Abstract

WILLCOX, JOYE KAY. Investigation of an Interactive Effect of Flavonoids on the Antioxidant Activity of Alpha Tocopherol. (Under the direction of George L. Catignani.)

The hypothesis tested was: dietary flavonoids interact with alpha-tocopherol (α-TOH) to improve its antioxidant capacity in a living system. This project began with a study of the flavonoid quercetin and α-TOH in an in vitro lipid environment, followed by a preliminary in vivo rat study feeding quercetin and α-TOH to determine the efficacy of this research and to develop and refine research methods. This was followed by the major study in which growing rats consumed tocopherol deficient or sufficient diets supplemented with quercetin or two additional flavonoids, catechin and epicatechin, a food source of flavonoids, cocoa, or a non-digestible polyphenolic compound, lignin. In both in vivo studies, plasma and various tissues were assayed to determine changes in concentration of α-TOH, vitamin A, and an oxidation product occurring when feeding these flavonoids in the presence and absence of tocopherol.

The in vitro system utilized the Oxidative Stability Instrument to determine if the combination of the flavonoid, quercetin, and alpha tocopherol provide an additive or synergistic effect in protecting a highly polyunsaturated oil from oxidation during exposure to controlled heating and aeration. From 0.2 to 20 mM concentrations of alpha tocopherol, the addition of 5, 10, and 20 mM quercetin provided enhanced

antioxidant protection, as measured by the OSI, Oxidative Stability Index. Quercetin was a more effective antioxidant in this system than alpha-tocopherol, which was in agreement with earlier in vitro research in our lab. However, the combination of tocopherol and quercetin produced less than an additive effect of each antioxidant alone.

The twelve week preliminary study involved feeding twenty-four male Sprague Dawley (SD) rats one of four AIN93-G treatment diets containing: no antioxidant, 5 g quercetin, 75 IU alpha tocopherol, or both antioxidants per kg of diet to determine differences in antioxidant effectiveness. The results from this preliminary study indicated that tocopherol is a more effective antioxidant than quercetin and that quercetin may have a mild positive effect on the antioxidant capacity of tocopherol at the levels fed.

The major study involved feeding seventy-two male SD rats AIN93-G diets sufficient or deficient in tocopherol alone or supplemented with one of five treatment compounds: 5 g. quercetin, 5 g. catechin, 5 g. epicatechin, 10 g. cocoa powder, or 10 g. lignin per kg of diet. In tocopherol sufficient groups, all test compounds significantly increased liver tocopherol as compared to the control. In plasma, tocopherol was significantly increased only in animals supplemented with quercetin and catechin. In adipose tissue, quercetin, catechin, and epicatechin significantly increased tocopherol levels. In the tocopherol deficient groups, tocopherol levels were significantly increased by all test

compounds in adipose tissue. Plasma and heart F2-isoprostanes were significantly increased in plasma and hearts of tocopherol deficient animals compared to tocopherol sufficient controls but none of the treatment compounds significantly decreased F2-isoprostanes beyond the protection displayed by α-tocopherol (sufficient vs. deficient). In conclusion: these antioxidant compounds, quercetin, catechin, and epicatechin at the levels fed were sufficient to spare tocopherol in these various tissues, but insufficient to alter lipid peroxidation as measured under these experimental conditions.

Investigation of an Interactive Effect of Flavonoids on the Antioxidant Activity of Alpha Tocopherol

by
Joye Kay Willcox

A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Nutrition

Raleigh

2002

Approved by:

_____	_____
Sarah L. Ash	Jimmy D. Garlich
_____	_____
Jack D. Odle	George L. Catignani Chair of Advisory Committee

Dedication

This work is dedicated to the memory of two very special men in my life, my wonderful father, Edward Tyson Willcox and my dear friend, Ottis C. Daniel.

Although Dad passed away during the first month of my tenure at NC State, his love, pride, and confidence in me along with several invaluable life lessons were guiding forces each step of the way to graduation. He taught me patience while fishing for bream on the Little Pee Dee River. He taught me perseverance in completing countless woodworking and garden projects. But, most importantly, Dad taught me the invaluable trait of looking for the bright side when times are tough. Those lessons, along with the unconditional love and pride he showed in every day of life, helped me to continue my studies and research until the job was done!

The Good Lord was certainly looking out for me when He allowed Ottis Daniel to walk into my life a few months before Dad died. "Mr. D" was my walking buddy, my closest confidante, and ardent cheerleader during the first five years of my graduate program. He started my school days off with a laugh each morning during our laps around the neighborhood block. He knew absolutely nothing about antioxidants, free radicals, and research methods, but he knew a lot about living and

getting the most out of each day. It was my privilege to share a small part of his life.

These two men are my heroes. As Veterans, as fathers who loved their families, and as individuals who believed in me even when I couldn't believe in myself, I salute you both with pride.

Biography

Joye Kay Willcox, the second of four daughters of Joyce and E.T. Willcox, grew up in Marion, South Carolina. She obtained her Bachelors Degree in Nutrition and Dietetics from Winthrop College in 1975 and a Masters of Nutritional Sciences from Clemson University in 1977. Upon completion of supervised work experience in dietetics, she completed requirements for becoming a Registered Dietitian. Her work experience as a dietitian included the S.C. Dept. of Mental Health, Georgia Department of Corrections, Veterans Administration Medical Center, and the U.S. Public Health Service assigned to the Bureau of Prisons.

Joye was commissioned as an Officer in the Army Reserves in 1980, transitioned to active duty with the U.S. Public Health Service for five years, and then to the Navy Reserves in 1996. She holds the rank of Lieutenant Commander and serves as the Health Promotions Coordinator of the Raleigh Naval and Marine Corps Reserve Center.

In 1996, prompted by a desire to change career paths from clinician to professor of dietetics, she began her doctoral program under the direction of Dr. George Catignani. Her thesis research involved an investigation of the ability of dietary flavonoids to influence the antioxidant effectiveness of alpha tocopherol *in vivo*. Her professional focus has, and will continue to be, promoting health and preventing disease through diet and lifestyle choices.

Acknowledgments

I would like to express deep gratitude to my major professor, Dr. George Catignani for his wise counsel and patient demeanor in directing my research program. He gave me enough autonomy to learn through experience, but enough direction to keep me on the right path. He provided insight, instruction, and at times when needed most, assurance of my ability to succeed.

