intakes of GF and GF plus WP.

3.3.2 Antioxidant Status

Copper and Zinc dependent SOD and selenium dependent GPX activities were determined using kits (Cat. No. SD 125, and RS 505, respectively), which were obtained from Randox Laboratories, Montreal, Canada. Erythrocyte SOD activity was measured in hemolysate with the use of a whole blood SOD control (Cat. No. SD126), that ensured accuracy of this procedure. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red dye (formazan). SOD present in the erythrocytes competes with I.N.T. for superoxide radicals, thereby preventing the production of formazan dye (Fig. 3.0). The intensity of color produced from this enzymatic reaction was measured by spectrophotometry (PERKIN - ELMER Lambda 3B UV/VIS specrtophotometer) at an absorbance maximum of 505 nm, which was indirectly associated with the level of available SOD within blood erythrocytes. The SOD activity was thus measured by the degree of inhibition of this reaction.

$$\text{Xanthine} \xrightarrow{\text{XOD}} \text{Uric acid} + O_2^{\cdot -}$$

$$\text{I.N.T.} \xrightarrow{O_2^{\cdot -}} \text{Formazan dye}$$

OR

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \text{ (no dye)}$$

Figure 3.0: An overview of the enzymatic reactions involved in the SOD procedure (adapted from Randox SOD method Cat. No. SD 125)

The GPX activity was measured on whole blood following the procedure described by Paglia and Valentine (1967). A GPX whole blood control was utilized in this method (Cat. No. SC 692), thereby ensuring precision of results. In this procedure GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH, the oxidised Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$ (Figure 3.1). The whole blood GPX concentration was thus assessed by the decrease in absorbance at 340 nm (UV) due to the oxidation of

NADPH to $NADP^+$, measured by spectroscopy (DIODE - ARRAY UV/VIS spectrophotometer, Hewlett Packard).

GPX

$$2GSH + ROOH \rightarrow ROH + GSSG + H$$

GR

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

Figure 3.1: The enzymatic reactions associated with the analysis of whole blood GPX activity modified from Randox procedure Cat. No. RS 505

Lipid peroxide levels in plasma were assessed using a highly sensitive thiobarbituric acid (TBA) assay (Yagi K, 1987). This method involved the reaction of TBA with lipid peroxides. The precipitation of lipid peroxides in the plasma with hydrophilic TBA-reactive substances, at a pH of 3, were required to determine the level of malondialdehyde (MDA) present in the blood. This reaction produced a red pigment, indicative of the level of MDA, which was quantified fluorometrically (PERKIN - ELMER luminescence spectrometer, LS50B). The plasma content of malondialdehyde (MDA) was thus expressed in terms of the level of plasma lipid peroxidation.

3.3.3 Plasma Levels of Antioxidant Nutrients

Plasma trace element status, including Cu and Zn, was assessed by an atomic absorption spectrophotometry method (Peaston 1973). The plasma samples were diluted 1 in 5 with 10% v/v propan-1-ol, so as to reduce the risks for contamination, error, and viscosity differences between plasma samples and aqueous standards. The analysis of copper and zinc was detected on a Perkin-Elmer 4000 atomic absorption spectrophotometer (model 0097686) at wavelengths 324 nm and 213.8 nm, respectively.

3.3.4 Blood Lipid Profiles

Total plasma cholesterol was determined by an enzymatic kit supplied by Sigma Diagnostics (Cat. No. 352-20). This procedure is based on a method described by Allain et al. (1974). In comparison to other non-enzymatic methods, no pre treatment of plasma samples were necessary, only one reagent was used, a reduced interference and a greater specificity for total cholesterol is associated with this enzymatic assay (Tonks 1967). Hence, due to the simple applications and accuracy of this procedure, this method was employed for the current study.

Cholesterol esterase first hydrolyzes cholesterol esters in the plasma to free cholesterol. Cholesterol oxidase oxidizes the free cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide is coupled with the chromogen, 4-

aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to produce a quinoneimine dye which has an absorbance maximum of 500 nm (VIS) (Figure 3.3). The color intensity produced was directly proportionate to the total cholesterol concentration in the samples. A PERKIN ELMER UV/VIS Spectrophotometer Lambda 3 B was used to measure the absorbance of samples against the reagent blank.

Cholesterol esterase

Cholesterol Esters + H_2O $\rightarrow$ Cholesterol + Fatty Acids

Cholesterol Oxidase

Cholesterol + O_2 $\rightarrow$ Cholest- 4 -en- 3 -one + H_2O_2

2 H_2O_2 + 4 - Aminoantipyrine + p-Hydroxybenzenesulfonate

$\downarrow$ Peroxidase

Quinoneimine Dye + 4 H_2O

Figure 3.3: An overview of the reactions involved with the enzymatic determination of plasma total cholesterol (modified from Sigma Diagnostic Cholesterol procedure Cat. No. 352-20)

Plasma HDL levels were quantified with an enzymatic kit obtained from Sigma Diagnostics (Cat. No. 352-3). Unlike other methods, such as the ultracentrifugation procedure, this precipitation method is considered simple and effective for determining plasma HDL (Warnick et al. 1982). This procedure involved the use of dextran sulfate and Mg ions to precipitate LDL and VLDL in plasma samples by the formation of insoluble complexes, thereby isolating the HDL fraction in the remaining supernatant. An enzymatic method was then applied (Cat. No. 352-20) to determine the HDL concentration in the clear supernatant.

Enzymatic analysis of plasma triglycerides were determined using a procedure obtained from Sigma Diagnostics (Cat. No. 336). This method was a modification of the method described by Bucolo and David (1973). Triglycerides are hydrolyzed by lipoprotein lipase to form glycerol and free fatty acids. Phosphorylation of glycerol by ATP, yielded glycerol - 1 - phosphate (G- 1 - P) and ADP, which was catalysed by glycerol kinase (GK). The G - 1 - P was then oxidized by NAD, and the catalytic activity of glycerol - 1 - phosphate dehydrogenase, to produce NADH and dihydroxyacetone phosphate. Subsequently, the simultaneous oxidation of NADH and reduction of 2-[p-iodophenyl]-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) occured to produce formazan (INTH), a coloured compound (Figure 3.4). Formazan colour intensity was thus quantified, by PERKIN ELMER Lambda 3B spectrophotometry (vis) at a wavelength absorbance maximum of 500 nm, which was directly proportionate to plasma triglyceride status.

