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EFFECTS OF ZINC OR CITRUS FLAVONOID SUPPLEMENTATION ON INDICES OF OXIDATIVE STRESS IN NON-INSULIN-DEPENDENT DIABETIC WOMEN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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ABSTRACT

Oxidative stress has been implicated in the development of atherosclerosis, a condition which leads to premature death in thousands of people each year. The mechanism by which free radicals increase susceptibility to the development of atherosclerosis is not yet known. However, oxidation of very low-density lipoprotein/low-density lipoprotein (VLDL/LDL), a marker of oxidative stress in vivo, may be critical to the pathogenesis of atherosclerosis.

Little research has examined the effects of zinc or citrus flavonoids, two potentially important antioxidants, on indices of oxidative stress. Therefore, the purpose of this study was to 1) determine if VLDL/LDL oxidation can be used to assess zinc deficiency-induced oxidative stress in a rat model, and 2) determine if zinc or citrus flavonoid supplementation can improve indices of oxidative stress, specifically VLDL/LDL oxidation, in women with non-insulin-dependent diabetes mellitus.

For the rat study, twenty-four rats were randomly assigned to one of three feeding groups: low zinc diet, adequate zinc diet pair fed to the mean intake of rats fed the low zinc diet, and adequate zinc diet fed ad libitum. Rats were maintained on their respective diet two weeks prior to sacrifice. For the human study, sixty postmenopausal NIDDM
women, between the ages of 40 and 60 years, were recruited for participation in this investigation. Subjects were randomly assigned to receive either a zinc supplement (30 mg zinc/d as amino acid chelated zinc), a flavonoid supplement (2000 mg flavonoid complex/d as two tablets, each containing the following complexes: 500 mg citrus bioflavonoids, 200 mg orange bioflavonoids, 200 mg lemon bioflavonoids, 50 mg quercetin, 50 mg rutin), or placebo for three weeks.

The lag time for copper-catalyzed VLDL/LDL oxidation was significantly shorter for rats fed a low zinc diet compared to an adequate zinc diet, while the oxidation propagation rate was significantly higher in the deficient rats. Based on body weight and plasma zinc, rats fed low zinc diet were moderately zinc deficient. The rat study demonstrated that VLDL/LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress.

Three weeks of zinc supplementation significantly increased indicators of zinc status, including plasma zinc and plasma 5’-nucleotidase activity, in women with NIDDM. In addition, insulin-like growth factor-1 (IGF-1), a potential functional indicator of the effects of zinc, significantly increased in subjects with initial values below 165 ng/mL while IGF-1 significantly decreased in subjects with initial values above 165 ng/mL. Despite the fact that zinc status was seemingly improved, zinc supplementation had no significant effect on VLDL/LDL oxidation.

Three weeks of citrus flavonoid supplementation did not significantly affect VLDL/LDL oxidation. Initial plasma vitamin C levels, measured as a possible effect of
citrus flavonoid supplementation, were very low in the diabetic patients. However, plasma vitamin C was unaffected by supplementation.

Plasma IgE, a potential marker of oxidative stress, was elevated drastically above normal in women with NIDDM. IgE levels were unaffected by zinc or citrus flavonoid supplementation. In addition, tumor necrosis factor (TNF) was elevated slightly above normal. TNF levels were also unaffected by zinc or citrus flavonoid supplementation. Myeloperoxidase activity was not elevated in the NIDDM patients. However, values tended to increase following three weeks of zinc supplementation.

This study strengthened the contention that patients with NIDDM may be prone to both zinc deficiency and low levels of plasma vitamin C. In addition, plasma IgE was elevated in patients with NIDDM and may be a useful marker of oxidative stress. Finally, because patients with NIDDM have a propensity toward poor antioxidant defense, poor zinc status and high oxidative stress, more rigorous antioxidant supplementation may be necessary to protect against the damaging effects of oxidative stress.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

A free radical is any molecule that contains one or more unpaired electron and is capable of independent existence (1). Oxygen containing species which are radicals, or easily produce radicals, are called reactive oxygen species (ROS). Aerobic metabolism by biologic systems inevitably results in formation of small amounts of ROS such as \( \bullet \text{O}_2 \), \( \text{H}_2\text{O}_2 \), \( \bullet \text{OH} \), and various unstable oxidized lipids. Many processes produce ROS including lipid peroxidation, radiation, quinone metabolism by redox-cycling, and xanthine oxidase, amine oxidase, cytochrome P450 and prostaglandin synthase enzymatic reactions. ROS can help to destroy invading organisms that may inhibit normal functioning of the cell as well as injured or damaged tissue. However, if the balance of ROS production/degradation becomes deranged, damage to biomolecules such as proteins, lipids, and nucleic acids occurs resulting in injury to cells, tissues, and organs.

ROS damage has been implicated in many disease processes. ROS could contribute to the initial lesions of diseases, and/or play a critical role in the development or exacerbation of diseases (2). For example, oxidation of low-density lipoprotein (LDL) is believed to be strongly associated with the development of atherosclerosis. Macrophages
do not take up native LDL to any great extent in vitro. However, when LDL has been modified by oxidation, it is taken up avidly by macrophages. This process could lead to the development of foam cells, an early stage in the development of atherosclerosis (3).

Normally, the production of ROS is balanced by complimentary detoxification reactions that quench radicals or limit their propagation (1). These reactions are mediated, in part, by a heterogeneous distribution of antioxidant protection systems originating from both endogenous and exogenous sources. Because endogenous sources of protection are limited, exogenous antioxidants may play an important role in ROS defense, especially when oxidative stress is exacerbated. Epidemiologic and biochemical data indicate that the nutritive antioxidants vitamin E, vitamin C, and selenium can significantly influence the oxidative status of biological tissues (4). However, there are other dietary constituents which may prove to be powerful antioxidants because of their diversity of function and/or abundance in food. Two such components could be zinc and flavonoids.

An imbalance between ROS accumulation and antioxidant levels, leading to increased oxidative stress, has been identified in non-insulin dependent diabetes mellitus (NIDDM) (5,6). ROS are formed at high rates in NIDDM, as indicated by the auto-oxidation of glucose and glycated proteins. In addition, hyperglycemia increases activity of the sorbitol pathway which also contributes to the formation of reactive species (5). As a result, patients with NIDDM may be particularly prone to complications associated with ROS.
Purpose

The purpose of this study was to 1) determine if VLDL/LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress in a rat model, and 2) determine the effects of supplementation with zinc or citrus flavonoids on indices of oxidative stress, especially LDL oxidation, in a female diabetic population.

Statement of the Problem

Oxidative stress has been implicated in the development of atherosclerosis, a condition which leads to premature death in thousands of people each year. The mechanism by which free radicals increase susceptibility to the development of atherosclerosis is not yet known. However, oxidation of LDL, a marker of oxidative stress in vivo, may be critical to the pathogenesis of atherosclerosis (7).

Numerous risk factors for the development of atherosclerosis have been identified, but many are difficult if not impossible to modify. Since current research has not yet yielded a conclusive explanation for the etiology of atherosclerosis, it seems likely that there may be other important risk factors yet to be identified. Modification of these potential risk factors may prove to be more successful than that of previously identified risk factors.

One of these potential risk factors may be marginal zinc deficiency which appears to be a widespread phenomenon, especially among older persons. Indirect evidence suggests that zinc has potent antioxidant potential (8). Therefore, zinc deficiency could lead to increased oxidative stress and thus increased risk of developing atherosclerosis.
Research indicates that flavonoids also have antioxidant properties (8,9). Epidemiological evidence suggests that a diet rich in flavonoids decreases the risk of developing cardiovascular disease. Due to the abundance of foods containing flavonoids, it is encouraging to note that increasing consumption of these compounds may not require a strictly regimented diet.

There is a lack of research which examines the effects of zinc or citrus flavonoid supplementation on radical-mediated oxidative injury in humans. Thus, examining the effects of these agents on indices of oxidative stress is of paramount importance.

**Objectives**

The objectives of this investigation are:

1) to use a rat model to determine if VLDL/LDL oxidation can be used to assess the antioxidant potential of zinc;

2) to determine if zinc supplementation will improve zinc status and indices of antioxidant function, including VLDL/LDL oxidation, in non-insulin-dependent diabetic women;

3) to determine if citrus flavonoid supplementation can improve indices of antioxidant function, including VLDL/LDL oxidation, in non-insulin-dependent diabetic women;

4) to determine if citrus flavonoid supplementation can increase plasma vitamin C, a potential antioxidant molecule, in non-insulin dependent diabetic women.
Hypotheses

1) In rats, a diet deficient in zinc will not produce a shorter lag time for VLDL/LDL oxidation compared to a diet adequate in zinc.

2) Zinc supplementation will have no effect on plasma zinc or functional indicators of zinc status (insulin-like growth factor and 5'-nucleotidase activity) in non-insulin-dependent diabetic women.

3) Zinc supplementation will have no effect on lag time for VLDL/LDL oxidation in non-insulin-dependent diabetic women.

4) Citrus flavonoid supplementation will have no effect on lag time for VLDL/LDL oxidation in non-insulin-dependent diabetic women.

5) Other indices of oxidative stress, such as plasma IgE and myeloperoxidase, will not be different in non-insulin-dependent diabetic women when compared to non-diabetic patients, and will not be affected by zinc or citrus flavonoid supplementation.

6) Tumor necrosis factor, which can be elevated by some types of oxidative stress, will not be affected by zinc or citrus flavonoid supplementation in non-insulin-dependent diabetic women.

7) Plasma vitamin C will not be different in the citrus flavonoid supplemented group compared to the placebo group.
Free Radicals and Disease

Free radicals and other oxygen-derived species are continually formed in cells either for specific metabolic purposes or by "accidents of chemistry" (2). The reactivity of free radicals varies with some capable of inflicting severe damage to biomolecules such as DNA, lipids, and proteins.

Ground state or triplet oxygen is technically a diradical, although it is poorly reactive due to spin restriction (10). Singlet oxygen is formed when sufficient energy is added to change the orbitals of one of the two unpaired electrons of triplet oxygen. The deleterious role that singlet oxygen plays is controversial. Spin restriction can also be removed by reduction reactions. The one-, two-, and three-electron reduction productions of oxygen are superoxide radical, hydrogen peroxide, and hydroxyl radical, respectively. Because of the prevalence of oxygen in biological systems, the oxygen-centered radicals are the most common type found. However, sulfur-, carbon-, nitrogen-, and phosphorus-centered radicals also exist which are capable of inflicting tissue injury.

The ability of transition metal ions to move electrons results in propagation of particularly toxic radical reactions (10). For example, the relatively nonreactive
superoxide anion may form the highly reactive hydroxyl radical in the presence of hydrogen peroxide and iron. The fact that there are highly efficient iron- and copper-sequestering proteins in the body indirectly indicates the importance of preventing these reactions from occurring.

Many processes can produce ROS including lipid peroxidation, radiation, xanthine oxidase reactions, cytochrome P450 reactions, quinone metabolism, as well as NADPH oxidase and myeloperoxidase secreted during phagocytosis. The work of Hochstein and Ernster (11) suggests that the most important event in the initiation and propagation of lipid peroxidation is the metal-catalyzed hydrogen abstraction from PUFA (12). ROS can also be formed by leakage of electrons from the electron transport system. It has been proposed that up to 2% of total mitochondrial oxygen consumption is used for the formation of ROS (13).

When ROS are generated, they are capable of interacting with lipid component PUFA forming lipid peroxyl radicals. Lipid peroxyl radicals, which have half lives of only seconds, can travel through the blood to disrupt other biomolecules (1). Lipid peroxidation results when peroxyl, hydroxyl, and/or alkoxyl radicals interact with fatty acid side chains, forming fatty acid radicals. These radicals most likely rearrange to conjugated diene structures which are then capable of reacting with molecular oxygen to yield conjugated diene peroxyl radicals (12).

Double bonds within fatty acids facilitate hydrogen abstraction, resulting in greater susceptibility of polyunsaturated fatty acids to radical attack. Oxidation of the PUFA carbon chain, cleavage and shortening of the carbon chain, and release of aliphatic
products result when lipid peroxidation is not terminated. These alterations not only disrupt the hydrophobic core of the membrane but can lead to adduct formation between the released aliphatic products and other molecules such as nucleosides (12). In addition, these aliphatic products can cross link two lipids or a lipid with a protein (14).

Although the involvement of free radicals in disease has only been verified in oxygen toxicity and carbon tetrachloride-induced liver damage, compelling evidence exists for their causative or contributory role in many disease processes (10). For example, chronic inflammation-induced tissue damage seems to mediated in part by ROS production. Such seems to be the case with rheumatoid arthritis, where inflammation actually becomes the disease process. In addition, ROS attack on DNA, RNA, and other portions of the cell is believed to be involved in both the promotion and progression stages of carcinogenesis. Evidence also suggests that ROS are important factors in milder forms of reperfusion injury such as myocardial stunning, but their contribution to more severe forms is most likely minimal.