The other members of my Graduate Committee were also true mentors and instrumental in making my program a success. Dr. Sarah Ash provided pivotal pep talks and reminded me often of my professional life beyond the lab. She also provided insight and example for developing my teaching skills informally, in the classroom, and during my participation as a Hewlett Initiative Graduate Fellow. Dr. Jim Garlich posed difficult, but important questions beyond the classroom that always kept me thinking! He exemplifies the essence of being a gentleman and a scholar in all he does. Dr. Jack Odle also provided thought provoking questions and a fine example for enthusiasm in my quest to understand the intricacies of energy metabolism. Although not an "official" member of my graduate committee, Dr. Leon Boyd was an excellent resource of chemicals, equipment, and information about lipids and flavonoids. His enthusiasm and energy were also a source of inspiration for me personally. I would also like to acknowledge Dr.

Jonathan Allen for his guidance and support.

Dr. L. Jackson Roberts, II of Vanderbilt University provided invaluable help with my research study. His expert advice and collaborative laboratory analyses are much appreciated.

The Biological Resource Facility personnel, especially B.J. Welker, provided excellent consultation and assistance during and after the preliminary animal study. Paula DeLong and Toni Grunther, along with several NCSU Vet School staff and Laboratory Animal Resource facility personnel, provided excellent consultation, support and hands-on assistance in the major study.

Dr. Francis Giesbrecht, Joy Smith and Dr.Jason Osborne provided excellent and frequent consultation regarding the statistical analysis of my data.

I am very grateful for the support, friendship, and technical assistance of Ruth Watkins. Ruth taught me the HPLC system from injection to detection and everything in between! She was also a valuable resource of information regarding routine to complex laboratory procedures and helping hands and assistance during my animal studies.

My research continued the Masters project of Dr.Keith Harris. Keith's notes, friendship and insights were greatly appreciated. Keith Hendricks provided much appreciated technical guidance during the OSI

experiment. Roger Thompson provided much direction and assistance with statistical questions and Dr. Fred Breidt kindly provided access to equipment in his lab.

Additionally, Heather Hickman provided helping hands and friendship as did my other dear friend, Dr. Heggie Allen. Another special friend, Dr. Debra Clare provided inspiration, insight, and on several occasions excellent editorial support in manuscript preparation. She also helped me laugh when I was too tired to think! My more recent association and friendship with Gary Matsey has forced me to think of life beyond graduate school. Our lunchtime runs at the gym were energy intensive and thought provoking to say the least! Other friends have provided encouragement along the way that made a huge difference in my life during this time. Although I hesitate to list these names in fear of leaving out someone very important, I must say a heartfelt "thank you" to: Michele Keziah, Paula Pharr, Penny Amato, Judy Cooper, Joshua Carlson, Annee Moxley, Lisa Sternhagen, and Dr. Teena Middleton. To misquote a song from the seventies, I truly "got by with a lot of help from my friends"!

Finally, and most important, I would like to thank my daughter, Elizabeth Bentley Armstrong, my mother, Joyce McMillan Willcox, my three sisters, Kakie Honig, Mary Tyson Miller, and Jane Salley, and my step children, Gaye Washburne and Joe Armstrong. They, and their

families believed in me and encouraged me every step of the way since my decision to enter this doctoral program. I have been truly blessed with close and wonderful friends who have also been instrumental in turning this venture into a success. (In chronological order) Bette, Gert, Sandi, Lynnell, Phyllis, Elyse, and Joan are the best friends anyone could have. I'm sure they are all tired of hearing the word "antioxidant", but their phone calls, prayers, "chats", and encouragement helped me more than they will ever know!

Other friends and loved ones helped me in different ways to get started and keep going. They know who they are and what they contributed. I will always be thankful for their friendship and support. I would also like to thank my friends and associates of NHCL Det.C and Raleigh Naval & Marine Corps Reserve Center who asked often about my progress and listened to my whining about rat parts, long days, and exams. My hometown Presbyterian and high school friends also made a big difference by asking and caring about my progress in completing this endeavor.

Although I designed the studies, ran the assays, analyzed and reported the results, and passed the exams, all those mentioned above, especially my daughter, have claim to a part of this Ph.D. degree. It would never have happened without their love and support. Many thanks.

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List of Symbols, Abbreviations, Nomenclature

(–SH–)	sulphydryl
(–S–S–)	disulphides
TE	tocopherol equivalents
TTP	tocopherol transfer protein
lμ	micromolar (i.e. micromoles l_1)
AA	ascorbic acid
AAPH	2,20-azobis(2-amidopropane)
ABAP	2,20-azobis(2-amidinepropane)dihydrochloride
ABTS	2,20-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)
acetyl CoA	coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AGE	advanced glycation end-products
ALS	amyotrophic lateral sclerosis
AR•	aroxyl radical (antioxidant radical)
ATBC	a-tocopherol, b-carotene cancer prevention study
AZT	azidothymidine
BHT	butylated hydroxyl toluene
cAMP	cyclic adenosine monophosphate

CARET	beta-carotene and retinal efficacy trial
CAT	catalase
CEHC	2,5,7,8-tetramethyl-2-(20-carboxyethyl)-6-hydroxychroman
CHAOS	Cambridge Heart Antioxidant Study
CHD	coronary heart disease
COPD	congested, obstructive pulmonary disease
CoQ	ubiquinone
CSF	cerebrospinal fluid
CVD	cardiovascular disease
CYP450	cytochrome-P450
cyto-C	cytochrome C
DNA	deoxyribonucleic acid
DRI	dietary reference intake
EC	epicatechin
ECG	epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGC	epigallocatechin
EGCG	epigallocatechin gallate
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EPA	eicosapentaenoic acid

Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FADH2	reduced .avine adenine dinucleotide
FRAP	Ferric Reducing Activity of Plasma
GC	gas chromatography
GISSI	Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico
GPX	glutathione peroxidase (tables only)
GR	glutathione reductase
GSH	glutathione (reduced)
GSHPx	glutathione peroxidase
GSSH	glutathione (oxidised)
GST	glutathione transferases
H_2O_2	hydrogen peroxide
HDL	high-density lipoproteins
HIV	human immunode.ciency virus
HOCl	hypochlorous acid
HOO•	hydroperoxyl radical
HOPE	Heart Outcomes Prevention Evaluation
HPLC	high performance liquid chromatography
IL	inter leukin
IU	international units