Lipoprotein Lipase

Triglycerides → Glycerol + Fatty acids

Glycerol Kinase

Glycerol + ATP → Glycerol-1-phosphate (G-1-P) + ADP

G-1-P dehydrogenase

G-1-P + NAD → DAP + NADH

Diaphorase

NADH + INT → Formazan + NAD

Figure 3.4: Schematic of the enzymatic determination of plasma triglycerides adapted from Sigma Diagnostic procedure (Cat. No. 336)

The plasma concentration of low-density-lipoprotein colesterol (LDL-C) (mg/dL) was accurately determined by a simple calculation that was validated by Friedewald et al. (1972). This method requires the accurate enzymatic determinations of plasma total cholesterol (TC), triglycerides (TG) and high-density-lipoprotein cholesterol (HDL-C). The plasma concentrations of TC, TG, and HDL can thus be used to calculate the plasma concentration of LDL cholesterol :

$$(TC) - (HDL\text{-}C) - (TG/5) = LDL\text{-}C$$

3.3.5 Statistical Analysis

The statistical significance of differences in erythrocyte SOD, whole blood GPX, plasma trace elements, plasma TBAR's, dietary intakes and blood lipids following dietary plant food supplementation was assessed by analysis of paired differences using one way ANOVA. The latter included Duncan's multiple range test,whereby each subject was compared to his own control values using SAS software (Version 6.12, SAS Institue, Cary, NC). The differences were judged to be statistically significant if the associated P value was < 0.05. The Pearson's correlation coefficient was used to quantify the strength of the association between plasma trace element status and erythrocyte SOD activity.

CHAPTER 4. RESULTS

Antioxidant and lipidemic responses to a plant extract called "Green Factors" concentrate (GF) were assessed in 15 male subjects (age: 51± 3.2) with hypercholesterolemia (> 5.2 mmol/L). To determine if the antioxidant and lipidemic responses to GF could be further improved, its supplemental effect was compared with the supplemental effect of GF plus germinated wheat powder (WP). The latter is known to be a concentrated source of antioxidant vitamins (see chapter 2.2). The study subjects underwent a 2 week baseline period at entry into the study. Following this period, they were given supplementation of GF (8.5 g twice daily) for 2 weeks followed by a combination of this plant food extract with WP (2.5g twice daily) for another two weeks. With the exception of the supplemental interventions, the subjects were instructed to maintain their usual pre intervention dietary intake throughout this study.

Due to scheduling conflicts, three subjects dropped out of the study, leaving 15 participants that completed the experiment. During the initial intake of GF, 2 out of the 15 subjects reported mild gastrointestinal discomfort, including lose stools and an increase in bowel motility, which subsided within the first 2 to 3 days of intake. No other adverse reactions were reported with the intake of the vegetable supplements. The particulars of the volunteers, at baseline, are presented in Table 4.1. The mean body mass index (BMI) of the participants was 29 ±1, reflective of a subject group that may be classified as overweight (Shils and Young 1990). After the supplemental

intakes, the BMI remained unchanged in comparison to the pre supplemental period (not tabulated). In addition, the baseline levels of mean total cholesterol was 6 ± 0.1, which suggests that this group of male participants are considered to be hypercholesterolemic (Canadian Consensus Conference on Cholesterol 1988).

Table 4.1: Baseline characteristics of the male subjects who participated in the current study [1,2]

	Value
Age (y)	51 ± 3
Body mass index (Kg/m^2)	29 ± 1
Total plasma cholesterol (mmol/L)[3]	6 ± 0.1

[1] Values are means ± SEM

[2] n = 15

[3] Total plasma cholesterol levels > 5.2 are categorized as hypercholesterolemic

Routine clinical blood chemistry measurements were conducted so as to determine the health status of the subjects at each treatment period. Basal measurements of hematological profiles, with particular references to hemoglobin and white blood cell counts, were found to be within the expected normal range for males. None of the supplemental intakes had any appreciable influence on these parameters (Table 4.2).

Table 4.2: The mean white blood cell and blood hemoglobin levels (±SEM) of 15 male subjects in response to "Green Factors" concentrate (GF) supplementation either alone or in combination with germinated wheat powder (WP)

Intervention period	White blood cells* (10^9/L)	Hemoglobin** (g/L)
Baseline	$5.6^a \pm 0.2$	$149^a \pm 0.7$
Post GF	$5.9^a \pm 0.2$	$149^a \pm 0.7$
Post GF+ WP	$5.9^a \pm 0.2$	$149^a \pm 0.7$
Washout	$5.8^a \pm 0.2$	$150^a \pm 0.7$

* Normal value 4 - 11

** Normal value 135 - 75

In each column, values not sharing a common superscript letter are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

4.1 "GREEN FACTORS" ALONE OR IN COMBINATION WITH GERMINATED WHEAT POWDER

Using food frequency questionnaires, the daily dietary intakes of total calories, dietary fiber and lipid were measured at the end of baseline, "green factors" (GF) and GF plus germinated wheat powder (WP) intakes. The purpose of measuring these intakes after the three study periods was to determine the consistency of the subjects' dietary intake during the treatments as well as to determine if these intakes would be modified by

the intake of the vegetable supplements. In comparison to baseline, the supplemental intake of GF for 2 weeks had no effect on the dietary intake of total calories or lipids (Table 4.3). On the other hand, the consumption of total dietary fiber was substantially elevated by 20 % post supplemental intake versus the pre intervention level. With the addition of WP to the intake of GF, the intakes of total fat and dietary fiber were unchanged in comparison to the supplementation of GF. The latter was also shown with the caloric intake, which was similar for both supplemental periods. The raised intake levels of total dietary fiber following supplementation of the plant food supplements, did not appear to be reflective of daily basal diet but the supplementation of GF and WP.