There is now extensive evidence that ROS play a critical role in the development of atherosclerosis (15). Adherence of monocytes to the endothelium overlaying cholesterol deposits initiates the formation of foam cells, a precursor stage to the fatty streak. Chemoattractants cause the infiltration of these monocytes though cell gap junctions into the subendothelial space, where they differentiate into macrophages. Macrophages, and to a lesser extent smooth muscle cells, take up lipoproteins and form foam cells. Through a series of poorly defined steps the fatty streak may develop into fibrous plaques, which eventually leads to clinically apparent coronary artery disease (3).
Only recently has an explanation for the uptake of lipoproteins by macrophages been proposed (16). LDL is the primary source of cholesterol in atherosclerotic lesions. However, macrophages have very few LDL receptors and those that are present are downregulated by increases in intracellular cholesterol. Therefore, it would seem that macrophages would not freely take up cholesterol and would do so less readily as the cell became engulfed. In fact, macrophages do not take up cholesterol to any great extent \textit{in vitro} even when incubated with high concentrations of LDL. Brown and Goldstein (16) were the first to suggest that because the role of the macrophage is to scavenge damaged material, perhaps the lipoprotein must first be “damaged” in order for it to be taken up by the macrophage. They soon identified a “scavenger receptor” that is not down-regulated by high intracellular LDL content. Thus, macrophages, via this receptor, can continue to take up LDL even as they become full.

Subsequent studies have shown that the damage that occurs to LDL that allows it to be taken up by macrophages is oxidation (17). The sequence of events leading to LDL oxidation is unclear, although it is known that it begins with either ROS hydrogen abstraction from PUFA within LDL or by direct enrichment of LDL with lipoperoxides. Both mechanisms result in seeding of LDL with lipoperoxides which decompose into reactive intermediates. These intermediates can then initiate oxidation in neighboring PUFA or in PUFA of nearby LDL particles. Decomposition of lipoperoxides \textit{in vitro} is dependent on the presence of transition metals such as copper or iron. Also, because the oxidation process is autocatalytic, theoretically a single hydrogen abstraction could result in oxidation of the entire LDL particle and of nearby particles (3).
There are many properties of oxidized LDL that make it more atherogenic than non-oxidized LDL (18). The most important factors are: 1) it has enhanced uptake by macrophages, 2) it is chemotactic for circulating monocytes and T-lymphocytes, 3) it inhibits tissue macrophage motility, 4) it is cytotoxic, 5) it renders LDL more susceptible to aggregation, 6) it can alter gene expression of neighboring arterial cells, 7) it can alter coagulation pathways, 8) it can alter arterial vasomotor properties, and 9) it can elicit autoantibody formation due to its immunogenicity (3).

Non-enzymatic glycation, as occurs in NIDDM, can also modify LDL in such a way that it is scavenged by macrophages (3). Furthermore, the auto-oxidation of glucose and glycosylated proteins increases ROS production and may contribute to the oxidation of LDL (19). Likewise, hyperglycemia increases activity of the sorbitol pathway, thus increasing triose phosphate formation. Auto-oxidation of triose phosphate subsequently produces two reactive species, α-oxaldehyde and H₂O₂ (5). These phenomenon, potentially resulting in increased oxidation of LDL, may help to explain the increased incidence of cardiovascular disease observed in NIDDM. In fact, higher plasma TBARS in diabetic patients with angiopathy than without angiopathy have been reported (20). Evidence of lipid peroxidation has been reported in several secondary complications of NIDDM (22). Sundaram et al. (5) reported increased lipid peroxidation within the first 2 years of diagnosis in 467 cases of NIDDM.

Several methods have been developed to estimate lipoprotein oxidation (12). It would seem logical to directly measure radical species since these are the agents that lead to LDL oxidation. However, these species are short-lived and disappear rapidly, yielding
primary and secondary lipid peroxidation products. Trapping agents can be used in vitro, but are difficult to apply to clinical specimens because the radical reaction has usually been terminated by the time of analysis. It is therefore more practicable to measure relatively stable substances that are products of the radical reaction.

The thiobarbituric acid reactive substances assay (TBARS) is a method of measuring malondialdehyde and/or malondialdehyde-like substance formation during lipid peroxidation (12). In addition, protein and lipid fluorescence (23,24) can be monitored for change as can lipoprotein antioxidant content (23,25). The laboratory of Esterbauer et al. uses copper-induced conjugated fatty acid hydroperoxide formation at 234 nm absorption. This method can be used to follow the kinetics of conjugated diene formation.

During the lag phase, few conjugated dienes are formed due to the protective action of antioxidants within the lipophilic PUFA (23). Thus, the greater the antioxidant content, the longer the lag phase. The propagation phase begins when the antioxidant scavenging potential of the PUFA is exhausted and conjugated diene formation increases at a rapid rate to a maximum value as the oxidation process rapidly accelerates. As conjugated dienes are formed during this phase, absorbance at 234 nm increases significantly. Finally during the decomposition phase, lipid hydroperoxides decompose to products such as 2-alkenals or 4-hydroxyalkenals.

In addition, measurement of other compounds has been used to estimate oxidative stress. Increasing evidence suggests that tumor necrosis factor (TNF) release by monocytes can stimulate an overproduction of oxygen-derived free radicals by neutrophils. Although TNF itself is a marker of inflammatory stress, elevated TNF values may suggest
increased oxidative stress due to its effect on free radical formation (26). In addition, TNF has been shown to be elevated in obese patients and may contribute to the insulin resistance of NIDDM (27).

Neutrophils contain the enzyme myeloperoxidase, which catalyzes the oxidation of halide ions. The hypohalous acids which are formed are powerful oxidizing agents which can produce damage similar to that produced by free radicals. Therefore, infiltration of neutrophils and release of hypohalous acids into tissue damaged by other factors may exacerbate injury. (10). Also, certain inflammatory cells release myeloperoxidase concomitant to superoxide release. As a result, large increases/decreases in myeloperoxidase may indicate changes in endogenous ROS production.

IgE production has also been proposed as a marker of oxidative stress. IgE is produced by B cells in response to IL-4 stimulation (28). Through a series of reactions, IgE can activate neutrophils. There is strong evidence implicating neutrophil activation in the exacerbation of tissue injury. Not only are they present at the site of injury, but their stimulation also results in protease, cytokine, and ROS release from the cell. Consequently, increased IgE levels may indicate increased oxidative stress via neutrophil activation. Supporting this contention, Qasim et al. (28) reported that N-acetyl-L-cysteine, a potent antioxidant precursor of intracellular glutathione, decreased both IL-4 and IgE production.
Antioxidants

Humans possess antioxidant defenses to protect against ROS. Because evidence supports a role of ROS in atherosclerosis development, antioxidants should play a crucial role in preventing LDL oxidation and its conversion to an atherogenic form (18). Initial trials examining the effects of antioxidants suggest that the atherogenic process can be retarded (29). Epidemiological surveys strengthen the hypothesis linking low plasma levels or dietary intakes of antioxidant vitamins (ascorbate, tocopherol, and B-carotene) with ischemic heart disease (30,31).

Successful resistance to oxidative damage is dependent on many factors including age, physiological state, and diet (8). Many nutrients have critical roles in reactions and can halt or limit lipid peroxidation. The most well-studied detoxification reactions are the radical scavenging reactions, such as those in which vitamin E participates. These reactions occur when an antioxidant reacts with an ROS, producing a stable and relatively harmless radical that can be recycled to a nonradical form. However, antioxidants may also confer protection by stabilizing organic free radicals and by preventing electron transfer between molecular oxygen and organic molecules.

Not only is there an abnormal production of ROS in NIDDM, but there is ample evidence that there are substantial antioxidant defense alterations which may contribute to increased susceptibility to oxidative damage (21). Lowered levels of antioxidants, including superoxide dismutase, catalase, reduced glutathione, vitamin C, and vitamin E, have been reported in patients with NIDDM (5). Lowered antioxidant defense in
combination with high ROS production may greatly increase susceptibility to ROS-induced damage in patients with NIDDM.

Research into antioxidant status and supplementation has been mainly concerned with vitamins C and E to the neglect of other food components with promising antioxidant potential (8). For example, although several mechanisms have been proposed by which zinc may serve as an antioxidant, it has been poorly investigated. In purified chemical systems, zinc clearly has antioxidant capacity. However, it is difficult to extrapolate these effects to biological systems \textit{in vitro} or \textit{in vivo}.

In another case, flavonoids have been investigated extensively in epidemiological studies, but little research has been conducted which examines the effects of flavonoid supplementation (30). Like zinc, there are several mechanisms by which they may serve as antioxidants. Moreover, it has been proposed that flavonoids may be responsible for the “French Paradox”, the observation that the French suffer relatively lower rates of coronary artery disease despite a high saturated fat diet.

Zinc

Zinc is a trace mineral with many important catalytic, structural, and regulatory roles (32). Zinc is essential to humans with a Recommended Daily Allowance of 15 mg/d for males over 10 years of age, 12 mg/d for females over 10 years of age, and 15 mg/d for pregnant women (33).

Although zinc is present in all fluids, tissues, and organs in the body, 85-95% is locked in skeletal muscle and bone where it probably rejoins systemic metabolism only in
situations of increased turnover or catabolism. The concentration of zinc in extracellular fluids is fairly low (approximately 1 ug/mL in plasma) and can be affected by many factors including exercise, stress, infection, estrogens, and others. As a result, plasma zinc concentration is not a reliable indicator of zinc status. In fact, plasma zinc concentrations can either be maintained during frank deficiency by the release of zinc from tissue catabolism (32), or drop dramatically as plasma zinc is compromised in order to spare tissue zinc (34).

Absorption and Utilization of Zinc

Zinc concentrations in the body are maintained within narrow limits (Aggett, 1995). Endogenous zinc is conserved by the intestine and liver when dietary intake of zinc is marginally adequate or deficient. Intestinal absorption of exogenous zinc increases through upregulation of carrier sites, increased enteropancreatic circulation, decreased pancreatico-biliary secretion, and, to a lesser extent, increased renal conservation. High dietary zinc, on the other hand, results in reduced absorption and increased gastrointestinal excretion (34).

Bioavailability of zinc is dependent both on properties of the diet and on physiological characteristics of the individual consuming the diet (35). High dietary content of phytates, calcium, copper, oxalates, polyphenols, and iron may decrease absorption. On the other hand, protein, albumin, histidine, and glutamic acid may increase absorption. Host characteristics such as elevated levels of corticosteroids and
prostaglandin \( E_2 \) may increase absorption of zinc while infection, stress, and malabsorption diseases may decrease absorption.

**Biochemical and Physiological Functions of Zinc**

The fundamental roles that zinc plays in cellular metabolism are diverse, including regulation of gene transcription systems, hormone receptors, signal transduction pathways, protein synthesis and turnover, carbohydrate metabolism, and many others (32). Zinc is a component of over 200 metalloenzymes or proteins including carbonic anhydrase, alcohol dehydrogenase, glutamic dehydrogenase, RNA and DNA polymerase, superoxide dismutase, metallothionein and thymulin (36). In addition, zinc may be required for the expression/production of growth hormone. Zinc is required for the synthesis of retinol binding proteins which carry hormone-vitamin A complexes to the cell nucleus. Activation of transcription of growth hormone probably occurs through effects of this complex on the promoter regions of the growth hormone gene.

**Zinc as an Antioxidant**

Indirect evidence suggests that high concentrations/dosages of zinc have antioxidant properties in biological systems both *in vitro* and *in vivo* (8). Moreover, mild dietary zinc deficiency has been reported to increase the susceptibility of experimental animals to oxidative stress (37, 38). Hammermueller et al. (39) demonstrated that that NADPH- and aminopyrene-dependent \( \text{H}_2\text{O}_2 \) production by liver and lung microsomes is elevated in zinc deficient rats. The authors postulated that uncoupling of the cytochrome
P-450 electron transport chain produced this effect. They also concluded that zinc deficiency results in membrane alterations that increase susceptibility to oxidative damage.

Dietary zinc is required for the synthesis of Cu,Zn superoxide dismutase (CuZnSOD), an enzyme that catalyzes the dismutation of superoxide anion radical. However, the increased oxidative stress observed in zinc deficient animals so far does not seem to be related to decreased CuZnSOD activity in the tissues (8). Similarly, pharmacological supplements of zinc do not produce a significant elevation of SOD activity in animals (40).

There are several mechanisms by which zinc may function as an antioxidant, but a precise role has yet to be elucidated (8). First, zinc may protect sulfhydryl groups against oxidation. Zinc-sulfhydryl binding has been demonstrated in studies examining δ-aminolevulinate dehydratase, an enzyme with four reactive sulfhydryl groups on each of its eight subunits (41). The ability of zinc to maintain an essential sulfhydryl group prevents intramolecular disulfide formation and stabilizes the molecule. Three mechanisms for zinc protection of sulfhydryl groups have been postulated: 1) zinc reduces sulfhydryl reactivity by direct binding, 2) chelation of zinc in close proximity to the sulfhydryl group reduces reactivity by stearic hindrance, 3) a conformational change occurs upon zinc binding that reduces the reactivity of the sulfhydryl group (41). Zinc has been shown to protect sulfhydryl groups in the enzyme dihydroorotase (42), DNA-binding proteins containing zinc fingers (43), and the protein tubulin (44). On the other hand, loss of zinc atoms occurs when the zinc in zinc metallothionein reacts with OH• and O2• leading to disulfide bond formation (45).
It has been proposed that zinc competes with iron for chelation by the organic ligand cysteine which, when bound by iron, can transfer electrons to oxygen and produce OH•. It has been shown in studies with iron, cysteine, and O₂ that zinc can decrease the amount of OH• bound by the spin trap 5,5-dimethyl-1-pyrroline N-oxide (46). By competing with iron for chelation by the organic ligand cysteine, it is likely that zinc also inhibits radical formation from other radical-generating systems. Because zinc has can bind both ADP (47) and NADPH (48), and inhibit NADPH oxidation (49), it is probable that ROS formation from other ROS-generating systems is inhibited. The iron catalyzed transformation of PUFA hydroperoxide to malondialdehyde may be inhibited by zinc through its competition with iron for oxygen ligand binding in the oxidized PUFA (50,51).