LDL	low-density lipoproteins
LPO l	ipid peroxidation
MDA	malonaldehyde
mg	milligrams
MI	myocardial infarction
MnSOD	Mn-superoxide dismutase
MONICA	Monitoring of Trends and Determinants in Cardiovascular Disease
MPO	myeloperoxidase
MW	molecular weight
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NICI	negative-ion chemical ionisation
NIH	National Institutes of Health
NO•	nitric oxide
NOS	nitric oxide synthase
ns	not significant
$\mathbf{O}_2^{\bullet-}$	superoxide radical
OH•	hydroxyl radical
$ONOO^-$	peroxynitrite
ORAC	oxygen radical absorbing capacity
ox-LDL	oxidized LDL
PDGF	platelet-derived growth factor

Pi	inorganic phosphorus
PKC	protein kinase C
PUFA	polyunsaturated fatty acid
RDA	recommended dietary allowance
RE	retinol equivalent
redox	cellular reduction and oxidation reactions
RNS	reactive nitrogen species
ROOH	alkylperoxide
RO•	alkoxyl radical
ROO•	Alkylperoxyl radical
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	TBA-reactive substances
TNF-a	tumour necrosis factor a
TOH	tocopherol
TO•	tocopheryl radical
TR	thioredoxin
TRAP	total radical-trapping antioxidant assay
UL	Tolerable Upper Intake Level
US FNB	US Food Nutrition Board

USA	United States of America
UV	ultraviolet
VLDL	very low-density lipoproteins
WBC	white blood cells
WHO	World Health Organization

Introduction

The prevention of chronic disease has prompted much scientific research. Population studies have shown that up to 80% of cardiovascular disease, 90% of type II diabetes, and approximately 30% of cancers could be avoided by diet and lifestyle changes (WHO/FAO, 2002). During the past few decades, scientific discovery has prompted debate as to whether oxidation, or more specifically, oxidative stress, is a primary cause or a secondary phenomenon of many chronic diseases, as well as the aging process itself. Consequently, much scientific curiosity and resources have focused on the role that antioxidants play in hindering oxidation, thereby delaying or preventing oxidative stress.

Because food is the most common source of antioxidants, complex interactions must be examined to determine the quality of individual antioxidants in the biological system. These interactions may involve the full array of both endogenous and exogenous antioxidant compounds. This doctoral research project has examined the ability of three major dietary flavonoids to spare and enhance the antioxidant capacity of alpha tocopherol (α-TOH) in vivo.

The literature review will be provided as a review article (Chapter 1) in manuscript form, a review of methodologies in measuring oxidative stress (Chapter 2), and a summary of tocopherol/flavonoid interactions (Chapter 3). These chapters are followed by four additional manuscripts describing and

reporting in vitro and in vivo findings (Chapters 4-7). This work will then close with a brief discussion and summary of conclusions.

Literature cited:

Joint WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases, 2002 draft.

Chapter One:

Antioxidants and Prevention of Chronic Disease

Joye K. Willcox, George L. Catignani, and Sarah L. Ash
Dept. of Food Science, Dept. of Animal Science
North Carolina State University

*Corresponding Author:
North Carolina State University
Box 7624
Raleigh, North Carolina 27695-7624, USA

Paper No. FSR _____ of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

Abstract
The generation of reactive oxygen species (ROS) and other free radicals (R•) during metabolism is a necessary and normal process that ideally is compensated by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle, and pathological situations, there can be an excess of radicals resulting in a situation of oxidative stress. Oxidative stress has been related to cardiovascular disease, cancer, and other chronic diseases that account for a major portion of deaths today. Antioxidants are compounds that hinder the oxidative processes and thereby delay or prevent oxidative stress. This review examines the process of oxidative stress and the pathways by which it relates to many chronic diseases. We will also discuss the role that endogenous and exogenous antioxidants may play in controlling oxidation and review the evidence thus far in understanding that relationship as it relates to the prevention of disease.

Key Words: antioxidants, chronic disease, aging

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The Genesis of Oxidation and the Production of Free Radicals

Oxygen is a toxic mutagenic gas that appeared in significant amounts in the Earth's atmosphere over 2.5 billion years ago. Oxygen was released into the atmosphere as water was split to obtain the hydrogen needed to drive metabolic reductions. This rise in atmospheric oxygen led to the formation of the ozone layer that filtered out enough of the solar UV radiation to allow living organisms to leave the sea and inhabit the land. At the same time, the more primitive anaerobic organisms died out with the exception of those that evolved a defense mechanism to protect themselves from the toxicity of oxygen. Other surviving organisms continued to evolve by using oxygen for metabolic transformations and energy production in mitochondria. Multicellular organisms developed as systems evolved to distribute oxygen in a controlled manner. Most eukaryotic cells have an oxygen gradient, decreasing from the cell membrane to the mitochondria in which oxygen is consumed during aerobic metabolism. Most human body cells are exposed to fairly low oxygen concentration, which may be regarded as an antioxidant defense mechanism (Halliwell and Gutteridge 1999).

Mammals have developed mechanisms to insure that oxygen is transported to all the cells that need it. Since oxygen solubility in water is limited, most of it is carried by hemoglobin that selectively binds and dissociates oxygen to allow appropriate distribution throughout the body.

About 85-90% of the oxygen taken up from the plasma by animal cells is utilized by the mitochondria to produce ATP (Halliwell, 1994). The essence of metabolic energy production involves oxidation of food materials, i.e., they lose electrons that are accepted by electron carriers such as NAD, FMN, and FAD. These reduced compounds in turn are re-oxidized by oxygen in the mitochondria, producing a large amount of ATP. This oxidation process must proceed in a stepwise fashion so that energy is released gradually as is achieved by the electron transport chain present in the inner mitochondrial membrane. The terminal enzyme in this electron transport chain, cytochrome oxidase, adds four electrons to oxygen:

$$O_2 + 4H^+ + 4\ e^- \rightarrow 2\ H_2O$$

However, this must be done in stages since oxygen can only accept one electron at a time. It is through this stepwise process that partially reduced oxygen species are generated as shown in Figure 1.