Table 4.3: Daily dietary intake (mean ± SEM) of study subjects (n = 15) in response to supplemental intakes of GF and GF plus WP

Nutrient	Baseline	Post GF	Post GF plus WP
Energy (C)	2003 ± 43^{a}	2091 ± 43^{a}	2109 ± 43^{a}
Total fiber (g)	24 ± 2^{a}	29 ± 2^{b}	30 ± 2^{b}
Total fat (g)	63 ± 1^{a}	66 ± 1^{ab}	67 ± 1^{b}

Values in each row not sharing the same superscript letter are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

Superoxide dismutase is an endogenous antioxidant enzyme responsible for the catalytic removal of superoxide anions. Erythrocyte SOD activity was thus measured

as an indicator of inhibition of superoxide radical formation. The supplemental intake of GF for 2 weeks significantly ($P < 0.05$) increased mean erythrocyte SOD activity by 11 % in comparison to baseline levels in the hypercholesterolemic subjects (Figure 4.1). An individualized analysis revealed that with the exception of one subject, all study participants displayed a positive response in the activity of the dismutase enzyme to GF, ranging from 2-20 % (Figure 4.2). After the addition of WP to the intake of the green supplement, the SOD activity was higher than the basal levels but unchanged compared to the values associated with GF intake (Figure 4.1). When the supplemental intakes were withdrawn for 2 weeks, the levels of erythrocyte SOD activity was decreased to its baseline level.

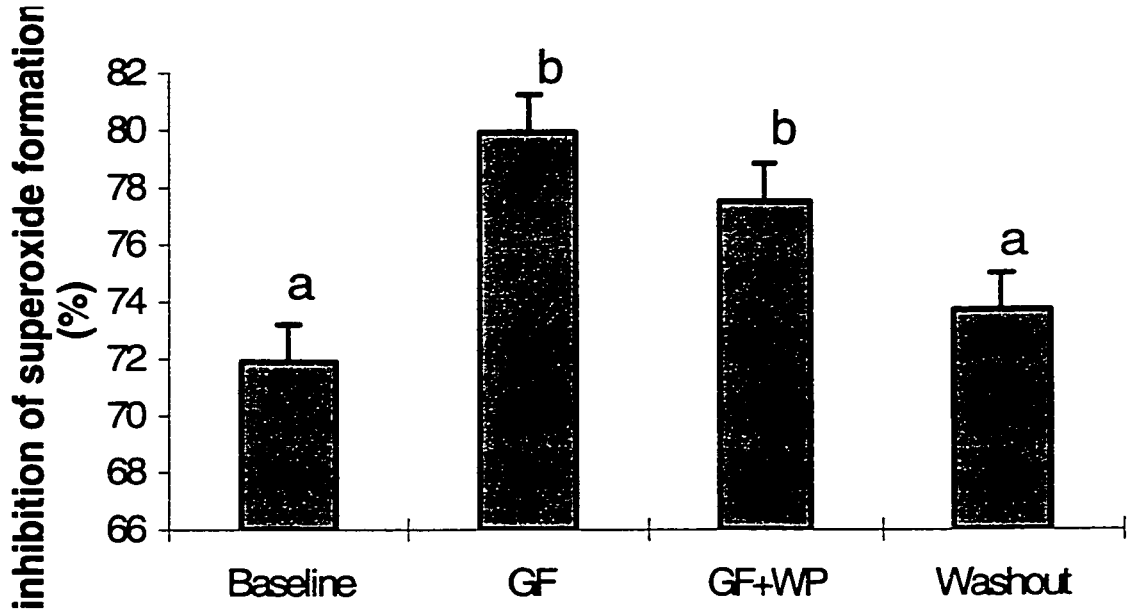

Figure 4.1: The mean percentage inhibition (± SEM) of superoxide radical formation in response to "green factors"(GF) supplemental intake for 2 weeks and GF plus germinated wheat powder for 2 weeks

* Columns not sharing the same superscript letter are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

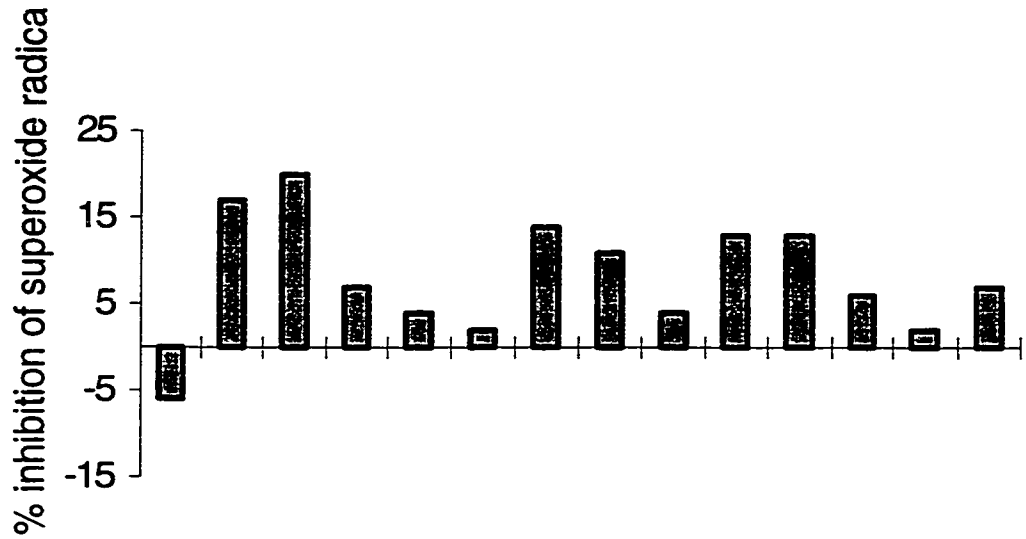

Figure 4.2: An individual analysis of the effect of "green factors" on erythrocyte SOD activity

* Each line represents the change in an individuals erythrocyte SOD activity (n=14) after "green factors" supplementation (2 wk) in the present study.

Since copper and zinc are integral parts of SOD, the plasma levels of these trace elements were determined in the study subjects. The supplemental intake of GF for 2 weeks resulted in a significant ($P<0.05$) increase in mean plasma zinc levels (Figure 4.3). According to the individual analysis, 11 out of 15 subjects (73 %) had displayed an elevated response in plasma Zn concentrations with the GF supplement (figure 4.4). When the supplemental intake of GF was combined with WP for 2 weeks, no additive effect was attained on the plasma zinc levels (Figure 4.3). After a washout

period of 2 weeks, however, plasma concentration of Zn was significantly reduced ($P < 0.05$) to its baseline level (ie. Pre-supplemental level).