Zinc may also indirectly exert antioxidant-like effects. Zinc may induce synthesis of metallothionein or other antioxidant proteins (52). Specifically, metallothionein can protect against hydroxyl radical-induced DNA damage in vitro by serving as a sacrificial scavenger for hydroxyl radicals. Zinc may also alter the metabolism of other minerals such as copper or iron or the enzyme systems that activate or detoxify ROS (53). In addition, zinc may stabilize plasma membranes and prevent overproduction of ROS (54). This may explain results observed by Parsons and DiSilvestro (38) who demonstrated that mild zinc deficiency produced high susceptibility to galactosamine-induced liver injury in rats. Because galactosamine treatment inhibits protein synthesis regardless of zinc status, the authors hypothesized that mild zinc deficiency results in membrane destabilization and exaggerated ROS production. Finally, zinc may inhibit ROS production through its effects on receptors that signal ROS production (55). Consistent with this idea, zinc-
deficient rats show diffuse, moderate inflammation as demonstrated by phagocytes that oversecrete ROS in vitro (56) and high ceruloplasmin activities (53)

**Zinc Deficiency Increases Susceptibility to Oxidative Damage**

Because zinc seems to play a critical role in protection from oxidative damage, deficiency of zinc would be expected to result in symptoms indicative of oxidative injury (8). Zinc deficiency does not, however, produce symptoms similar to those of vitamin E and selenium deficiency, nor does it produce membrane composition changes that would be expected following oxidative damage. Nevertheless, several studies (56,57,60,61) have reported increased ROS production in membranes or tissues from zinc-deficient animals. Indices of lipid peroxidation such as malondialdehyde, conjugated dienes, and lipid hydroperoxides are elevated in zinc-deficient animals (60). In rats, zinc deficiency is associated with elevated ROS production by tissue organelles and phagocytic cells in vitro (8,57).

Many studies support the hypothesis that zinc deficiency results in increased oxidative stress. Mild zinc deficiency in rats decreases resistance to stress-induced injury thought to be mediated by ROS (37,53). Pucheu et al. (58) identify the decrease in plasma zinc concentrations observed following myocardial infarction as one of the possible causes of reperfusion injury following thrombolytic therapy. Reperfusion injury has recently been attributed to the cytotoxic effects of ROS (59). In a rat model, Pucheu et al. (58) reported that a diet deficient in copper, zinc, selenium, and manganese increases myocardial tissue sensitivity to oxidative stress following a period of global zero-flow
ischemia and reperfusion. Male rats, following a 3 week zinc-deficient diet (4 ppm), had significantly greater lipid peroxide levels as measured by TBARS and conjugated dienes following coronary occlusion than did rats fed a zinc-adequate diet (60 ppm)(60). Similarly, Oteiza et al. (61) reported that weanling male rats fed a zinc deficient diet (0.5 µg Zn/d) had significantly higher levels of TBARS in testes homogenates and greater 8-Oxo-2′-deoxyguanosine levels in testes DNA than did pair fed and ad libitum controls. They concluded that zinc deficiency alters ROS production and detoxification beyond those effects associated with zinc-deficiency-induced changes in iron metabolism. The investigators hypothesized that the oxidative damage that occurs in zinc deficient animals could be the result of impairments in the oxidant defense system (e.g., superoxide dismutase) and/or repair processes such as poly(ADP) ribose polymerase activity.

Kok et al. (62) suggested that low serum zinc is associated with increased risk of cardiovascular disease. Faure et al. (63) found increased levels of VLDL and LDL and enhanced production of MDA by the LDL in rats fed an 8-week diet deficient in zinc (0.2 ppm). These results suggested that zinc deficiency increases lipoprotein fragility and susceptibility to peroxidation, possibly leading to increased atherogenic risk. In addition, de Lorgeril et al. (64) reported that lipid peroxidation is involved in the accelerated coronary artery disease that develops in most, if not all, heart transplant recipients. Thirteen of the thirty heart transplant recipients studied had very low (less than 10 µmol/L) zinc levels. In stepwise multivariate regression, zinc was a significant (p<0.004) predictor of lipid peroxide levels. These results provide support for the hypothesis that zinc is a physiologic antioxidant in a well-defined clinical condition. However, serum zinc
is not always a reliable indicator of zinc status because values can be influenced by many factors other than zinc deficiency.

Tissues isolated from zinc-deficient animals do not always have increased levels of oxidation or peroxidation products (65). It has been hypothesized that the endogenous antioxidant defense system may be capable of protecting against certain oxidative stress caused by zinc deficiency but, when additional oxidative stress is added, the system is overwhelmed (8). For example, in zinc-deficient rats exposed to hyperoxia, Taylor et al. (66) reported increased lung damage compared to control and pair-fed groups. They suggested that, although some of the damage was the result of decreased feed intake, a greater proportion was the result of a zinc-deficient diet. A similar overwhelming of antioxidant defenses by high ROS production concomitant to marginal zinc deficiency could result in high oxidative stress observed in patients with NIDDM.

**Zinc Supplementation and Susceptibility to Oxidative Damage**

In isolated, activated human neutrophils, zinc decreases superoxide anion radical production (67). Zinc also decreases malonaldehyde and radical production in cultured hepatocytes (68). Similarly, Richard et al. (69) reported lower basal lipid peroxidation as measured by TBARS in human skin fibroblasts grown for 21 d in culture media added with different zinc concentrations (100, 125, and 150 μM). They suggest that zinc replaces iron or competes with iron at the level of cell uptake. However, it remains to be determined whether the decrease in lipid peroxidation is directly due to increased
intracellular zinc levels, from decreased intracellular iron, and/or perhaps increased
synthesis of metallothionein.

Although hypochlorous acid-induced mobilization of dimercaptopropanol-zinc has
been hypothesized to induce cell injury (70), Lapenna et al. (71) propose that zinc can be
protective by inhibiting transition metal-driven lipid peroxidation. They demonstrated that
zinc mobilized from thiolate bonds by hypochlorous acid in vitro can induce protection
toward iron-mediated oxidation of linolenic acid and deoxyribose, possibly reflecting the
capacity of zinc to complete for the iron at a variety of binding sites. This antioxidant
effect was noted only when hypochlorous acid interacted with the dimercaptopropanol-zinc
complex so that the zinc was “free” in solution. Thus, they concluded that it is the “free”
zinc that possesses antioxidant properties. In addition, zinc inhibited linolenic acid
peroxidation (35%; p<0.0001) more efficiently than deoxyribose oxidation (15%; p<
0.01). The authors postulated that this observation could be due to an interaction of zinc
with iron and double bonds of PUFA which could inhibit the formation of iron-oxygen-
enoic acid complexes, compounds which have been proposed in initiate lipid peroxidation.

Filipe et al. (72) found that zinc inhibits spontaneous lipid peroxidation, as
measured by both copper-induced TBARS and conjugated dienes, in rat brain whole
homogenates at concentrations of 200 μM zinc. Additionally, copper-induced lipid
peroxidation was decreased in human plasma by 27% and 49% when incubated with 20
μM zinc (72). The authors propose that this decrease may be the result of competition
between zinc and copper for binding to critical biological sites which influences ROS
generation or lipid peroxidation. Coudray et al. (60) however found that although zinc can
inhibit the Fenton reaction catalyzed by the citrate-iron complex in vitro, it does not occur in plasma and probably does not play a significant role in vivo. They propose that the protection against free radicals offered by zinc is a result of its modulation of superoxide dismutase.

The antioxidant property of zinc is fairly apparent when added in vitro at free zinc concentrations of 0.01-0.10 mM, or in high-dose injections, but is difficult to assess at physiological concentrations which are much less than 0.015 μM (73). In addition, intracellular free zinc concentrations in most tissues are also likely very low due to the high-affinity sequestration of zinc in biological systems (74). Also, cells have the ability to regulate and compartmentalize zinc (69). However, evidence does exist that zinc also offers antioxidant protection in vivo. Both oral (75) and injected zinc (76) have been shown to protect rat liver from carbon tetrachloride toxicity. Because the liver plays a central role in the metabolism of zinc, it is hypothesized that accumulation of zinc in this organ confers protection.

Whether or not low serum zinc levels result in a predisposition to cardiovascular disease is controversial. However, zinc is present in human atherosclerotic lesions (77). Under conditions of elevated oxidative stress, such as in the artery wall where iron and copper may be released from their sequestered states, “free” zinc may be released from plasma proteins or from damaged cells (78). Bedi et al. (79) reported that atherosclerotic lesion development was decreased in cholesterol-fed rabbits by zinc administration. However, these effects could not be separated from those produced by the 78% decrease in serum cholesterol. Wilkins et al. (78) reported a 50% reduction in LDL
uptake by mouse macrophages with 3 μM zinc administration to the culture media. A
dose-dependent reduction in LDL modification was also observed when endothelial cells
were incubated with zinc. The investigators hypothesize that the inhibitory effect of zinc
is due to the displacement of copper or iron from binding sites on the LDL particle or by
displacing iron from cysteine and inhibiting ROS formation.

**Zinc and NIDDM**

NIDDM is accompanied by perturbations in micronutrient absorption, uptake by
tissues, and excretion, possibly resulting in a worsening of the oxidative balance and
decreased ability to combat endogenously produced ROS (80). Kinlaw et al (81) reported
impaired intestinal absorption of zinc in NIDDM patients. Hyperzincuria is also a
consistent observation among NIDDM (81,82). Although the underlying mechanism
responsible for hyperzincuria in NIDDM is not fully known, it is related to hyperglycemia
*per se* and not to significant glycosuria. It has been suggested that excessive urinary
excretion of zinc results from interference by glucose of active transport of zinc into renal
tubular cells. Studies evaluating plasma zinc in NIDDM patients are inconclusive, with
some reporting normal (83) or high (84) plasma zinc levels. On the other hand, Sjorgren
et al. (85) reported large decreases in plasma zinc levels concomitant to increased
excretion rates in NIDDM patients. However, these studies did not report zinc status
according to the type of diabetes and plasma zinc is not necessarily a good indicator of
zinc status.
Accelerated atherosclerosis is the leading cause of death in diabetic patients (86). This phenomenon may be related to increased or uncontrolled oxidative activity and/or impaired activity of endogenous antioxidant defense systems. Not surprisingly, markers of oxidative damage are elevated in diabetic patients and are associated with the development of complications (87). Jain et al. (88) reported increased lipid peroxidation assessed by TBARS and malondialdehyde in erythrocytes of diabetic subjects. The amount of lipid peroxidation was significantly correlated with levels of glycosylated hemoglobin, an index of elevated blood glucose. In addition, Hunt et al. (89) reported concomitant peroxidation and glycosylation in diabetes. Glucose can enolize and thereby reduce molecular oxygen, yielding ROS. Glucose, as a result, may increase oxidative stress. In addition, evidence drawn from experiments in vitro suggests that glycosylated proteins, which are increased in NIDDM, may form superoxide anions under physiological conditions (90). Superoxide has been shown to reductively mobilize iron from ferritin (91) and from mitochondria (92) and may further increase oxidative stress in NIDDM patients. Indirect evidence exists that hydrogen peroxide formation is also increased in diabetic patients which may increase release of iron from heme proteins such as hemoglobin and myoglobin (93). As a result, it is likely that the highly reactive hydroxyl radical may be formed in vivo. Increased activity of the aldose reductase catalyzed formation of sorbitol from glucose, a pathway with increased activity in NIDDM, may decrease NADPH, a reducing cofactor for glutathione reductase, and thus impair antioxidant capacity (94).
It is clear that oxidative stress is elevated in diabetic patients and may lead to the increased LDL oxidation observed in the blood of diabetic animals. This may also be due to the increase in triglycerides in all classes of particles in diabetics with hypertriglyceridemia, fatty acids associated with triglycerides are more susceptible to oxidation than those associated with cholesterol in the LDL particle, or LDL glycosylation may accelerate oxidation (95).

Few studies have examined the effect of antioxidant supplementation in diabetic patients. In one exception, ascorbic acid and tocopherol have been shown to decrease glucose auto-oxidation, reduce the covalent linking of glucose to serum proteins in vitro, and inhibit serum protein glycation in vivo (96). Even fewer studies have investigated the effects of zinc supplementation. In one study, Winterberg et al. (97) reported a decrease in cholesterolemia and glycemia with 20 d of zinc supplementation (50 mg/d) in NIDDM patients. However, fewer studies have specifically evaluated the effects of zinc supplementation on indices of lipid peroxidation.