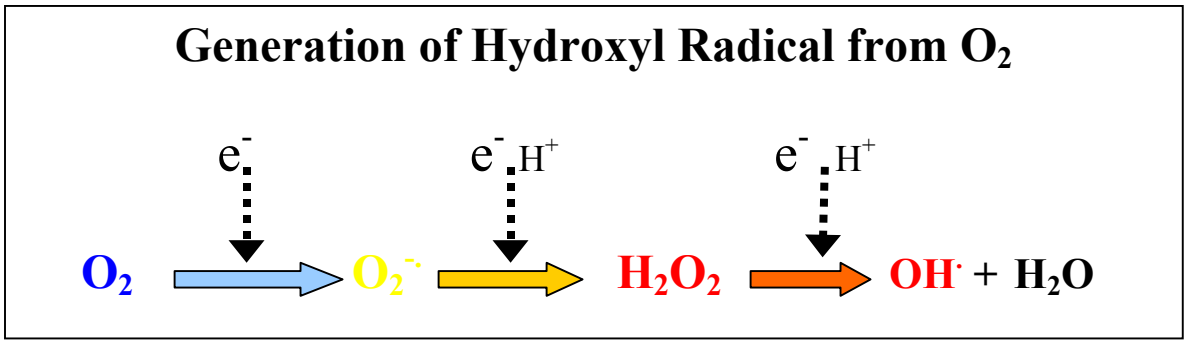

Figure 1 Step-wise generation of Hydroxyl Radical from Oxygen.

Because cytochrome oxidase has a very high affinity for oxygen, it is able to sequester most of these damaging partially reduced oxygen species until they can be converted to water. However, the system is not perfect and many oxygen species leave the chain as free radicals.

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. An unpaired electron is one that occupies an orbital by itself. The presence of unpaired electrons makes free radicals highly reactive in seeking another electron to fill the orbital.

When a single electron is added to the ground state O_2 molecule, the superoxide radical is produced ($O_2^{\cdot -}$). Several organic molecules oxidize in the presence of O_2 to produce the superoxide radical, including glyceraldehydes, the reduced flavins, adrenaline, L-dopa, dopamine, cysteine, etc. These auto-oxidations are catalyzed by the presence of metal ions such as iron and copper.

The most important source of superoxide radicals in vivo are the electron transport chains present in many bacterial membranes, within mitochondria, endoplasmic reticulum, and nuclear membranes in eukaryotic cells. Most of these radicals are produced by the leakage of electrons on O_2 prior to the terminal cytochrome oxidase step, shown in Figure 2. This leakage is increased as the O_2 concentration increases. It is estimated that 1-3% of the O_2 reduced in mitochondria may form the superoxide radical (Turrens, 1997).

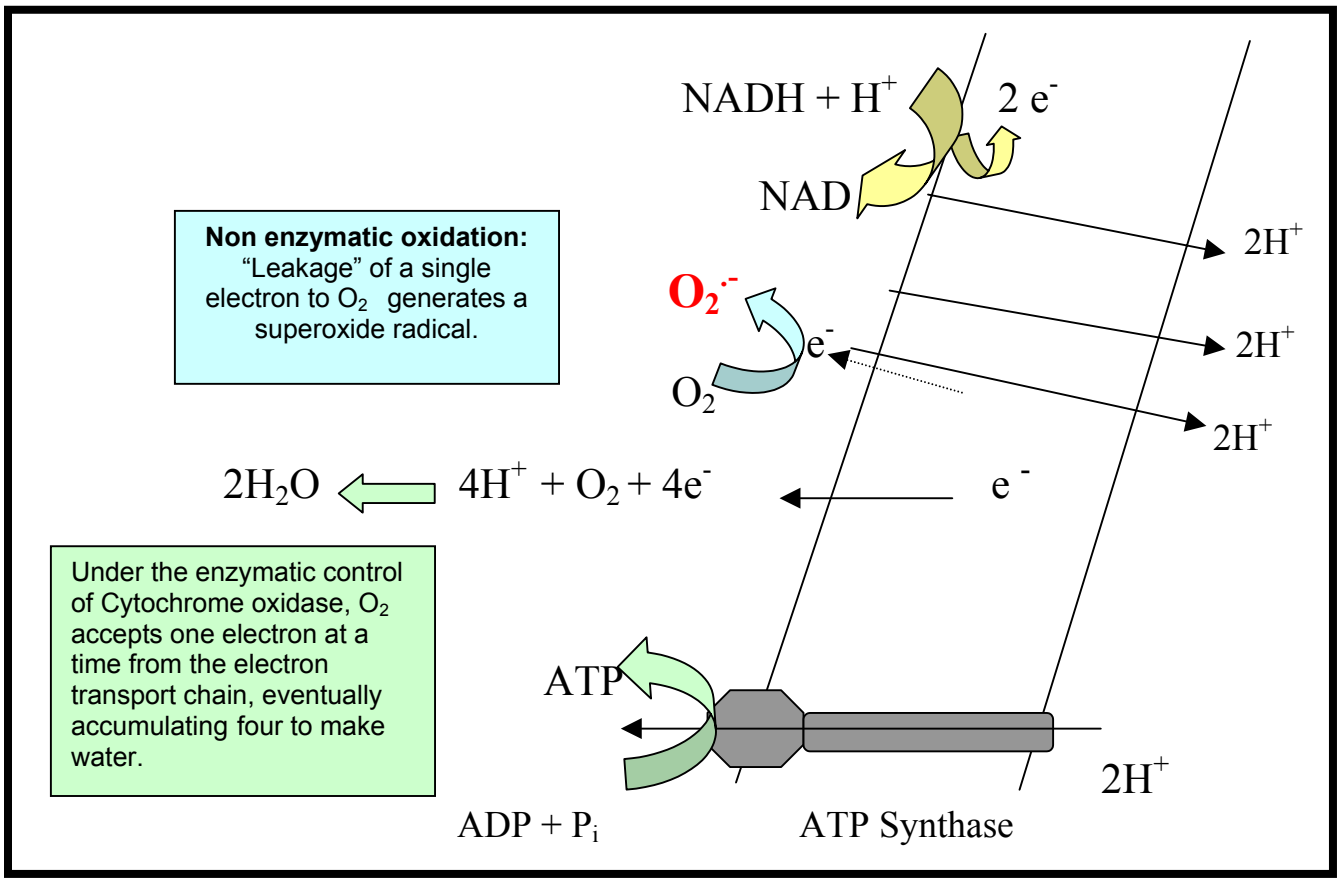

Figure 2. Generation of Superoxide from Electron Transport Chain.