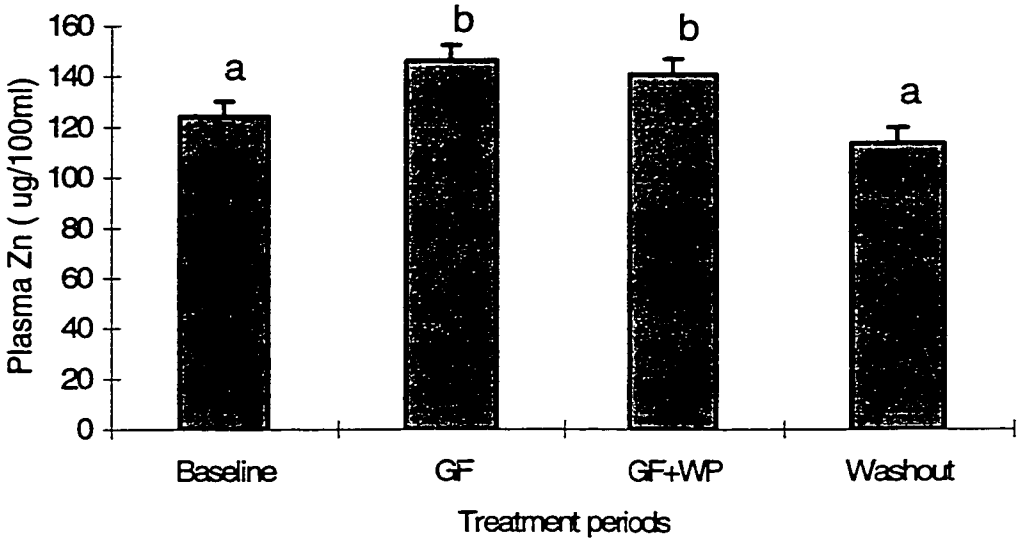

Figure 4.3: The response of mean plasma zinc concentrations (± SEM) with supplemental intakes of GF and GF plus WP in hypercholesterolemic subjects
Columns not sharing the same superscript letter are significantly different at $P < 0.05$ which was analyzed by Duncan's multiple range test

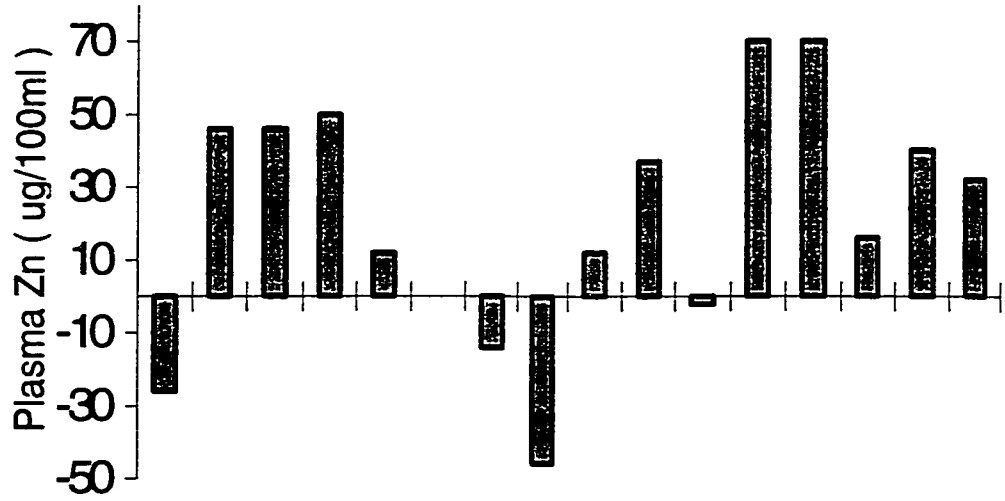

Figure 4.4: An individual Analysis of the effect of "green factors" intake on plasma Zn levels (μg/100ml)

Unlike the effects observed for Zinc, the mean plasma copper concentrations were not influenced, at a statistically significant level by the intake of either GF or GF plus WP supplementation (Table 4.4).

Table 4.4: Mean (± SEM) plasma Cu status in 15 male subjects, in response to "green factors" (GF) alone and in combination with germinated wheat powder (WP) for 2 weeks.

	Plasma Cu (ug/100ml)	
Diets	Mean	SEM
Baseline	106.2[a]	± 2.0
GF	107.2[a]	± 2.0
GF plus WP	109.2[a]	± 2.0
Washout	108.5[a]	± 2.0

Columns not sharing the same superscript letter are significantly different at $P < 0.05$ (Duncan's multiple range test)

The correlation between erythrocyte SOD activity and plasma concentrations of either Zn or Cu is shown in table 4.5. Neither Zn nor Cu concentration in plasma was significantly correlated with the antioxidant enzyme activity.

Table 4.5: Pearson correlation coefficient between plasma trace elements (Cu and Zn) and erythrocyte SOD activity

	Baseline		Post GF		Post GF+WP	
	r	p-value	r	p-value	r	p-value
SOD vs. Zn	0.18	0.54	-0.10	0.76	0.12	0.68
SOD vs. Cu	0.18	0.53	0.14	0.64	0.30	0.30

SOD: copper-zinc dependent superoxide dismutase.

Glutathione peroxidase (GPX) is a selenium dependent antioxidant enzyme which is involved in the removal of H_2O_2. In contrast to SOD, the mean whole blood GPX activity remained unaffected by the intake of GF alone or in combination with WP (Table 4.6). There were, however, a small proportion of the study participants (27 %) that showed a positive response to post supplemental intake with GF (Figure 4.5).