Patients with NIDDM experience many metabolic perturbations in addition to those related to ROS production. It is possible that zinc deficiency contributes to the development or exacerbation of these conditions and that supplementation could potentially alleviate these symptoms. For example, obesity is associated with low levels of circulating growth hormone (GH). Insulin-like growth factor 1, a stimulator of GH release, has been shown to be decreased (98) or normal (99,100) in obese subjects. There is evidence that zinc regulates circulating IGF-1 concentration (101). Decreased circulating IGF-1 has been observed in zinc deficient animals (102) and humans (103),
resulting in decreased lipolysis which may contribute to or perpetuate metabolic abnormalities associated with obesity (104). Several investigators have reported increased circulating IGF-1 concentrations following zinc supplementation (101,105,106). Because of the obesity, hyperglycemia (an inhibitor of IGF-1 secretion), and susceptibility to zinc deficiency associated with NIDDM, zinc supplementation may be beneficial in increasing circulating IGF-1 levels.

**Flavonoids**

Flavonoids are nonnutritive, low molecular weight polyphenolic compounds with diverse chemical structure and characteristics (Figure 1) (107). They are found ubiquitously in plants and vegetables, nuts, seeds, flowers, bark, and citrus fruits. As a result, they are consumed regularly as part of the human diet. Over 4,000 different flavonoids have been identified. They include two major groups of related compounds, the anthocyanins (catechins) and the anthoxanthins (flavonols, flavones, and flavanones). The most widely investigated classification of flavonoids is the flavonols, especially quercetin, which has been shown to have positive physiological effects.

Little information is available regarding flavonoid intake of the average American diet. Until recently, it was believed that the average intake of all dietary flavonoids was about 1 g/d (108). However, this value is based on food analysis techniques now considered inappropriate (109). Moreover, flavonoid intake was based on whole food analysis and estimates of the average American diet extrapolated from the Organization
for Economic Cooperation and Development food consumption statistics. It is now known that this method overestimated food intake.

The only investigation which has utilized more advanced methodologies to determine major flavonoid content in food was conducted in the Netherlands (110). The flavonols quercetin, kaempferol, myricetin, and the flavones luteolin and apigenin were analyzed in 28 vegetables, 9 fruits, and beverages commonly consumed in The Netherlands. The average flavonoid intake in The Netherlands as determined by a cross-check dietary history was estimated to be approximately 25.9 mg/d. The major dietary flavonoid consumed was quercetin (16 mg/d), followed by kaempferol (4 mg/d), luteolin (0.92 mg/d), and apigenin (0.69 mg/d). The greatest dietary sources of flavonoids were tea (61% of intake), onions (13% of intake), and apples (10%). Because flavonoid intake was estimated from the content of only five flavonoids in Dutch foods, total flavonoid consumption in this population may be higher. In addition, the flavonoid content of foods in The Netherlands may not represent the flavonoid content of foods consumed in other countries. Analysis of flavonoid content of foods consumed in other countries is required to determine flavonoid consumption in other populations.

Chemical Structure of Flavonoids

Flavonoids are based on a flavan nucleus (111) with a basic chemical structure C_6-C_3-C_6 skeleton (112). Two aromatic rings are joined by an aliphatic three-carbon chain. Hydroxyl, methyl, or sugar groups are attached at various points on the skeleton. The relative orientation of the attached moieties determines the biochemical activity of the
Figure 1. Chemical structure of select flavonoids.
classifications. Many occur naturally as flavonoid glycosides with carbohydrate substitutions such as D-glucose, L-rhamnose, glucorhamnose, galactose, lignin, and arabinose (113). The most common flavonoid flavonoid and its metabolites. The structure of the various flavonoids varies within glycosides in the human diet are quercetin, the quercetin glycoside rutin, and robinin (107). Very few plant species have been examined for their flavonoid content and therefore quantification of all flavonoids is incomplete (114).

Absorption and Utilization of Flavonoids

Little is known regarding the absorption and metabolism of flavonoids; however, they are believed to be relatively nontoxic and, if absorbed and biologically active in vivo, have a wide range of biological effects. In rats, a few flavonoids have been examined and found to be excreted either unchanged or as flavonoid metabolites in the urine and feces. Bravo et al. (115) reported little fermentation by the gut flora and excretion of less than 5% of ingested catechin and tannic acid in rats consuming 0.5 g flavonoid/d for 3 weeks. These results suggest that extensive absorption of the flavonoids had occurred. Further research by Bravo et al. (116) indicated that flavonoids do not interfere with the digestion and absorption of other nutrients but may alter lipid metabolism by stimulating bile acid excretion.

Human studies that have examined flavonoid absorption, metabolism, and excretion are conflicting. Some report that flavonoids are absorbed after oral
administration (117) while others report poor absorption, with few flavonoids reaching the general circulation unchanged at measurable concentrations (118). Das et al. (117) reported that phenols were detected within 6 h in the plasma of patients receiving a 92.3 mg/kg dose of catechin. These phenolic compounds were detected in the urine as both free and conjugated forms. Approximately 19% was excreted unchanged in the feces. Gugler et al. (118), on the other hand, reported no measurable concentration of flavonoid or its metabolites in the plasma or urine of subjects who received 4 g of quercetin. Approximately 53% of the dose was recovered in the feces. As a result, the researchers concluded that 1% or 40 mg had been absorbed. Because normal dietary flavonoid intake is between 23 and 170 mg/d (110), absorption of 40 mg is not unreasonable. However, because these studies examined the effects of pharmacological doses of catechin or quercetin, rather than normal dietary intakes (23-170 mg/d), it is difficult to extrapolate their results to smaller intakes. In addition, the absorption and metabolism of other flavonoids have not been investigated. It is unlikely that humans consume dietary flavonoids individually, or a large percentage of their total intake from a single source.

**Flavonoids as Antioxidants**

Interest in flavonoids has arisen since quercetin was shown to inhibit carcinogen-induced tumors in rats (119) and inhibit colonic cell proliferation *in vitro* (120). In addition, several mechanisms by which flavonoids can be antioxidant *in vitro* have been demonstrated. First, flavonoids can scavenge superoxide anions and hydroxyl radicals and
thus inhibit initiation of lipid peroxidation (120). Secondly, Afanas'ev et al. (121) propose that flavonoids terminate radical reactions by donating hydrogen atoms to the peroxy radical, forming flavonoid radicals. These flavonoid radicals can then react with free radicals and terminate the propagating chain of lipid peroxidation. Thirdly, flavonoids act as metal chelators and inhibit the superoxide-driven Fenton reaction, an important source of ROS (121,122). Finally, flavonoids can also inhibit the pro-oxidant enzymes lipoxygenase and cyclooxygenase (4). Unfortunately, no clear evidence exists that flavonoids exert antioxidant activity in vivo.

There are several structural features of flavonoid that affect the inhibition of lipid peroxidation. First, flavonoids that have a hydroxyl group on the flavan nucleus, such as catechin, quercetin, myricetin, morin, and fisetin, are more potent inhibitors of lipid peroxidation than those that lack a hydroxyl group (122). Secondly, double bonds on the flavan nucleus increase the antiperoxidative effects of flavonoids and hydrogenation of these bonds decrease the effect (123). Thirdly, some studies (124,125), but not others (126,127), have demonstrated that a carbonyl group on the flavan nucleus is necessary for the antiperoxidant activity of flavonoids. In addition, hydroxyl radical scavenging activity increases with the number of hydroxyl groups on the flavan nucleus (124). Other factors such as the pattern of hydroxylation (127), the presence of a sugar moiety (124) and the presence of methoxyl groups (127) also affect the antiperoxidative activity of flavonoids.

Phenolic compounds exhibit anticarcinogenic activity in several animal models (128). Their anticarcinogenic activity is believed to result from their antioxidant
properties. However, their ability to decrease the bioavailability of carcinogens, inhibit arachidonic acid metabolism, and inhibit protein kinase C activity may also render them anticarcinogenic (4). Green tea extracts, which contain the flavonoids catechin and quercetin, have been postulated to increase antioxidant enzyme activity, specifically glutathione peroxidase and catalase (129). This anticarcinogenic activity has been demonstrated in inhibition of colon, esophagus, lung, liver, mammary, and skin cancers (4,130).

Limasset et al. (131) measured the ability of various flavonoids to inhibit ROS production by stimulated polymorphonuclear neutrophil leukocytes (PMNs). NADPH-oxidase was activated by stimulating PMNs with either N-fMetLeuPhe (a bacterial peptide, FMLP), phorbol myristate acetate (a protein kinase C activator, PMA), or opsonized zymosan (OZ). ROS production was measured by chemiluminescence. Limassett et al. (131) reported that the citrus flavonoids hesperidin and naringin were specific inhibitors of FMLP activation, which suggests that they interfere with the lipid environment of the membrane FMLP receptor or with the receptor itself. They also reported that quercetin inhibited protein kinase C, possibly by competing with ATP for the catalytic site. Because protein kinase C is believed to phosphorylate specific cellular proteins and enzymes that trigger cellular activation, inhibition of protein kinase C would limit neutrophil activation. However, overall the most active compounds were apigenin, morin, fisetin, and the citrus flavonoid kaempferol. Interestingly, flavane, flavanone, aceacetin, and hesperidin methyl chalcone were inactive. The flavanones, primarily found in citrus fruit, are reported to have the poorer antioxidant activity, with hesperidin the only
flavonone that possesses any significant antioxidant character. In contrast, Wang (132) showed no effect of hesperidin and niringin on the autooxidation of linoleic acid and postulated that neither antioxidant activities.

Blackburn et al. (133) also reported positive effects of quercetin on ROS formation. They reported decreased FMLP-induced degranulation and superoxide production in human neutrophils following incubation with quercetin. They suggest that quercetin inhibits the activity of protein kinase C, tyrosine kinase, phosphorylase kinase or Ca\(^{2+}\)-dependent phosphodiesterase and thus inhibits neutrophil activation. Quercetin has also been shown to exhibit higher antiradical activity towards hydroxyl radicals, peroxyl radicals, and superoxide anions in iron-loaded hepatocytes than catechin or diosmetin (122). Morel (122) demonstrated that quercetin was able to scavenge hypochlorous acid generated by myeloperoxidase more effectively than rutin, although rutin has been shown to efficiently form inert complexes with iron and inhibit iron-dependent lipid peroxidation (122). Similarly, Wang et al. (132) reported that both quercetin and rutin inhibit the autooxidation of linoleic acid. Quercetin feeding (3 μg/mL) in mice also decreases NADPH- and ascorbate-dependent lipid peroxidation of liver microsomes in a dose dependent manner. This effect was observed with a concomitant increase of catalase and glutathione reductase. Hepatic SOD and glutathione peroxidase were not affected by quercetin feedings. Additionally, quercetin is capable of removing iron already inside hepatocytes, thus inhibiting iron-dependent lipid peroxidation in vitro (122) and may act as an antiinflammatory agent (134).
In human epidemiology studies, flavonoids have been associated with low rates of coronary heart disease (CHD) (130). This observation was first noted in segments of the French population who have similar atherogenic risk factors, including saturated fat intake and plasma cholesterol concentrations, to segments of the U.S. population, but have a much lower incidence of CHD. It has been postulated that this effect results from the consumption of large quantities of red wine that contains abundance of flavonoids including catechins, flavonols and anthocyanins (4). However, the ethanol content could also contribute to the positive effect of red wine on CHD.

Flavonoids from red wine, which contains a high concentration of quercetin, have been shown to increase serum antioxidant capacity by 18% (9). They also inhibit macrophage- or copper-induced LDL oxidation *in vitro* (136) more effectively than α-tocopherol, which suggests that they may be potent inhibitors of atherosclerosis (137,138). However, de Rijke et al. (139) reported that 550 mL of low-alcohol red wine consumed daily for 4 weeks did not affect the susceptibility of LDL to Cu²⁺-induced oxidative modification. They concluded that the French paradox may be due to factors other than the effect of wine on LDL oxidation.

The Zutphen Elderly Study (135) is the only published epidemiological study to date which examines dietary flavonoid intake and the incidence of CHD. The average flavonoid intake of 805 men aged 65-84 years was 26 mg/d, with major contributors being tea (61%), onions (13%), and apples (10%). A significant inverse relationship was found between dietary flavonoid intake and CHD mortality. A weaker inverse relationship was found between flavonoid intake and myocardial infarction (MI). The predominant
flavonoid consumed in foods was quercetin. Tertiles of quercetin intake gave similar relative risks for CHD and MI as tertiles of total flavonoid intake. Adjustment for other dietary factors and for non-dietary factors such as blood lipids, obesity, and physical activity did not attenuate the relationship between flavonoid intake and CHD. The researchers hypothesize that diets with a high flavonoid content may protect LDL against oxidation by free radicals.