Superoxide production increases when the integrity of the mitochondrial electron transport chain organization is compromised. The production of these oxygen radicals may thus result in damage to the proteins, lipids, and DNA in the respiring mitochondria, leading to mutations in mitochondrial DNA that have been associated with a wide range of human diseases (Shigenaga, 1994).

In addition to the electron transport system release of electrons, the liver endoplasmic reticulum may generate superoxide radicals through the desaturase enzyme system. In this system, desaturase introduces C=C bonds

into fatty acids. The reaction requires O_2, NADH or NADPH, and cytochrome b_5. Electrons are transferred from NAD(P)H to cytochrome b_5 by a flavoprotein enzyme. Then the reduced cytochrome b_5 donates electrons to the desaturase enzyme. Both cytochrome b_5 and the flavoprotein can leak electrons on to O_2, to produce the superoxide radical.

The production of superoxide radical occurs within all aerobic cells and is dependent on oxygen concentration. The superoxide radical is relatively innocuous, but at physiological pH approximately 1% will be protonated to exist as peroxyl radical (**$HO_2\cdot$**), a more reactive radical (Borg 1993). Superoxide can decrease the activity of certain enzymes including some antioxidant defense enzymes including catalase, glutathione peroxidase and several in the energy metabolism scheme such as NADH dehydrogenase. See Figure 3.

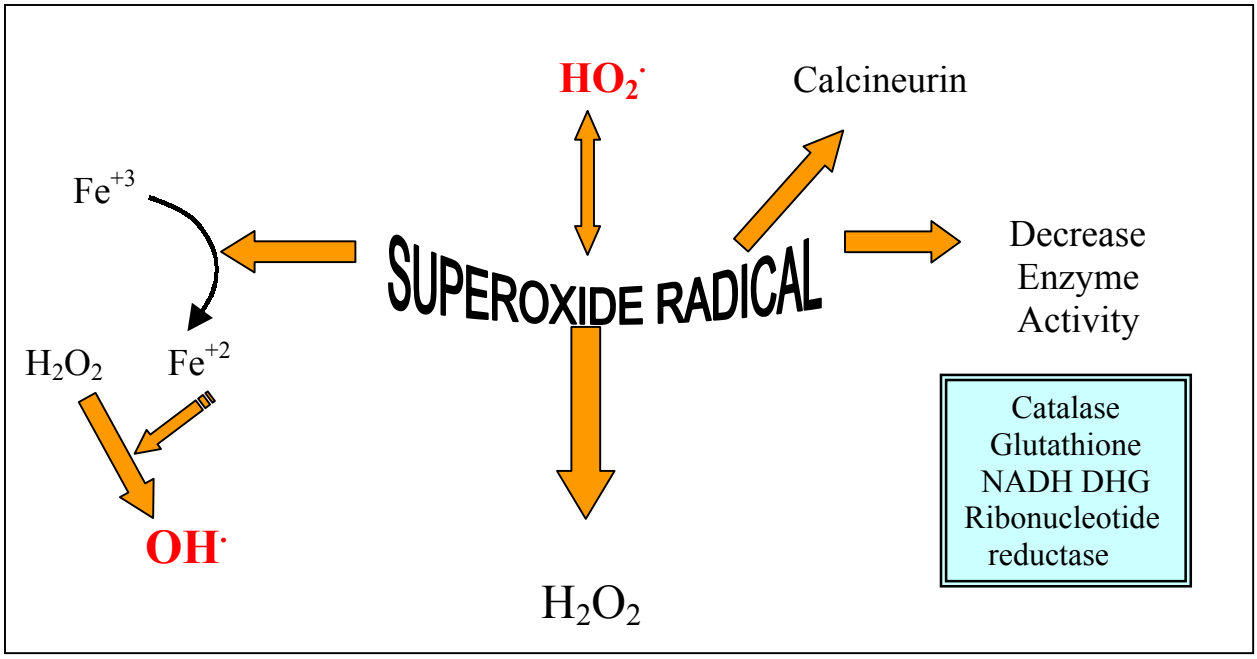

Figure 3 Targets and Fates of Superoxide Radical.

Another enzymatic target of superoxide damage is the ribonucleotide reductase that makes the precursors required for DNA synthesis. It may also damage calcineurin, a protein involved in signal transduction.

Aside from direct damage, superoxide can be more cytotoxic by generating more reactive species, such as hydrogen peroxide, **H_2O_2**, by the addition of one more electron. Hydrogen peroxide is not a radical since the additional electron fills the orbital, but it can attack some enzymes such as glyceraldehyde-3-phosphate dehydrogenase, an enzyme in the glycolytic pathway. See Figure 4.