Table 4.6: The influence of GF intake alone and in combination with WP on the levels of whole blood GPX in 15 male subjects with hypercholesterolemia

	GPX levels (U/L whole blood)	
Diets	Mean	SEM
Baseline	122.0[a]	± 7.3
GF	105.9[a]	± 7.3
GF+WP	112.2[a]	± 7.3
Washout period	113.4[a]	± 7.3

Columns not sharing the same superscript letter are significantly different at $P < 0.05$ (Duncan's multiple range test)

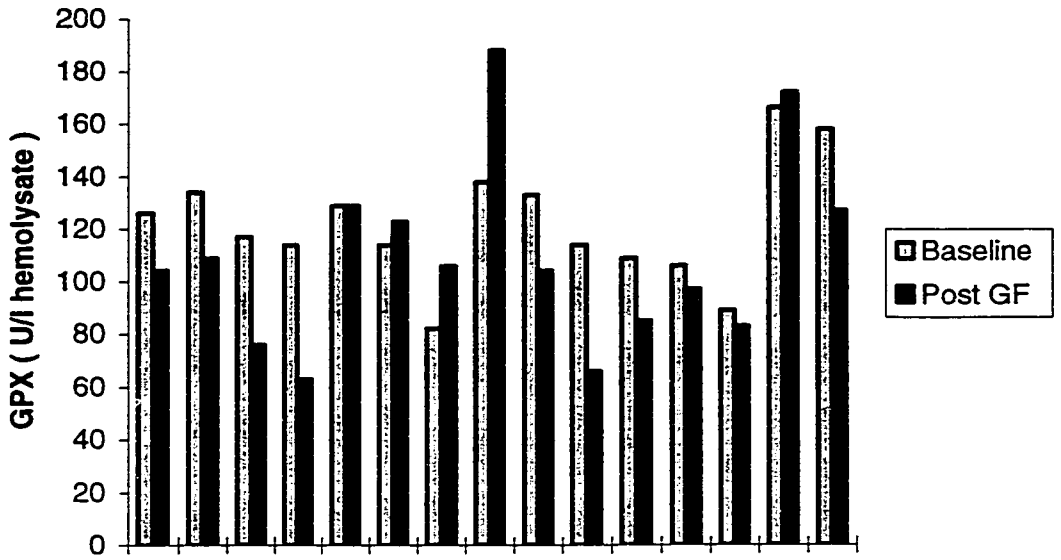

Figure 4.5: An individual analysis of the response in GPX activity after the intake of GF for a period of 2 weeks

The plasma level of malondialdehyde (MDA) is an indicator of lipid peroxidation. The supplemental intake of GF for 2 weeks had lowered the mean plasma MDA by 11 %, when compared to the mean baseline level (Table. 4.7). Although this reduction was not statistically significant, the reduction in plasma MDA was evident among 71 % of the participants (Figure 4.6). The addition of WP to the supplemental intake of GF failed to show any further reductions of plasma MDA concentrations (Table 4.7). After the 2 week cessation of supplemental intake, a trend of increased levels of plasma MDA was evident among 10 of the 15 study participants (Figure 4.7).

Table 4.7: The effects of the intake of supplemental intakes of GF and GF in combination with WP on plasma levels of TBARS (mean ± SEM) in male subjects with hypercholesterolemia

	Malondialdehyde (nmol/ml)	
Diets	Mean	SEM
Baseline	2.9[a]	± 0.2
GF	2.6[a]	± 0.2
GF+WP	2.5[a]	± 0.2
Washout	3.0[a]	± 0.2

Columns not sharing the same superscript letter are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

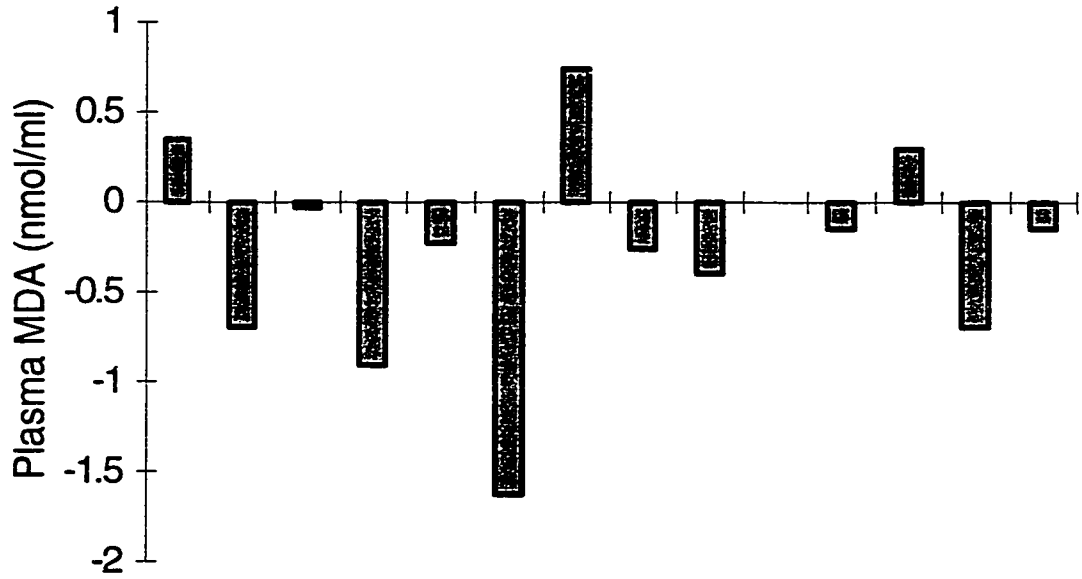

Figure 4.6: An individual analysis of the effects of "green factors" intake on the levels of plasma TBARS in 14 male subjects with hypercholesterolemia