De Whalley et al (140) found that the flavonoids fisetin, morin, and quercetin were potent inhibitors of LDL modification by mouse macrophages. These flavonoids also inhibited the cell-free oxidation of LDL mediated by copper sulfate. Protection of LDL was hypothesized to be the result of inhibition of lipid hydroperoxide generation and protection of α-tocopherol (and possibly other endogenous antioxidants) from oxidation. The inhibition of CHD may be due not only to the inhibition of LDL oxidation, but to the ability of flavonoids to inhibit cyclooxygenase and lipoxygenase, thus inhibiting thromboxane and leukotriene formation (141). However, the ability of flavonoids to inhibit LDL oxidation in vivo is dependent on their absorption, metabolism, and especially the association between flavonoid and lipoprotein.

The mechanisms by which flavonoids inhibit LDL oxidation are unknown. Flavonoids may decrease ROS formation (121,124) or delay LDL oxidation by being oxidized themselves and protecting α-tocopherol (140). Alternatively, flavonoids may donate a hydrogen atom to regenerate the α-tocopherol radical (137) or chelate divalent metal ions and reduce the formation of free radicals induced by the Fenton reaction (121). Flavonoids such as catechin, rutin, and quercetin not only inhibit LDL oxidation, but also
the cytotoxicity of oxidized LDL (141). In addition, cells preincubated with these flavonoids are resistant to the cytotoxicity of previously oxidized LDL.

Some research has examined the effects of flavonoid drugs on the prevention of inflammatory-related conditions. For example, PMN production of ROS following stimulation by phorbol myristate acetate is decreased after administration of Daflon 500 mg, a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin prescribed for its antiinflammatory action (142). Although diosmin inhibits superoxide formation, hesperidin alone and Daflon 500 mg did not. However, hesperidin and Daflon 500 mg both inhibited H₂O₂ formation. The researchers hypothesize that hesperidin and Daflon 500 mg inhibit myeloperoxidase activity. Struckmann (143), on the other hand, reported a dose-dependent inhibition of superoxide production by rat neutrophils following administration of Daflon 500 mg. Similarly, Jean et al. (144) demonstrated that Daflon 500 mg inhibited both superoxide and H₂O₂ production by zymosan-stimulated mouse macrophages. Daflon 500 was also able to scavenge H₂O₂ in cell-free systems. Daflon 500 mg has also been shown to reduce the hyperglycemia induced by alloxan injection in the rat (144). It was hypothesized that Daflon 500 mg protected the β-cells of the pancreas from the destructive effects of ROS.

**Flavonoids and NIDDM**

There is a lack of data concerning the relationship between flavonoids and NIDDM. Because of the elevated oxidative stress experienced by these patients and the data supporting the role of flavonoids as antioxidants, further research is warranted. Bone
et al. (145) demonstrated that epicatechin can alter insulin secretion and islet cell replication in isolated islet cells. However, epicatechin did not improve diabetic symptoms in streptozotocin-diabetic or spontaneously diabetic BB/E rats. It should be noted that these results are from an insulin-dependent diabetic model and that this study did not examine the effects of epicatechin in a NIDDM model.

Secondary benefits of flavonoid supplementation may occur in patients with NIDDM. Flavonoids inhibit aldose reductase and may be beneficial in inhibiting sorbitol accumulation (7). There is also evidence that flavonoids, specifically quercetin, may inhibit the breakdown of ascorbate to dehydroascorbate (146). Because a high turnover and low plasma levels of ascorbate have been reported in patients with NIDDM (147), flavonoid supplementation may be beneficial in normalizing ascorbate levels. The maintenance of normal ascorbate levels in NIDDM patients may be especially important because ascorbate functions as an important component of cellular defense against oxygen toxicity and lipid peroxidation mediated by ROS. In addition, administration in vitro and in vivo of ascorbic acid reduces sorbitol accumulation in human erythrocytes (148). Moreover, 500-1,000 mg/d supplementation of ascorbate has been shown to reduce hypercholesterolemia (149) and cutaneous vascular fragility (150) in patients with low dietary intake of ascorbate.

The sparing of ascorbate may occur through inhibition of ascorbate photooxidation (151,152), inhibition of ascorbic acid oxidase (152), or chelation of copper and other trace metals, resulting in the retardation of metal-catalyzed oxidation (7). Another protective mechanism is based on the ability of flavonoids to scavenge ROS. Quercetin and rutin
have been found to have greater ascorbate protective activity than other flavonoids (153). However, hesperidin had significant protective capacity and increased the tissue ascorbate concentration \textit{in vitro} and \textit{in vivo}. Nonetheless, question arose as to whether the hesperidin preparation was contaminated with other flavonoids. Finally, flavonoids such as quercetin and hesperidin have been shown to enhance the reduction of dehydroascorbate by glutathione.
CHAPTER 3

MATERIALS AND METHODS

Animals and Treatments

Twenty-four male, Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, Indiana), initially weighting 175-199 g were housed individually in stainless steel cages, and provided with deionized water *ad libitum*. Rats were randomly assigned (n = 8 per group) to one of three feeding groups: low zinc diet (ICN Biochemicals, Cleveland, Ohio), adequate zinc diet (4 ppm zinc as zinc carbonate) pair fed to the mean intake of rats fed the low zinc diet, and adequate zinc diet fed *ad libitum*. Rats were maintained on their respective diets for two weeks prior to sacrifice. Blood was collected via cardiac puncture into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as preservative. Measurement of plasma zinc, isolation of the non-HDL fraction of plasma, and the VLDL/LDL oxidation assay were performed in the same manner as for the NIDDM subjects (see below). The animal protocol was approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.
Human Subjects and Treatments

Sixty non-insulin-dependent diabetic women were recruited from the practices of Dr. David Frid (Cardiology, The Ohio State University) and Dr. Charles Katz (Endocrinology, private practice) for participation in this investigation. All subjects were postmenopausal and between the ages of 40 and 60 years. Subject characteristics are presented in Table 1. Participants gave their written informed consent on a form approved by the Biomedical Sciences, Human Subjects Review Committee of The Ohio State University (Appendix).

Subjects were randomly assigned to receive either a zinc supplement (30 mg zinc/d as amino acid chelated zinc), a flavonoid supplement (2000 mg flavonoid complex/d as two tablets, each containing the following complexes: 500 mg citrus bioflavonoids, 200 mg orange bioflavonoids, 200 mg lemon bioflavonoids, 50 mg quercetin, 50 mg rutin), or placebo. Subjects were instructed to take the assigned treatment daily for three weeks and asked to refrain from all other supplement use during this time. Prior to initiation of supplementation, approximately 10 mL of blood was collected from an antecubital vein into vacutainers containing EDTA as preservative. Because of constraints due to subject recruitment, samples were not drawn from fasting participants. A second appointment was scheduled for three weeks following the initial blood draw. To reproduce absorptive conditions as accurately as possible, this appointment was scheduled for the same time of day as the first blood draw. Subjects were instructed not to take the assigned treatment on the day of the second blood draw. At the second appointment, all unused supplement/placebo was collected and counted in order to assess compliance. If five or
more capsules were returned, the subject would be dropped from the study for noncompliance. However, it was not necessary to drop any subjects.

**Analytical Measurements**

Blood samples were centrifuged at 1500 x g for 15 minutes. Two milliliters of plasma was removed and stored at 5° C for no more than 24 hours. This aliquot was used for the VLDL/LDL oxidation assay. The remaining plasma was aliquoted and frozen at 0° for future analysis.

**Plasma zinc**

Samples from subjects receiving the zinc supplement and placebo were diluted 1:1 with deionized water. Plasma zinc was analyzed by flame atomic absorption spectrophotometry (Spectra AA5, Varian Techtronpty Limited, Mulgrave, Victria, Australia). Plasma zinc concentration was determined by comparing sample absorbencies to those of the standard curve.

**Plasma 5’-nucleotidase activity**

Plasma 5’-nucleotidase activity was analyzed in samples from subjects who received the zinc supplement and placebo. 5’-nucleotidase catalyzes the hydrolysis of inosine 5’-monophosphate, yielding inosine. Purine nucleoside phosphorylase converts inosine to hypoxanthine, which is then oxidized to urate by xanthine oxidase. For each mole of hypoxanthine oxidized, two moles of hydrogen peroxide are liberated for conversion to uric acid. Hydrogen peroxide is measured spectrophotometrically at 510 nm by the oxidation of the chromogenic system, 3,5-dichloro-2-hydroxybenzenesulfonic acid.
acid/4-aminophenazone in the presence of peroxidase, forming a red quinoneimine dye (156).

To prepare the stock solution, 9.237 g of barbital sodium, 585 mg of dichlorobenzenesulfonyl chloride, and 797.4 mg of disodium hydrogen phosphate-2-hydrate were dissolved in 800 mL of deionized water and heated gently for 1 hr. One mL (946 mg/L) of ferrocyanide solution was added to the mixture and the volume brought to 1 L with deionized water. The final stock solution was stored at room temperature for no more than 2 months.

The reaction mixture was prepared by dissolving 4.413 g of β-glycerophosphate, 4.55 mg of 4-aminoatipyrine, and 13.72 mg of manganese acetate-4-hydrate in 48 mL of stock solution. The pH was adjusted to 7.6 at 37°C with 1 mol/L HCl. 5.6 mg peroxidase, 332 μL of xanthine oxidase, and 44.8 μL of nucleoside phosphorylase were then added to the mixture and the final volume brought to 50 mL with stock solution.

The starting reagent was prepared by dissolving 1.148 g of inosine 5’-monophosphate in 10 mL deionized water.

The assay was performed by pipetting 0.5 mL of reaction mixture and 0.05 mL of plasma into a 1 cm path length cuvette. When the serum blank no longer displayed a change in absorbance (approximately 10-15 minutes), 0.01 mL of starting reagent was added. When the absorbance change became linear (approximately 20 minutes), the absorbance was recorded for 8 minutes at 510 nm and 37°C. 5’-nucleotidase activity was determined by the following formula:
$5'$-nucleotidase activity (U/L) = \Delta A/\text{min} \times 153^*$

* the dye extinction coefficient given by xanthine as substrate

**Plasma insulin-like growth factor-1 (IGF-1)**

Plasma IGF-1 values were determined in samples from subjects who received the zinc supplement and placebo. Analysis was performed by radioimmunoassay at the Clinical Research Center at The Ohio State University.

**Human sex hormone binding globulin (SHBG)**

Plasma SHBG was measured by radial immunodiffusion (The Binding Site, San Diego, California) in samples from subjects who received the citrus flavonoid supplement or placebo. This method involves the diffusion of antigen (SHBG) from a cylindrical well through an agarose gel which contains a mono-specific antibody to SHBG.

The assay was performed by pipetting 10 µL of plasma or standard into each well in the agarose-coated plate. Plates were incubated at room temperature for 72 hours to allow for antigen diffusion through the agarose gel. The square of the diameter of the ring that was formed by this diffusion was related linearly to the concentration of SHBG.

**Plasma vitamin C concentration**

Plasma vitamin C concentration was measured in the citrus flavonoid-supplemented group and placebo group by the reduction of dinitrophenylhydrazine (DNP) by vitamin C (158). The stock standard was prepared as 1 mg/mL ascorbic acid in deionized water. The working standard was prepared as 10 µg/mL in deionized water and prepared fresh daily. The DNP reagent was prepared by combining 2.0 g of 2,4-dinitrophenylhydrazine, 250 mg of thiourea, 3.0 mL of 10 mg/mL CuSO$_4$$\cdot$5H$_2$O, and 50
mL of concentrated H$_2$SO$_4$ in 150 mL of H$_2$O and filtering through glass wool using an aspirator vacuum. This reagent was made daily.

The assay was performed by adding 1.6 mL 10% TCA to 0.4 mL of the H$_2$O blank, standard, or plasma and chilling for 30 minutes in ice water bath. The mixture was centrifuged at 2000 rpm for 10 minutes. One mL aliquots were then pipetted into culture tubes and 0.4 mL dinotrophenylhydrazine reagent added. The blank, standards, or plasma aliquots were capped and incubated at 37°C for 3 hours. Culture tubes were then chilled in an ice water bath and 1 mL of cold 65% H$_2$SO$_4$ added. Samples or standards were allowed to stand for 30 minutes at room temperature before absorbance was read at 520 nm. Plasma vitamin C concentration was determined by comparing the sample absorbencies to those of the standard curve.

**Plasma cholesterol**

Total cholesterol concentration was measured, using a kit from Sigma Chemical Company (St. Louis, MO), for standardization of VLDL/LDL concentrations for the oxidation assay. The cholesterol assay is based on the hydrolysis of cholesterol by cholesterol esterase, forming cholesterol and fatty acids. Cholesterol is then oxidized to cholest-4-en-3-one and H$_2$O by cholesterol oxidase. Hydrogen peroxide is coupled with the chromagen, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to form a quinoneimine dye. The absorbance is then read against a blank, and cholesterol concentration in mg/dL determined using the formula:

$$\frac{A_{\text{TEST}}}{A_{\text{CALIBRATOR}}} \times 200^*$$

* concentration in mg/mL of the cholesterol standard
VLDL/LDL oxidation

The VLDL/LDL oxidation assay was performed on all samples. Non-HDL (LDL, VLDL, and possibly lipoprotein a) was isolated by MgCl$_2$ and dextran sulfate precipitation as described by Finley et al (159). The precipitation reagent was prepared by combining equal volumes of a 20 g/L solution of dextran sulfate (Sigma) with a solution of 2 mol/L MgCl$_2$ (Sigma) and adjusting the final pH to 7.0 with 0.1 N NaOH.