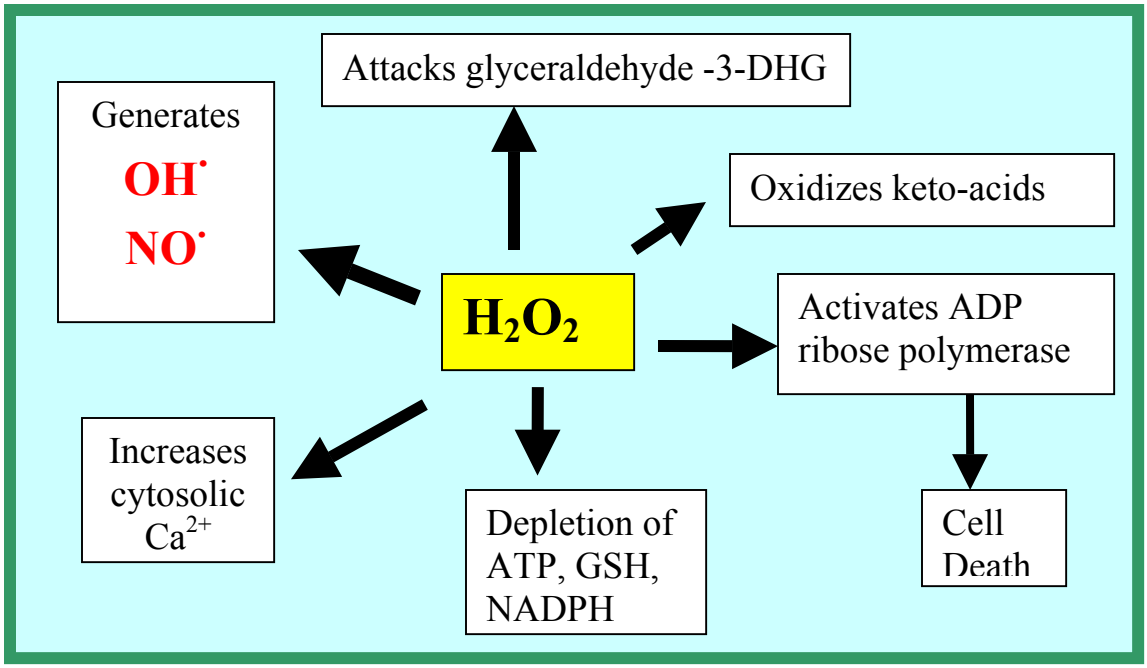

Figure 4 Targets and Actions of Peroxides in vivo.

It can also oxidize certain keto-acids such as pyruvate. H_2O_2 leads to depletion of ATP, reduced glutathione, and NADPH. It induces a rise in free cytosolic Ca^{2+} and activates a polymerase that leads to cell death. H_2O_2 can cross cell membranes to react with iron and copper ions to form much more damaging species such as hydroxide radical (OH·) and peroxynitrite (NO·) (Roberford and Calderon, 1995).

Free radicals can also be generated by activated leukocytes as part of the immune response. Macrophages and neutrophils possess the enzyme NADPH oxidase which can catalyze the one-electron reduction of oxygen to superoxide which generates the other oxygen radicals discussed. Another enzyme found in leukocytes, myeloperoxidase, generates hyperchlorous acid (HOCl) from H_2O_2. These reactive oxygen species (ROS) are used in the immune response to kill ingested or extracellular bacteria. Unfortunately, their actions are not limited to their intended purpose and they may contribute to the detrimental effects seen by the free radical-induced oxidative process (Punchard and Kelly, 1997).

Tissue damage will also uncouple electron transport chains and release compartmentalized reactions to generate free radicals. Ischemia, as occurs during myocardial infarction and organ transplants, can produce free radicals through the action of xanthine oxidase. This enzyme converts hypoxanthine and xanthine to uric acid thereby producing superoxide which may in turn produce peroxide and, with the input of reduced iron, make a hydroxyl radical.

This plays a major role in the tissue damage seen in ischemia/reperfusion injury (Punchard and Kelly 1997).

Other environmental factors can lead to the production of free radicals, as shown in Figure 5.

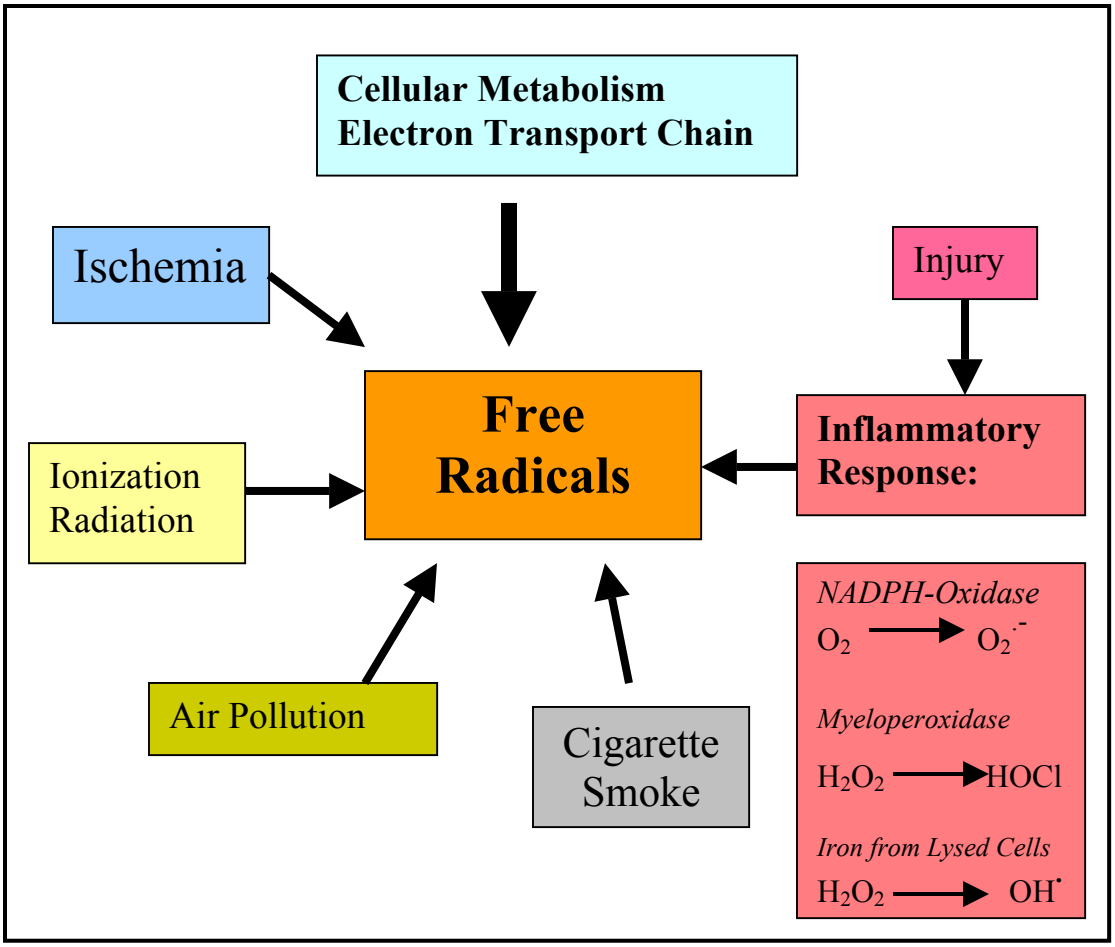

Figure 5 Summary of Sources of Free Radicals.