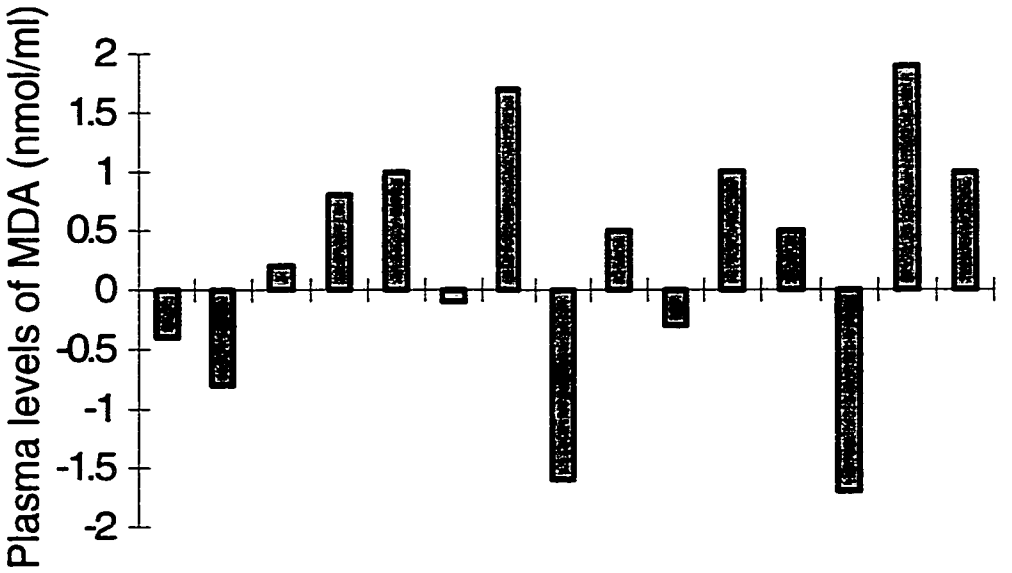

Figure 4.7: An individual analysis of the withdrawal of GF + WP supplementation and its effects on plasma levels of malondialdehyde (MDA)

The influence of the supplemental intake of GF and GF plus WP on the mean blood lipid levels of the hypercholesterolemic subjects are summarized in table 4.8. The supplemental intake of GF resulted in significant decreases ($P < 0.05$) in both total cholesterol and LDL-cholesterol concentrations in the plasma. The plasma concentrations of HDL-cholesterol and triglycerides, however, remained unaffected in the presence of the supplemental intake (Table 4.8). Although the mean HDL-cholesterol concentration was unresponsive to GF, its ratio with LDL-cholesterol was significantly ($P < 0.05$) elevated by 8 % versus the basal level (Figure4.8). Combining germinated wheat powder with "green factors" as a dietary supplement failed to show any additional effect on the lipid responses (Table 4.8) to GF alone,

except for the mean plasma HDL concentration, which was decreased ($P < 0.05$). The lipidemic response to the supplemental intake remained unchanged following a washout period of 2 weeks. In contrast, the mean washout HDL/LDL ratio was significantly lower ($P < 0.05$) than the ratio associated with the GF supplementation (Figure 4.8).

Table 4.8: The effect of supplemental intake of GF (2 weeks) and GF plus WP (2 weeks) on blood lipid status (mean ± SEM) in hypercholesterolemic male subjects (n = 15).

Diets	T-Chol (mmol/L) Mean	HDL-Chol (mmol/L) Mean	LDL-Chol (mmol/L) Mean	Triglycerides (mmol/L) Mean
Baseline	6.03 ± 0.1^{a}	1.06 ± 0.2^{ab}	3.84 ± 0.1^{a}	2.49 ± 0.1^{a}
GF	5.71 ± 0.1^{b}	1.11 ± 0.2^{a}	3.48 ± 0.1^{b}	2.47 ± 0.1^{a}
GF+WP	5.65 ± 0.1^{b}	1.03 ± 0.2^{b}	3.52 ± 0.1^{bc}	2.41 ± 0.1^{a}
Washout	5.96 ± 0.1^{ab}	1.04 ± 0.2^{ab}	3.79 ± 0.1^{ab}	2.49 ± 0.1^{a}

T-Chol: Total cholesterol; HDL-Chol: high density lipoprotein cholesterol; LDL-Chol: low density lipoprotein cholesterol

Mean values within a column not sharing the same superscript letter were significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

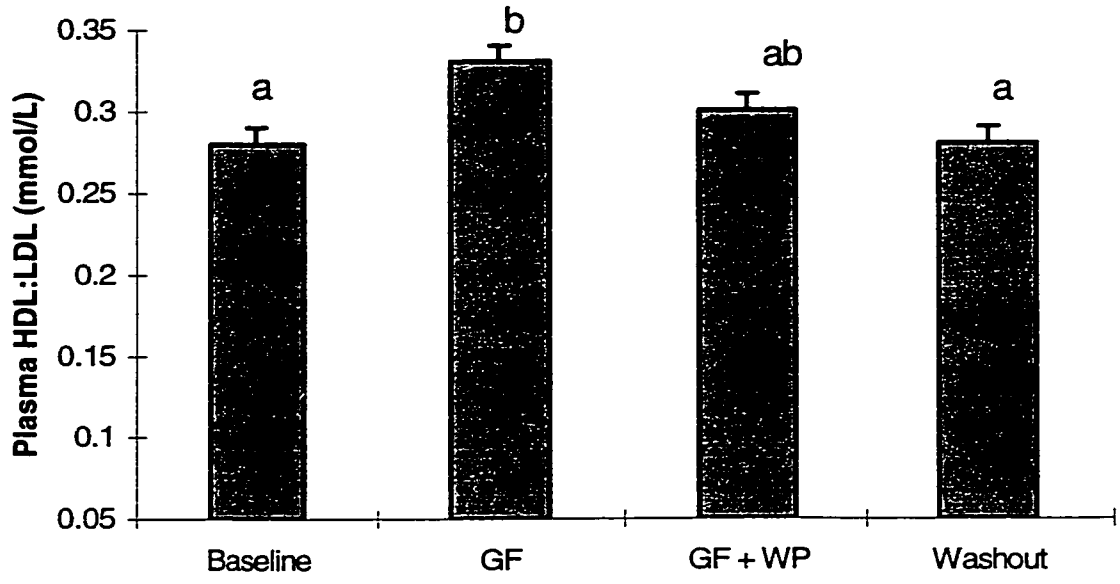

Figure 4.8: The mean (± SEM) HDL/LDL ratio in response to supplemental intakes of GF and GF plus WP

Columns not sharing the same superscript letter are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test

CHAPTER 5. DISCUSSION

High intake of fruit and vegetables has been found to provide antioxidant properties (Lampe 1999). For this reason, the availability of fruit-and vegetable-based supplements have been increasing in the commercial market (Zammer 1995). However unlike whole fruit and vegetables, the antioxidant effects of many of these plant-based supplements are largely unknown. "Green factors" concentrate (GF) is sold as a vegetable substitute and has been readily available in the health food industry for several years. The present clinical study was undertaken essentially to assess the antioxidant and lipidemic responses to GF and to determine if the combined intake of GF plus germinated wheat powder (WP) would further increase these responses.