The 0.9% PBS solution was prepared by combining 250 mL of 0.15 mol/L NaCl (Jenneile Enterprises, Cincinnati, Ohio) and 250 mL of 10 mmol/L NaH$_2$PO$_4$ (Mallinckrodt, Paris, Kentucky) and adjusting the pH to 7.0 with 1 N NaOH. The 4.0% PBS solution was prepared by combining 250 mL of 0.68 mol/L NaCl (Jenneile Enterprises) and 250 mL of 10 mmol/L NaH$_2$PO$_4$ and adjusting the pH to 7.0 with 1 N NaOH.

The CuSO$_4$ solution was prepared by adding 12.4 mg CuSO$_4$$\cdot$5H$_2$O to 10 mL of H$_2$O, yielding a 8 mM solution.

The assay was performed by precipitating the non-HDL fraction from 2 mL of plasma and 2 mL of 4% PBS by adding 0.4 mL of precipitation reagent, vortexing for 1 minute, and centrifuging at 1500 x g for 10 minutes. EDTA was then removed by suspending the non-HDL pellet in 4 mL of 0.9% PBS and reprecipitating with 0.2 mL of precipitation reagent, vortexing for 1 minute, and centrifuging at 1500 x g for 10 minutes. The non-HDL pellet was then dissolved in 1.5 mL of 4% PBS and cholesterol concentration determined by the method described previously.
The cholesterol concentration of the non-HDL fraction was adjusted to 75 \( \mu \text{g/mL} \) and oxidation of the non-HDL fraction initiated by the addition of 2 \( \mu \text{L} \) of \( \text{CuSO}_4 \) to 2 mL of the diluted non-HDL solution. Conjugated diene formation was monitored by incubating samples at 37°C and reading absorbency at 234 nm every 10 minutes as described by Esterbauer (160). The lag phase was determined by drawing a line tangent to the line representing the propagation phase of the curve and extrapolating this line through the horizontal (time in minutes) axis. The time interval between the addition of copper to the non-HDL solution and the extrapolation point was defined as the lag time.

The direct effects of plasma zinc on VLDL/LDL oxidation were assessed in 4 pooled plasma samples derived from 6 rats maintained on a zinc-deficient diet. Zinc (10 \( \mu \text{g/mL} \)) was added to 2 mL of plasma, producing a concentration of zinc similar to that found in rats maintained on a zinc-adequate diet, before isolation of the non-HDL fraction. VLDL/LDL oxidation lag time and propagation rate were compared between these samples and those derived from animals maintained on an adequate zinc diet.

**Immunoglobulin E (IgE)**

IgE concentration was measured by enzyme linked immunoadsorbent assay (ELISA) in all samples. The kit was obtained from The Binding Site (San Diego, California) and is based on the “sandwich method”. This assay method implements an amplification system in which the alkaline phosphatase reaction provides a cofactor that activates a redox cycle, leading to the formation of a colored product. Color development is based on the concentration of IgE in the sample.
One hundred microliters of plasma or standard were incubated in microwells pre-coated with an anti-IgE monoclonal antibody to which IgE will bind. Following a 90 minute incubation at 2° C, the wells were washed to remove all unbound proteins. Anti-human IgE peroxidase conjugate (100 μL) was then added to each well to “sandwich” the IgE immobilized during the first incubation. Plates were incubated at 2° C for 90 minutes. The IgE peroxidase conjugate bound to the captured IgE and excess conjugate was removed by a further washing of the wells. The bound conjugate was visualized using 3,3′,5,5′-tetra-methylbenzidine (100 μL) which turned yellow on addition of stopping solution. Optical density was read at 450 nm and sample IgE concentration determined from the standard curve.

**Tumor necrosis factor-α (TNF-α)**

TNF-α concentration was measured by ELISA in all samples. The kit was obtained from R&D Systems (Minneapolis, Minn.) and is also based on the “sandwich method” described previously.

Two hundred microliters of plasma or standard were incubated in microwells precoated with a monoclonal antibody specific for TNF-α. Following a 20 hour incubation at 2° C, the wells were washed to remove all unbound protein. TNF-α conjugate (200 μL) was then added to the wells to “sandwich” the TNF-α immobilized during the first incubation. Plates were incubated for 4 hours at room temperature and again washed. Substrate solution (50 μL of nicotinamide adenine dinucleotide phosphate, NAD) was added to the wells and incubated at room temperature for 60 minutes before 50 μL of amplifier solution was added to initiate color development. In this amplification
system, alkaline phosphatase dephosphorylates the reduced form of NAD. The NADH subsequently serves as a specific cofactor that activates a redox cycle driven by the secondary enzyme system consisting of alcohol dehydrogenase and diaphorase. NADH reduces a tetrazolium salt by the action of diaphorase, producing a colored formazan dye and NAD\(^+\). Optical density was read at 490 nm and sample TNF-\(\alpha\) concentration determined from the standard curve.

**Myeloperoxidase (MPO)**

Myeloperoxidase concentration was measured by ELISA in all samples. A kit was obtained from Calbiochem (San Diego, CA). This method uses biotinylated polyclonal anti-MPO as antibody. The final step of the assay is based on biotin-avidin coupling which uses avidin covalently-linked to alkaline phosphatase. MPO concentration is determined by adding the alkaline phosphatase substrate p-nitrophenylphosphate. Color development is based on the concentration of myeloperoxidase in the sample.

One hundred microliters of plasma or standard was added to microwells precoated with a primary monoclonal antibody to myeloperoxidase. Following a 2 hour incubation at 37\(^\circ\) C, the wells were washed to remove all unbound protein. Biotinylated polyclonal anti-myeloperoxidase (100 \(\mu\)L) was added to each well and incubated for 1 hour at 37\(^\circ\) C. Plates were washed and avidin-alkaline phosphatase solution (100 \(\mu\)L) was then added to each well. Following a 1 hour incubation at 37\(^\circ\) C, p-nitrophenylphosphate (100 \(\mu\)L) was added to each well. Optical density was read at 405 nm and sample myeloperoxidase concentration determined from the standard curve.
Statistical Analysis

All statistical analysis was performed using Statistical Analysis (SAS) software. For the rat data, results were expressed as means ± standard error of the mean. Between group means were compared by 2-way analysis of variance (ANOVA) and Fisher's least-significant-difference (LSD) multiple comparisons method. For the NIDDM data, results were expressed as means ± standard deviations. Differences from pre- to post-supplementation were compared for each group using paired t-tests. Statistical significance was set for all analysis at $p \leq 0.05$. 
Table 1. Human subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>ZINC</th>
<th>FLAVONOIDS</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>AGE (YEARS ± SD)</td>
<td>60.0 ± 12.0 (45 - 72)</td>
<td>55.3 ± 7.7 (49 - 69)</td>
<td>54.9 ± 8.4 (48 - 80)</td>
</tr>
<tr>
<td>WEIGHT (LBS ± SD)</td>
<td>197.2 ± 56.0 (160 - 270)</td>
<td>200.2 ± 31.7 (171 - 265)</td>
<td>184.4 ± 34.4 (140 - 270)</td>
</tr>
<tr>
<td>NUMBER OF YEARS DIABETIC (± SD)</td>
<td>13.4 ± 10.1 (2 - 22)</td>
<td>10.1 ± 10.4 (1 - 20)</td>
<td>9.5 ± 7.0 (1 - 17)</td>
</tr>
<tr>
<td>NUMBER OF SUBJECTS TAKING INSULIN</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>NUMBER OF SUBJECTS TAKING ORAL GLYCEMIC AGENTS</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
CHAPTER 4

RESULTS

Rat Study

The purpose of this study was to determine if, in a rat model, VLDL/LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress. Based on body weight and plasma zinc (Table 2), rats fed a low zinc diet were moderately zinc deficient.

Copper-induced VLDL + LDL oxidation lag time was significantly shorter for rats fed the low zinc diet compared to those fed the zinc adequate diets (Figure 2, Table 3). In addition, the oxidation propagation rate was significantly higher in the low zinc group than in the adequate zinc groups. Addition of zinc to plasma samples had no significant effect on lag time or propagation rate (Table 4). The additional zinc normalized the zinc content of the plasma from the deficient animals.

Human Study

The purpose of this study was to determine if zinc or citrus flavonoid supplementation improves indices of oxidative stress, especially VLDL/LDL oxidation, in women with NIDDM.
Diet | Body Weight (g ± SD) | Plasma Zinc (µg/mL ± SD)
--- | --- | ---
Adequate Zinc | 238 ± 6<sup>a</sup> | 11 ± 2<sup>a</sup>
Pair-Fed | 215 ± 4<sup>b</sup> | 11 ± 1<sup>a</sup>
Low Zinc | 197 ± 3<sup>c</sup> | 7 ± 1<sup>b</sup>

Values expressed as means ± SEM
Different superscripts in the same column denote significant differences (p < 0.001, 2-way ANOVA + LSD).

Table 2. Effects of diet on zinc status in 8 rats.
Figure 2. Example of LDL oxidation curve
<table>
<thead>
<tr>
<th>Diet</th>
<th>Lag Time (minutes ± SD)</th>
<th>Propagation Rate (pMoles/min/mg cholesterol ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate Zinc</td>
<td>73 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pair-Fed</td>
<td>80 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low Zinc</td>
<td>42 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts in the same column denote significant differences (p < 0.001, 2-way ANOVA + LSD)

Table 3. Effects of zinc intake on lipoprotein oxidation in 8 rats.
Table 4. Effects of zinc addition *in vitro* on lipoprotein oxidation in 6 rats.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Lag Time (minutes ± SD)</th>
<th>Propagation Rate (pMoles/min/mg cholesterol ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate Zinc</td>
<td>85 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low Zinc</td>
<td>55 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low Zinc + Zinc</td>
<td>46 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts in the same column denote significant differences (p < 0.01, paired t-test)
Zinc Supplementation

Initial plasma zinc values were low in the NIDDM patients, although there was a great deal of variation. Amino acid-chelated zinc was chosen as the supplement form because it is better absorbed and may be less likely to interfere with copper status than other forms of zinc supplements. The zinc supplement was well-absorbed in the NIDDM patients as indicated by the strong rise in plasma zinc observed following three weeks of zinc supplementation (Figure 3). Additionally, initial plasma 5'-nucleotidase activity was extremely low in all of the NIDDM patients (Figure 4). Bales et al. (154) demonstrated that this zinc-dependent enzyme may be a sensitive indicator of mild zinc deficiency in healthy elderly subjects. 5'-nucleotidase activity significantly increased following zinc supplementation but was still well below normal. The combination of low plasma zinc and low 5'-nucleotidase activity suggests that the NIDDM patients were somewhat zinc deficient. As would be expected, there was a moderate correlation ($r = 0.54$) between plasma zinc and plasma 5'-nucleotidase activity.

Plasma IGF-1 concentration was measured as a secondary functional indicator of zinc status. Conceivably, zinc supplementation could raise IGF-1 values because zinc is required for dimerization of growth hormone, an inducer of IGF-1. On the other hand, zinc supplementation may lower IGF-1 values that have been elevated in response to increased stress (156). IGF-1 concentration was significantly affected by zinc supplementation (Table 5). Twelve of the twenty zinc-supplemented subjects had initial IGF-1 values below 165 ng/mL. In these subjects, zinc supplementation significantly increased IGF-1 values. In the eight subjects whose initial IGF-1 values were above 165
ng/mL, zinc supplementation significantly lowered IGF-1 values. Because plasma IGF-1 values were measured as an indicator of the general effects of zinc supplementation, a strong correlation would be expected between plasma IGF-1 and plasma zinc. There was a fairly weak correlation (0.30) between the two.

Despite evidence that the NIDDM patients were moderately zinc deficient and that zinc deficiency increases the susceptibility of LDL to oxidation (Figure 3, Figure 4), zinc supplementation had no effect on LDL oxidation. Although LDL oxidation lag time in the NIDDM patients tended to increase following zinc supplementation, the increase was not significant. The propagation rate was also not affected by zinc supplementation (data not shown).

Zinc supplementation also did not significantly affect the IgE and TNF values. The NIDDM patients examined in this investigation exhibited abnormal values for these parameters. IgE was elevated drastically above what is considered normal (Table 6) and TNF was elevated slightly above what is considered normal (Table 7). TNF values were moderately correlated with the LDL oxidation lag time in the zinc supplemented group ($r = 0.41$). On the other hand, myeloperoxidase activity was within the normal range. It was not significantly affected by zinc supplementation but tended to increase over the three week period (Table 8).