Exposure to ultraviolet radiation can generate free radicals, as can air pollution and cigarette smoke. Nitrogen dioxide, one of the major oxidants in smog, is also found in cigarette smoke. Two free radicals are found in cigarette smoke, one in the tar portion and the other in the gaseous phase. The

principal radical, NO·, found in the tar portion, is capable of reducing oxygen to superoxide radical. The much more reactive oxygen and carbon-centered radicals are found in the gas phase (Chow 1993).

Diseases Associated with Oxidative Stress

Under ideal circumstances the body would be in a steady state with free radicals produced and quenched by the endogenous antioxidants. However, it has been determined that this balance is not perfect because oxidative damage occurs to DNA, proteins, lipids, and small molecules in living systems under ambient oxygen states. Oxidative stress refers to the situation of a significant imbalance between free radicals and the antioxidant defense system. The resulting harm is termed oxidative damage.

Oxidative stress can result from :

- Diminished antioxidants, e.g. vitamin E, urate
- Diminished antioxidant defense enzymes, e.g. glutathione peroxidase, catalase
- Increased levels of oxidation products, e.g. malondialdehyde, DNA damage

Oxidative stress can give rise to:

- Adaptation with changes in gene expression to elevate antioxidant defenses.
- cell injury leading to DNA strand breakage

- changes in cell behavior with increase or decrease of cell proliferation (fibrosis)
- cell death through necrosis (swelling/rupture) or apoptosis

Cells can normally deal with mild oxidative stress by up-regulating the synthesis of antioxidant defense mechanisms through changes in gene expression. However, at higher levels of oxidative stress cell injury may occur when adaptation is not adequate for the build up of oxidation products. This leads to oxidative damage to all types of biomolecules including DNA, proteins, and lipids that have been associated with many diseases. The target of oxidative damage varies depending on the characteristics of the cell and the type and degree of stress imposed. Whereas some diseases may be caused by oxidative damage to proteins, DNA, and lipids, oxidative stress may be a consequence and not a cause of the primary process in many human diseases. As a secondary event, however, oxidative stress plays an important role in furthering tissue damage in several diseases. Tissue damage by infection, trauma, toxins, temperature extremes, and other causes usually lead to formation of increased amounts of free radicals that contribute to disease pathology (Halliwell and Gutteridge 1999). The imbalance of reduction-oxidation homeostasis appears to be one of the processes that regulate gene expression in many pathological conditions (Haddad, 2002).

Cardiovascular Disease

Cardiovascular disease is the leading cause of death in the United States and Europe. Most cardiovascular events are secondary to atherosclerosis, a disease of arteries involving a local thickening of the vessel wall, mainly evident in mid-sized muscular arteries. Three types of pathological processes are generally recognized: foam cells, fatty streaks, and fibrous plaques. A stroke or myocardial infarction occurs when the lumen of the vessel becomes completely occluded, usually by a thrombus forming at the site of a plaque (Ross 1992].

Atherosclerotic lesions are thought to be initiated by emigration of monocytes into the arterial inner core (tunica intima), recruited by adhesion molecules, possibly in response to arterial endothelium injury. A variety of factors have been implicated in causing this initial injury, including mechanical damage from flow stress worsened by high blood pressure, viral infection (herpes viruses and cytomegalovirus), exposure to blood-borne toxins such as xenobiotics from cigarette smoke and elevated levels of normal metabolites, such as glucose, cholesterol, or homocysteine (Halliwell and Gutteridge, 1999).

The fatty streak is the earliest and most common atherosclerotic lesion seen in cardiovascular disease. It is made up of aggregates of foam cells in the subendothelial portion of the vessel wall. These foam cells contain large deposits of oxidized LDL cholesterol engulfed by macrophages, which were

formerly monocytes circulating in plasma before diffusing through the intimal wall of the blood vessel. In vitro work has shown that the receptors of these scavenger macrophages identify oxidized LDL and uptake it in an unregulated manner, thereby transforming the macrophage into a lipid laden foam cell as shown in figure 6. These transformed macrophages are considered as precursors to the development of the occlusive plaque of atherosclerosis (Westhuysen 1997).

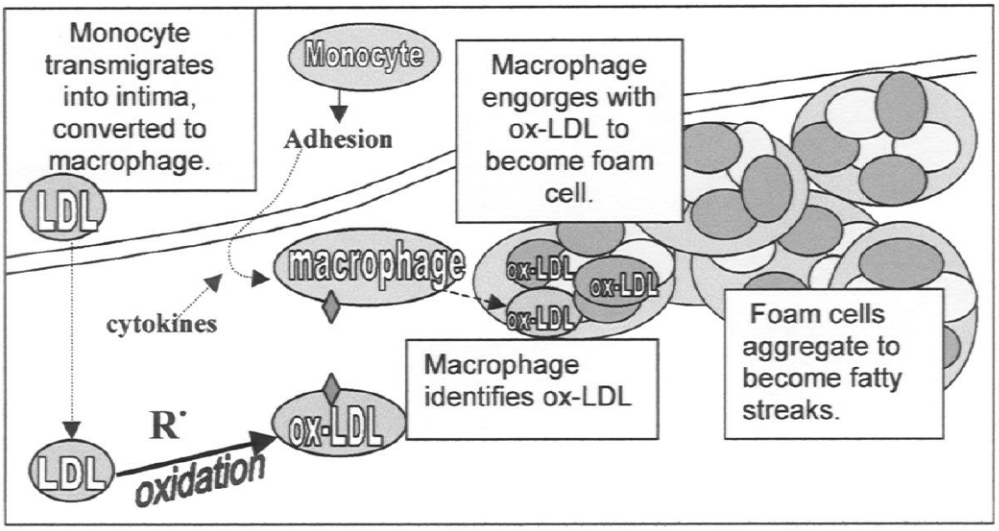

Figure 6. Proposed Scheme for Oxidative Development of the Fatty Streak.