An improved antioxidant status was evident with the supplemental intake of GF in a select group of hypercholesterolemic male subjects. Since the dietary intakes, as shown by consistant levels of caloric intakes with each dietary period, were found to be unchanged, it appeared that the changes observed with the antioxidant and lipidemic parameters measured were due to the supplemental intakes as opposed to the basal intakes. The latter is also reflected by the similarities in BMI associated with the pre supplemental intakes as well as the post supplemental intakes of GF plus WP. A micronutrient analysis of the GF supplement (Chapter 2) indicated that it contained substantial levels of antioxidant nutrients, including vitamins C and E, carotenoids, copper, and zinc. The GF supplement was thus considered to be an

antioxidant-rich vegetable supplement with the potential to increase the dietary intake of the latter nutrients. These findings are in parallel with other studies in which fruit and vegetables have been found to provide rich sources of antioxidants to the daily diet (Rauma et al. 1995, Cao et al. 1998).

Of the parameters measured to determine antioxidant status, the erythrocyte SOD activity was found to be most responsive to the GF intake. The SOD activity was thus increased by 11 % after a 2 week intake of GF. This association was further supported by the fact that after the withdrawal of supplementation, the levels of dismutase activity were reduced to its pre-supplemental level. This antioxidant enzyme quenches superoxide radicals, thus reducing the degree of oxidation (McCord 1995, Bannister and Rotilio 1987, Ornoy et al. 1999). The increased level of SOD, therefore, suggests a greater level of endogenous antioxidant defense associated with the supplemental intake of GF (Delmas-Beavieux et al. 1995). Similarly, in another study, long term adherents to a strict uncooked vegan diet were reported to have a significantly higher level of mean erythrocyte SOD activity in comparison to omnivores. The increased activity of the antioxidant enzyme was also attributed to the higher intake of antioxidant-rich food in the vegetarians (Rauma et al. 1995). Thus the SOD-response to GF is in accordance with that reported in relation to a fresh fruit and vegetable intake (Hininger et al. 1997).

The SOD enzyme requires Zn for its antioxidant role. Sprouts and vegetables are generally considered to be good sources of the trace element (Lampe 1999). GF

contains 1.2 mg of zinc per daily serving which contributes approximately 20 % of its daily requirement (Nutrition Recommendations 1990). Hence GF becomes an important source of dietary zinc. The zinc content of the green supplement was reflected by its raised plasma concentrations in the study participants receiving the supplement (8.5g twice daily) for 2 weeks. As seen with SOD, after the washout period the mean plasma value for the trace element was reversed to its baseline level, further confirming GF's potential as a dietary source of zinc. The GF-associated increase in plasma zinc levels may have influenced the SOD activities in erythrocytes. Low zinc status has been known to affect SOD activity (Rauma et al.1995, Richard et al. 1991). On the other hand it has been found that trace elements, particularly copper and zinc, may potentially induce the activities of the SOD enzyme. Sufficient levels of both dietary Cu and Zn were reported to be associated with achieving maximum SOD activities (Roughead et al. 1999). Furthermore, calves fed supplemental Cu and Zn, 25 ppm and 100 ppm respectively, showed a higher level of SOD activity versus control animals (Mates et al. 1999). Although the mean plasma copper level was not changed in the study subjects, a significantly ($P < 0.05$) elevated level of plasma zinc was evident in association with the intake of GF concentrate.

It has been suggested that zinc may act directly and indirectly as an antioxidant. Acute intake of dietary zinc has been shown to protect sulfhydryl groups from free radical attack. As well, long term intake of zinc induces other endogenous antioxidants such as the metallothioneins (DiSilvestro 2000). Zinc salts have also been reported to exert radical scavenging properties in vitro (Bagchi et al. 1997).

Incubations with 10 μM, 25 μM, 50 μM, 100 μM, and 200 μM of Zn DL-methionine have thus caused an approximate inhibition of superoxide anion production by 6%, 18%, 40%, 52% and 58%, respectively (Bagchi et al. 1997). It seems possible that the increased mean plasma Zn levels found with the supplemental intakes of GF may have acted to scavenge superoxide radicals, thereby sparing SOD enzymes, and thus potentially elevating the erythrocyte levels of the enzyme.

Although 75% of the study participants exhibited increased levels of both plasma zinc and erythrocyte SOD post GF intake and that their mean levels were found to be significantly ($P < 0.05$) elevated, the correlation between the two indices was not found to be statistically significant. This finding is contrary to other studies showing a positive correlation between the two antioxidant indices (Zbronska et al. 1995). Unlike the present study, other studies included a greater number of subjects, and the intervention period was much longer (Magalova et al. 1999). Future research requires a larger study population taking the GF concentrate for a longer period of time so as to further explore the relationship between trace elements and superoxide dismutase activity.

Glutathione peroxidase (GPX) is another important antioxidant index, which is active in removing hydrogen peroxide thereby preventing the generation of free hydroxyl radicals (Yagi 1987). Unlike SOD activity, dietary intake of the green food supplement did not alter the activities of whole blood GPX. In agreement with these results, it was reported that a higher intake of fruit and vegetables altered the activities

of SOD but not GPX (Rauma et al. 1995). However, other studies have found significant changes in the levels of GPX with the higher intake of antioxidant rich food which was attributed to the improved status of selenium (Aydemir et al. 2000, Bartfay et al. 1998, Lampe 1999). GPX activity is dependent on plasma selenium status and has been found to be positively correlated to the plasma concentrations of the latter trace mineral (Richard et al. 1991, Lampe 1999). Hence, given the lack of selenium content within the green food supplement (see chapter 2), it is of no surprise that GPX status was not significantly altered. In support of these findings, most fruit and vegetables are not particularly high sources of selenium, which would explain the lack of this trace element in the GF concentrate (Lampe 1999).

The plasma concentrations of thiobarbituric acid-reactive substances (TBARS) are often used to determine the lipid oxidation status (Yagi 1987). Reduced plasma TBARS have been reported in human subjects receiving high dietary fruit and vegetable intakes, indicating decreased lipid oxidation (Bruce et al. 2000, Rauma et al. 1995,Vang et al. 1997). Supplementation with dried fruit and vegetable juice extracts from sources such as carrots, beets, parsley, kale, spinach, broccoli, cabbage, apples, oranges and tomatoes have also been reported to decrease plasma lipid peroxides from 16.85 to 3.13 umol/L in 15 subjects (Lampe 1999). Furthermore, an increased intake of fruit and vegetables from 4 to 9 servings per day has been shown to significantly reduce breath pentane levels, reflecting inhibition of oxidative stress (Jacob 1999). The plasma levels of TBARS were not modified by the intake of GF for 2 weeks, at least, at a statistically significant level. There was, however, a

decreasing trend in plasma TBARS concentrations found among 79% of the GF supplemented subjects.