Between-group subject characteristics were not significantly different from each other. However, regression analysis was used to determine if the number of years a patient was diabetic was a significant predictor of oxidative stress. The number of years a
Figure 3. Effect of zinc supplementation on plasma zinc in non-insulin-dependent diabetic women

* Significantly different from pre-supplementation (p < 0.05, paired t-test)

Plasma Zinc (μg/mL)

- Zinc
- Placebo

* "Pre-supplementation"
* "Post-supplementation"
Figure 4. Effect of zinc supplementation on plasma 5'-nucleotidase activity in non-insulin-dependent diabetic women

* Significantly different from pre-supplementation (p < 0.05, paired t-test)
<table>
<thead>
<tr>
<th>Group</th>
<th>Initial IGF-1 Value (ng/mL)</th>
<th>N</th>
<th>Pre Zinc IGF-1 (ng/mL ± SD)</th>
<th>Post Zinc IGF-1 (ng/mL ± SD)</th>
<th>Mean Change (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>≤ 165</td>
<td>12</td>
<td>113.0 ± 31.5</td>
<td>154.2 ± 50.1</td>
<td>+ 43.5</td>
</tr>
<tr>
<td></td>
<td>&gt; 165</td>
<td>8</td>
<td>193.6 ± 21.9</td>
<td>152.3 ± 61.7</td>
<td>- 40.9</td>
</tr>
<tr>
<td>Placebo</td>
<td>≤ 165</td>
<td>15</td>
<td>94.8 ± 32.4</td>
<td>112.3 ± 30.8</td>
<td>- 16.8</td>
</tr>
<tr>
<td></td>
<td>&gt; 165</td>
<td>5</td>
<td>245.5 ± 88.0</td>
<td>268.2 ± 102.0</td>
<td>- 5.3</td>
</tr>
</tbody>
</table>

*Significantly different from pre-supplementation (p < 0.05, paired t-test)

Table 5. Plasma insulin-like growth factor-1 values in 40 non-insulin-dependent diabetic women.
patient was diabetic was not a significant predictor of LDL oxidation lag time, plasma myeloperoxidase activity, or plasma IgE levels. In addition, when data from those patients whose diabetes was controlled with insulin was analyzed separately from those whose diabetes was controlled by oral glycemic agents, results were not different from those obtained when both subgroups were analyzed together.

**Citrus Flavonoid Supplementation**

Plasma SHBG was measured as a possible general indicator of the efficacy of citrus flavonoid supplementation. In the subjects who received the citrus flavonoid supplement, there was no change in plasma SHBG (Figure 5). SHBP values were extremely variable.

Plasma vitamin C values were also not affected by citrus flavonoid supplementation, although values were significantly lower (0.23 mg/dL) than what is observed in healthy subjects (Table 6). In addition, the vitamin C-sparing effect of flavonoid-supplementation that has been reported by some investigators *in vitro* (106, 149) was not observed in this study.

LDL oxidation lag time was not significantly affected by citrus flavonoid supplementation, although it tended to increase over the supplementation period (Table 7). The propagation rate was also not affected by citrus flavonoid supplementation (data not shown).

IgE (Table 8) and TNF (Table 9) values were not significantly affected by citrus flavonoid supplementation. Myeloperoxidase activity was also not significantly affected following supplementation (Table 10).
Plasma sex hormone binding globulin (mg/mL)

□ Pre-supplementation
□ Post-supplementation

Figure 5. Effect of citrus flavonoid supplementation on plasma sex hormone binding globulin in non-insulin-dependent diabetic women

(Paired t-test)
<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Supplementation Vitamin C (μmol/L ± SD)</th>
<th>Post-Supplementation Vitamin C (μmol/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>13.06 ± 3.7</td>
<td>14.2 ± 4.2</td>
</tr>
<tr>
<td>Placebo</td>
<td>13.6 ± 3.8</td>
<td>13.6 ± 4.0</td>
</tr>
<tr>
<td>Normal (non-diabetic)</td>
<td>23 - 85</td>
<td></td>
</tr>
</tbody>
</table>

(Paired t-test)

Table 6. Plasma vitamin C in 40 non-insulin-dependent diabetic women.
<table>
<thead>
<tr>
<th>Group</th>
<th>Lag Time (minutes ± SD) Pre-Supplementation</th>
<th>Lag Time (minutes ± SD) Post-Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>69.9 ± 21.4</td>
<td>74.3 ± 16.1</td>
</tr>
<tr>
<td>Citrus Flavonoid</td>
<td>67.9 ± 15.2</td>
<td>73.1 ± 15.7</td>
</tr>
<tr>
<td>Placebo</td>
<td>69.8 ± 20.2</td>
<td>67.5 ± 14.5</td>
</tr>
</tbody>
</table>

(Paired t-test)

Table 7. Effects of zinc or citrus flavonoid supplementation on LDL oxidation in 60 non-insulin-dependent diabetic women
<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Supplementation (IU/L ± SD)</th>
<th>Post-Supplementation (IU/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>10 - 350</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>749.4 ± 947.8</td>
<td>481.1 ± 685.6</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>968.0 ± 623.5</td>
<td>821.8 ± 430.5</td>
</tr>
</tbody>
</table>

(Paired t-test)

Table 8. Effect of zinc or citrus flavonoid supplementation on plasma immunoglobulin E levels in non-insulin-dependent diabetic women
<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Supplementation (pg/mL ± SD)</th>
<th>Post-Supplementation (pg/mL ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.47 - 5.5</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>5.8 ± 3.5</td>
<td>4.6 ± 2.7</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>5.8 ± 2.7</td>
<td>5.4 ± 3.2</td>
</tr>
</tbody>
</table>

(Paired t-test)

Table 9. Effect of zinc or citrus flavonoid supplementation on plasma tumor necrosis factor levels in non-insulin-dependent diabetic women
<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Supplementation (ng/mL ± SD)</th>
<th>Post-Supplementation (ng/mL ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>10 - 300</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>12.9 ± 0.63</td>
<td>17.0 ± 0.62</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>13.6 ±1.89</td>
<td>18.9 ± 3.5</td>
</tr>
</tbody>
</table>

(Paired t-test)

Table 10. Effect of zinc or citrus flavonoid supplementation on plasma myeloperoxidase levels in non-insulin-dependent diabetic women
CHAPTER 5

DISCUSSION

Antioxidant supplementation has been shown to decrease markers of oxidative stress in both animal (5,39,75,79) human models (80,97,110,112). This study demonstrated that supplementation with the antioxidants zinc and citrus flavonoids had no effect on indices of oxidative stress in women with NIDDM. The rat study verified that VLDL/LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress. However, this oxidation was not affected by zinc supplementation in the human study. Although other indices of oxidative stress, including IgE and TNF, were not affected by either supplement, it is noteworthy that both were elevated above normal values in the NIDDM patients used in this study.

Rat Study

Zinc deficiency has been shown to increase oxidative stress in animal models (60,61,63). The rat data, which indicate that VLDL/LDL oxidation is increased in marginally zinc-deficient animals, support these results. These results also support the hypothesis that zinc decreases VLDL/LDL oxidation, seemingly through antioxidant
mechanisms. This hypothesis is supported by several observations. First, pair fed animals had normal oxidation values. Therefore, reduced feed intake could not account for the increased VLDL/LDL oxidation observed in the zinc deficient animals. Second, addition of zinc to plasma samples from zinc deficient animals did not change the oxidation of LDL. As a result, zinc plays some role in protecting LDL from oxidation besides just its presence in the oxidation reaction mixture. Third, although addition of large amounts of zinc (i.e., 3 μM) to some lipoprotein oxidation assays can increase resistance to oxidation (78), the amount that would be present in this study would have been only about 0.1 μM. Because blood samples were drawn into tubes containing EDTA, most of the endogenous zinc present in the sample would be chelated and removed with the EDTA. Fourth, zinc deficiency probably does not increase the susceptibility of LDL to oxidation through changes in lipid composition. In humans with genetic zinc deficiency, the lipid linoleic acid concentration is inversely proportional to the dosage of zinc with which the patient is supplemented (168). Thus, it would seem that lowering linoleic acid would increase resistance of LDL to oxidation. This does not appear to be the case. Finally, zinc deficiency did not increase the susceptibility of LDL to oxidation by decreasing activity of the antioxidant enzyme extracellular superoxide dismutase (ECSOD). Both moderately zinc deficient rats (DiSilvestro, R, unpublished results) and severely deficient rats (170) have been shown to have only small decreases in ECSOD activity.
Zinc Supplementation

This study demonstrated that women with NIDDM may be at high risk for zinc deficiency. Plasma zinc was low in all patients examined, although there was a great deal of variation. As a result, plasma zinc may be a particularly unreliable indicator of zinc status in diabetics because of high rates of flux through the serum caused by high excretion rates (85). Nevertheless, the extremely low 5'-nucleotidase values that were observed support the contention that subjects were zinc deficient. Zinc status appeared to increase following three weeks of supplementation, as indicated by increases in both plasma zinc and plasma 5'-nucleotidase activity. However, despite the two-fold increase in 5'-nucleotidase activity, values were still well below normal. A longer supplementation period might normalize the values; however, the study was designed to keep the supplementation period short to avoid the confounding effects of changes in diabetic symptoms that might be encountered with longer studies.

IGF-1, measured as an indicator of the general effects of zinc supplementation, was normalized following the supplementation period. Changes in IGF-1 concentration as a result of zinc supplementation may be indicative of several phenomenon. Zinc depletion has been shown to attenuate circulating IGF-1 in rats fed a zinc-deficient (0 ppm) diet while zinc supplementation restores IGF-1 values to normal (161). Furthermore, administration of IGF-1 intravenously to diabetic rats has been shown to increase energy expenditure and lipid oxidation (162). Therefore, zinc supplementation may be beneficial in a population susceptible to zinc deficiency by increasing circulating IGF-1 levels. In this respect, increased levels of IGF-1 may be beneficial. On the other hand, there appears to
be a close relationship between IGF-1 synthesis and the development of atherosclerosis in experimental animals (164). Two observations relate IGF-1 synthesis to CHD. First, smooth muscle cells have been shown to synthesize and secrete an IGF-1-like peptide. Secondly, IGF-1 is regulated by platelet-derived growth factor. In addition, it has been shown that following balloon-denudation carotid injury and other acute-injury models, there is a large increase in IGF-1 synthesis. Therefore, IGF-1 may be involved in some way in ROS-mediated injury. As a result, decreases in IGF-1, possibly mediated by decreased oxidative stress, may be associated with a decreased risk for CHD.

Despite evidence for normalization of IGF-1 by zinc supplementation, many problems arise when measuring IGF-1 in diabetics. Patients with poorly controlled diabetes have been shown to have low-normal or high levels of IGF-1 (164). These discrepancies may be due to differences in assay methodology, interference of IGF-binding proteins in IGF radioimmunoassays, or failure to standardize patient treatment protocols. As a result, cross-study comparisons of IGF-1 are difficult. It appears that other factors besides improved zinc status can modulate IGF-1 values. For example, precise control of blood glucose levels may also result in normalization of IGF-1 (165). It has been proposed that metabolic deterioration first results in decreased synthesis of IGF-1 and subsequently in decreased plasma levels. Secondarily, loss of negative feedback regulation results in hypersecretion of growth hormone which would stimulate overproduction of IGF-1, thus increasing plasma levels. Glucose control was not monitored in the patients used in this investigation, so it is not possible to ascertain if this is the reason for the
normalization of IGF-1. However, the short supplementation time period should have minimized the chance for changes in glucose control.

Decreases in VLDL/LDL oxidation were not observed in the NIDDM patients following zinc supplementation. It has been suggested that zinc supplementation may be beneficial only in subjects who are zinc deficient (58). However, as indicated by the extremely low 5'-nucleotidase activities, subjects were at least marginally zinc deficient. Despite the fact that 5'-nucleotidase increased significantly following zinc supplementation, values were still below normal. It is possible that zinc status was not sufficiently improved to reduce the high oxidative stress experienced by NIDDM patients. The zinc was obviously well-absorbed as indicated by the strong increase in plasma zinc; therefore, malabsorption cannot account for the lack of effect. Because plasma samples were not obtained from fasted subjects, fluctuations in VLDL concentration may conceivably interfere with interpretation of LDL oxidation assays. However, approximately half of the studies examining LDL oxidation do not obtain samples from fasted subjects. There are several reasons that this should not pose a problem when interpreting the results of the oxidation assay. First, all samples were standardized to 75 mg/mL cholesterol. Second, preformed lipid hydroperoxides and PUFAs seem to be the strongest predictors of the LDL oxidation lag time, factors that are most likely unaffected by fasting or nonfasting. Third, the myeloperoxidase and IgE results are concordant with the LDL oxidation results. If nonfasting samples were problematic, one would expect disagreement among these results. Additionally, large dose zinc supplementation can lead to a copper deficiency, which may subsequently increase oxidative stress. Although
copper status was not measured in these patients, it seems unlikely that such a small zinc dosage administered for three weeks could cause a copper deficiency. Perhaps a longer supplementation period and/or higher dosage of zinc is required to improve zinc status in NIDDM patients enough to have an effect on oxidative stress.

Because zinc supplementation did not significantly affect VLDL/LDL oxidation, it is not surprising that other indices of oxidative stress were also not affected. Research suggests that IgE production can be used as a reliable indicator of oxidative stress (170,171). Additionally, antioxidant supplementation has been shown to reduce IgE levels that have been raised in response to oxidative injury (170). Supplementation with N-acetyl-L-cysteine (NAC), an antioxidant precursor of intracellular glutathione, and glutathione have been shown to decrease IgE production in a dose dependent manner (170). It was hypothesized that these antioxidants act on T cells by increasing resistance to oxidative stress-mediated apoptosis, cytotoxic properties, synthesis and turnover of interleukin-2 receptors, interleukin-2 production, and proliferation. Because zinc also acts on T cells and may be able to modulate cytokine production, theoretically it may be able to affect IgE in a similar manner. On the other hand, elevated IgE levels may have reflected the influence of abnormal carbohydrate metabolism on IgE synthesis, rather than oxidative stress alone (171).