Elevated plasma cholesterol is one of the risk factors for cardiovascular disease (CVD) (Frost et al.). Dietary intake of the GF supplement for 2 weeks resulted in significant reductions in plasma total cholesterol and LDL cholesterol, and was accompanied by an increased ratio of HDL to LDL-cholesterol. In agreement with these findings, epidemiological evidence reveals that populations that consume greater quantities of plant food have lower plasma cholesterol concentrations (Anderson and Hanna 1999). Food of plant origin, such as fruit and vegetables, are generally considered to contain substantial amounts of dietary fiber (Anderson 1986). Diets high in fiber content are thus known to modify blood lipid status (Appleby et al. 1999). Two weeks of supplemental intakes of GF increased significantly ($P < 0.05$) the intake of dietary fiber by 20 % of that provided by the basal diet. It is, therefore, possible that the greater intake of dietary fiber associated with the GF supplementation, was responsible for its hypolipidemic effects (Haskell et al. 1992, Davidson et al., Marlett et al.1994). The latter is supported by another study that reported reductions in serum cholesterol and LDL cholesterol levels by 13 and 14 %, respectively, in hypercholesterolemic men that supplemented their diet with oat bran for 10 days (Anderson 1986).

The addition of an antioxidant rich food source such as germinated wheat powder (WP) to the supplemental intake of GF concentrate failed to show any additive effects

on antioxidant status, with particular reference to SOD activity, TBARS, and plasma concentrations of copper and zinc. These results are similar to a recent study where total antioxidant capacity was maximized at a dose level of 60 mg of supplemental vitamin C/day for a period of 2 weeks. An increase in the dose level to 6 g of vitamin C/day had no further effect (Anderson and Phillips 1999). It is possible that the antioxidant status reached its maximum peak level with the GF supplementation. The combination of this supplement with WP did not incur any additive effect on the antioxidant status. These results are also supportive of a recent study reporting that the extent to which antioxidant concentrations can be elevated within tissues, is limited (Rauma et al. 1995). The underlying mechanism responsible for limiting the tissue saturation of antioxidants is unclear, however, factors such as absorption and excretion of dietary antioxidants may play a role in this process (Anderson and Phillips 1999). For instance, the absorption of vitamin C in the small intestine is facilitated by an active carrier-mediated transport system. This system works most efficiently at low concentrations, however, when mucosal concentrations of the vitamin is greater than 6 $mmol^{-1}$, the latter transport system becomes saturated; thus, potentially explaining the decrease in the percentage of vitamin C absorbed with increasing intake of the antioxidant vitamin (Basu and Dickerson 1996). In addition, with increasing intake of vitamin C the kidney tubules also become saturated thereby elevating the amount of vitamin C excreted in the urine (Basu and Dickerson 1996). It is therefore possible that the addition of WP to the intake of GF had not further altered antioxidant status due to antioxidant saturation of body tissues, which may

have consequently affected the absorption and excretion of the antioxidants provided by the supplemental plant concentrates.

No additional effect on blood lipids was evident with the combined intake of GF plus WP. This was expected since the intake of dietary fiber was not significantly changed with the addition of the WP supplement. Perhaps a higher intake of the latter supplement would have exerted an additional hypolipidemic response since the intake of dietary fiber would have also been increased (Ripsin et al. 1992).

In summary, the present study revealed that the intake of GF concentrate increased the antioxidant status of the male hypercholesterolemic subjects. Both exogenous and endogenous antioxidants, including erythrocyte SOD activity and zinc status, were positively affected by the supplemental intake. The mechanism responsible for this effect is not fully understood, however, it is likely that the increased plasma levels of Zn in response to GF intake may have played a critical role in influencing the other antioxidant parameters. In addition, a hypolipidemic response was apparent with the supplemental intervention. These results were expected since the GF supplement provided additional intake of dietary fiber. The latter findings are in accordance with the beneficial properties associated with the intake of fresh fruit and vegetables (Appleby et al 1999, Lampe 1999), suggesting that GF may be a potential vegetable substitute. The lack of an additive antioxidant effect with the addition of WP suggests that both endogenous and exogenous levels of antioxidants, with particular reference to SOD and plasma Zn, were already saturated with the intake of GF

concentrate. Hence, indicating that antioxidant status may have a peak level that may not be exceeded with additional intake of antioxidant rich food concentrates.

Conclusion

Out of the antioxidant parameters tested, erythrocyte SOD activity and plasma concentrations of zinc appeared to be most responsive to the 2 week supplemental intake of GF(8.5g twice daily) in a select group of hypercholesterolemic (>5.2 mmol/L) male subjects. In parallel, the plasma concentrations of total and LDL-cholesterol were also reduced while the HDL/LDL ratio was increased with the supplementation of GF. Although these results indicate that GF may be a potential vegetable substitute, long term studies with a larger study population are required to further explore the antioxidant and lipidemic responses to GF before conclusions can be made about its use as a dietary supplement.

The addition of another antioxidant rich dietary supplement (WP) to the supplemental intake of GF did not further affect the antioxidant status of the study participants. These results suggest that with the intake of GF, the plasma concentrations of zinc and erythrocyte activity of SOD may have reached their maximum level. Indeed other studies on antioxidants have displayed similar results (Anderson and Phillips 1999). Hence, the achievable antioxidant status, particularly SOD and zinc status, with supplemental intake of antioxidant rich vegetable concentrates may have a level of

saturation, and once this level is reached, additional intake of antioxidants may not further modify these levels. In order to test this hypothesis, future investigations should implement a randomized cross over experimental design. Using this design, one group of study subjects would start with supplemental intakes of GF and the other with WP for the first 2 week period, followed by the addition of WP to the GF supplemental group, and GF to the WP supplemental group for an additional 2 weeks. This randomized design would help to draw conclusions about the proposed antioxidant saturation concept.

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