Myeloperoxidase levels were within the normal range in the NIDDM patients used in this study. Therefore, the elevated oxidative stress experience by NIDDM patients is most likely mediated by factors other than myeloperoxidase. However, zinc supplementation tended to raise values, which may suggest that immune function was
improved following supplementation. Because zinc plays an important role in immunity, it is logical that immune parameters are affected by supplementation. No other indices of immune function were examined, so it cannot be determined if immunity was in fact improved.

Although TNF values were slightly elevated above normal, this elevation was probably not the result of an inflammatory condition. Insulin-dependent diabetes may be an inflammatory disease, but NIDDM most likely is not (172). It has been hypothesized that advanced glycosylation end-products (AGEs) can bind to macrophages which then stimulate the release of TNF. Because the production of AGEs is increased in uncontrolled diabetes, it is likely that stimulation of TNF production is also increased. However, indices of long-term glycemic control were not measured in this study. Nevertheless, the lack of effect on TNF levels by zinc supports this contention. In animal studies, responses to inflammatory stimuli in vitro and in vivo, including TNF production, are decreased by dietary intake of zinc (173). If the elevation of TNF had been related to inflammation, one would expect a decrease following zinc supplementation. In addition, elevated TNF values have been associated with obesity (27). Because obesity is a hallmark of NIDDM, it is logical that TNF would be elevated in patients with NIDDM, and not depressed by zinc.

**Citrus Flavonoid Supplementation**

Citrus flavonoid supplementation in NIDDM patients did not decrease VLDL/LDL oxidation. These results contrast with studies that report decreases in LDL oxidation in
vitro following flavonoid supplementation (9,122,132). However, these studies did not specifically examine the effects of citrus flavonoids. Instead, catechin and quercetin were the most commonly investigated flavonoids. Although the citrus flavonoid supplement used in this study contained quercetin, it was probably a very small percentage of the supplement. However, the total quercetin content of the supplement is not known because, despite the fact that the label indicates that 5% quercetin was added to the supplement, it may have also have been added as a complex with other flavonoids. Also, work in vitro may or may not be applicable to work in vivo.

In addition, citrus flavonoid supplementation did not affect other indices of oxidative stress. Citrus flavonoids have not been shown to influence T cell activity and may therefore be unable to influence IgE production. However, examination of other flavonoids that contain sulphhydryl groups similar to those on NAC and glutathione may yield more promising results. Also, if myeloperoxidase does not mediate elevated oxidative stress in NIDDM patients, it is not surprising that the citrus flavonoid supplement had no effect on myeloperoxidase activity because it has no known function in the immune system. Finally, previous research has not examined the effects of citrus flavonoids on TNF levels. Based on their known mechanism of action, a direct reduction of TNF production following supplementation does not seem likely. As mentioned previously, it cannot be determined how much of the supplement was actually absorbed. If absorbed, the citrus flavonoids may not posses the antioxidant activity of the other flavonoids. Because patients with NIDDM experience high levels of oxidative stress, a longer supplementation period may be necessary to significantly decrease oxidative stress.
To determine if citrus flavonoid supplementation produced any physiological effects, levels of SHBG were examined. Prior work suggests that flavonoid supplementation can increase plasma levels of SHBG (157). In contrast, the present study found no effect of citrus flavonoid supplementation. In previous work, Cassidy et al. (158) measured SHBG following soya flavonoid supplementation, not citrus flavonoid supplementation. In addition, they used young, healthy women as subjects rather than patients with NIDDM. The bioavailability of citrus flavonoids is not known. As a result, it is possible that either citrus flavonoids do not modulate SHBG or that not enough citrus flavonoids were absorbed to produce changes in SHBG levels. Additionally, marked obesity, as seen in patients with NIDDM, is associated with decreased levels of SHBG (166). It may be that the effects of obesity on SHBG levels interfere with or mask the effects of citrus flavonoid supplementation.

Vitamin C sparing was also examined as a possible effect of flavonoid supplementation. These results must be viewed with caution however, because samples were not deproteinized immediately following the blood draw. Instead, they were frozen at 2°C for up to two months and deproteinized prior to analysis. As a result, loss of vitamin C could have occurred. Nonetheless, the results that were obtained on vitamin C levels are in agreement with others who report low levels in patients with NIDDM (96, 167). However, levels were much lower than what is reported by other investigators. Additionally, supplementation with citrus flavonoids did not result in sparing of vitamin C, as evidenced by a lack of change in vitamin C concentration following the supplementation period. There are several possible explanations for this lack of effect. Foremost may be
problems resulting from the storage of the samples. On the other hand, perhaps the sparing effect was overwhelmed by the tendency toward very low plasma levels of vitamin C observed in this group of patients. Moreover, the inability of the flavonoid supplement to reduce oxidative stress may be associated with its inability to spare vitamin C (i.e., increased ROS consumption of vitamin C). On the other hand, the lack of a sparing effect could also be due poor supplement absorption or a supplementation period that was too short.

Summary and Conclusions

The purpose of the rat study was to determine if LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress. Male, Sprague Dawley rats were randomly assigned to one of three feeding groups: low zinc diet, adequate zinc diet (4 ppm zinc as zinc carbonate) pair fed to the mean intake of rats fed the low zinc diet, and adequate zinc diet fed ad libitum. Rats were maintained on their respective diets for two weeks prior to sacrifice. Blood was collected via cardiac puncture and analyzed for plasma zinc and VLDL/LDL oxidation.

The purpose of the human study was to examine the effects of zinc or citrus flavonoid supplementation on indices of oxidative stress, specifically VLDL/LDL oxidation, in women with NIDDM. Sixty postmenopausal non-insulin-dependent diabetic women, between the ages of 40 and 60 years, were recruited for participation in this study. Subjects were randomly assigned to one of three groups: zinc supplementation (30 mg zinc/d as amino acid chelated zinc), flavonoid supplementation (200 mg flavonoid
complex/d), or placebo. Blood samples were drawn at the end of the three-week supplementation period for comparison to those that were drawn prior to supplementation. Samples drawn from patients who received the zinc supplement were analyzed for zinc status assessors, VLDL/LDL oxidation, and other indices of oxidative stress. Samples drawn from patients who received the flavonoid supplement were analyzed for plasma vitamin C, VLDL/LDL oxidation, and other indices of oxidative stress. Samples drawn from patients who received the placebo were analyzed for zinc status assessors, plasma vitamin C, VLDL/LDL oxidation, and other indices of oxidative stress.

The rat study demonstrated that VLDL/LDL oxidation is a good indicator of zinc deficiency-induced oxidative stress. In addition, three weeks of supplementation with either zinc or citrus flavonoid had no effect on indices of oxidative stress, including VLDL/LDL oxidation, in women with NIDDM. Specifically, the following conclusions were drawn about accepting or rejecting the proposed hypotheses:

1) In rats, a zinc-deficient diet produced a shorter lag time for VLDL/LDL oxidation compared to a diet adequate in zinc (reject).

2) Three weeks of zinc supplementation increased plasma zinc and plasma 5’-nucleotidase activity in women with NIDDM (reject). Three weeks of zinc supplementation increased IGF-1 in subjects with initial values below 164 ng/mL and decreased IGF-1 in subjects with initial values above 165 ng/mL (reject).

3) Three weeks of zinc supplementation had no effect on lag time for VLDL/LDL oxidation in women with NIDDM (accept).
4) Three weeks of citrus flavonoid supplementation had no effect on lag time for VLDL/LDL oxidation in women with NIDDM (accept).

5) Plasma IgE levels were elevated above normal (reject) and myeloperoxidase activity was normal in women with NIDDM (accept). Neither was unaffected by zinc or citrus flavonoid supplementation (accept).

6) Tumor necrosis factor was not affected by zinc or citrus flavonoid supplementation in women with NIDDM (accept).

7) Plasma vitamin C was not different in the citrus flavonoid supplemented group compared to the placebo group (accept).

Although this study demonstrated no benefits of zinc or citrus flavonoid supplementation on indices of oxidative stress, several interesting observations can be made. First, this study strengthened the contention that patients with NIDDM may be prone to both zinc deficiency and low levels of plasma vitamin C. Secondly, plasma IgE was elevated in patients with NIDDM, a disease associated with high oxidative stress. As a result, this study supports other research that suggests that IgE can be used as a marker of oxidative stress. Thirdly, because patients with NIDDM have both a propensity toward poor endogenous antioxidant defense and high oxidative stress, they may be particularly prone to the damaging effects of ROS. As a result, antioxidant supplementation may be particularly beneficial in this population. However, because of the combination of low endogenous antioxidant levels and high oxidative stress, more rigorous antioxidant supplementation may be required to significantly reduce ROS-induced damage.
It is possible that variations in experimental design would have produced more positive results. For example, a longer supplementation period may have significantly reduced oxidative stress, while increasing zinc status and plasma vitamin C toward normal. In addition, use of flavonoids such as catechin or quercetin, with known antioxidant activity \textit{in vitro}, may have produced beneficial results. Finally, larger supplement dosages may be required in a diabetic population. However, because of the danger of toxicity, this approach should be viewed with caution.

Possible areas of further consideration include determination of the bioavailability of the citrus flavonoids. In addition, toxicity studies should be performed on the citrus, and other, flavonoids before they are advocated for general use. Also, because this research supports the contention that patients with NIDDM are prone to zinc deficiency, research is required to assess other benefits of improving zinc status in this population. Furthermore, indices of oxidative stress should be examined following longer zinc and citrus flavonoid supplementation periods. Finally, further research is necessary to determine the antioxidant activity of both zinc and the citrus flavonoids and the role they play in CHD prevention.
CONSENT TO INVESTIGATIONAL TREATMENT

I, ________, hereby authorize Dr. Robert DiSilvestro, or assistants of his choosing, to perform 2 blood drawings of under 1 ounce each upon (Myself)

The experimental portion of the treatment is:

Daily consumption of zinc supplement (30 mg), or a flavonoid supplement (about 1 g of citrus flavonoids), or an amino acid placebo, for 3 weeks. I will not know whether I receive the zinc, flavonoid, or amino acid placebo. This will be decided by a random process. The participants' last names will be listed by alphabetical order and given a number based on their place on the list. Even numbers will be assigned zinc treatment and the odd numbers will be assigned placebo. Neither supplement dosage is considered toxic. Blood will be drawn before and after the amino acid placebo, flavonoid, or zinc supplementation periods for a total of 2 drawings.

This is done as part of an investigation entitled:

Effects of zinc or citrus flavonoid supplementation on indices of oxidative stress in non-insulin-dependent diabetic women

1. Purpose of treatment:

To determine whether increasing zinc or flavonoid intake will decrease indices oxidative stress in a typical group of adult Type II diabetic women.
2. Possible alternative procedures or treatment:

Nonparticipation.

3. Discomforts or risks reasonably to be expected:

Due to blood drawing: minor discomfort, bruising, fainting, and very minor risk of infection.
Due to supplements: none anticipated.

4. Possible benefits for subjects/society:

For society, this project will provide new insight into the relationship of diet and side effects of diabetes. The individual subjects will learn if raising their own zinc or flavonoid intake will decrease oxidative stress. Also, the individual subjects will be paid $80 for participation. Supplements will be supplied free of charge.

5. Anticipated duration of subject's participation:

3 weeks, with 2 visits. The first visit will be for drawing blood and receiving supplements; the second visit will be to return unused supplements and for the second blood draw.

I hereby acknowledge that Dr. DiSilvestro has provided information about the procedure described above, about my rights as a subject, and he answered all questions to my satisfaction. I understand that I may contact him at Phone No. 292-6848 should I have additional questions. He has explained the risks described above and I understand them; he has also offered to explain all possible risks or complications.

I understand that, where appropriate, the U.S. Food and Drug Administration may inspect records pertaining to this study. I understand further that records obtained during my participation in this study that may contain my name or other personal identifiers may be made available to the sponsors of this study. Beyond this, I understand that my participation will remain confidential.

I understand that I am free to withdraw my consent and participation in this project at any time after notifying the project director without prejudicing future care. No guarantee has been given me concerning this treatment.

I understand in signing this form that, beyond giving consent, I am not waiving any legal rights that I might otherwise have, and I am not releasing the investigator, the
sponsor, the institution, or its agents from any legal liability for damages that they might otherwise have.

In the event of injury resulting from participation in this study, I understand that immediate medical treatment is available at University Hospital of The Ohio State University. I also understand that the costs of such treatment will be at my expense and that financial compensation is not available. Questions about this should be directed to the Human Subjects Review Office at 292-9046.

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.

Date:___ Time __AM/PM Signed ________________________________

(Subject)

I certify that I have personally completed all blanks in this form and explained them to the subject before requesting the subject to sign it.

Date: ___ Signed ________________________________

(Signature of Project Director or his authorized representative)
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