In contrast, native or non-oxidized LDL uptake is under high homeostatic regulation. Uptake is down regulated by an excess of intracellular cholesterol.

The following mechanisms have been observed:

- Increased recruitment of circulating monocytes into the vessel intima by the chemoattractant force of oxidized LDL (Frostegard et al. 1991).
- Enhanced rates of oxidized LDL uptake and degradation by the macrophage through the receptor (Henriksen et al. 1981).
- The oxidation of the LDL particle inhibits macrophage exodus from the artery (Quinn et al. 1987).
- Cellular injury caused by oxidized LDL (Cathcart et al. 1985).

These observations have led to the hypothesis that in vivo LDL may be oxidized by free radicals, leading to the atherosclerotic process. This has not yet been proven to occur in vivo. However, LDL from human atherosclerotic lesions has been proven to contain lipid peroxidation products not found in normal vessels (Pratico et al. 1997). Elevated amounts of oxidized LDL are present in blood of patients with atherosclerotic disease (Holvoet et al. 1995), and antibodies against oxidized LDL react with rabbit and human atherosclerotic lesions (Yla-Herttuala et al. 1989). The presence of autoantibodies against oxidized LDL have been correlated with atherosclerosis. (Palinski et al. 1990).

The Initiation of Lipid Peroxidation

Dietary fats, after being digested, absorbed, and packaged in lipoproteins, are transported around the body. The more cholesterol rich lipoprotein, LDL, contains approximately 2700 fatty acids about half of which are polyunsaturated fatty acids (PUFA), that are very sensitive to oxidation (Esterbauer, 1991). Polyunsaturated fatty acids are also found in cell membranes. These membranes consist of a phospholipid bi-layer in which the hydrophilic heads point outside towards the aqueous environment on both sides of the membrane, whereas the fatty acid tails point inwards to produce a hydrophobic membrane interior. Polyunsaturated fatty acid side chains mainly determine fluidity of the cell membrane. Fluidity decreases with lipid peroxidation (Arora et al. 2000). Membrane fluidity is essential for proper function of biological membranes, including the action of the important LDL receptor.

Increased concentrations of superoxide, hydrogen peroxide and the presence of metal ions can create conditions favorable for the production of **hydroxyl radical (OH·)** which results from the addition of one additional electron to H_2O_2.

The hydroxyl radical is the most reactive radical ($k=10^9 M^{-1}s^{-1}$) and can react with almost every type of molecule found in living cells (Halliwell and Gutteridge, 1992). The hydroxyl radical reacts by hydrogen abstraction, addition, and electron transfer from non-radical molecules, thus initiating the

oxidation of macromolecules such as lipids resulting in a lipid radical (L·). This lipid radical in the presence of oxygen generates a **lipid peroxyl radical** (**LOO·**) which can continue the chain of oxidation by attacking another lipid to make the next lipid radical as the oxidation process continues.

As shown in Figure 7, lipid peroxidation begins when a methylene group (-CH2-] of the PUFA is attacked by a free radical to abstract a hydrogen atom and an electron. An adjacent double bond weakens the attachment of the hydrogen atoms present on the next carbon, especially if there is a double bond on either side of the methylene group.

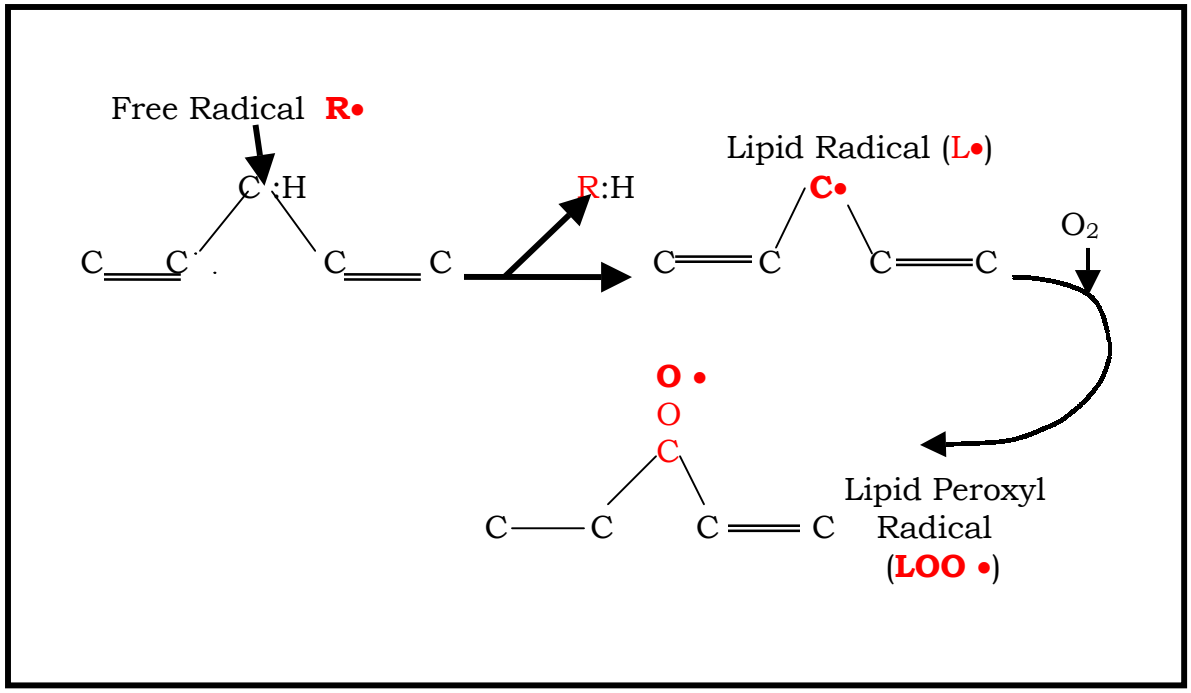

Figure 7 Initiation of Lipid Peroxidation

With subsequent rearrangement and reaction with more oxygen, lipid peroxyl radicals (LOO·) are formed. The lipid peroxyl radicals can oxidize

adjacent lipids in the cell membrane or LDL molecule. Now oxidatively modified, the LDL cholesterol particle is identified by the receptor of the macrophage and engulfed, leading to foam cell formation as discussed above.

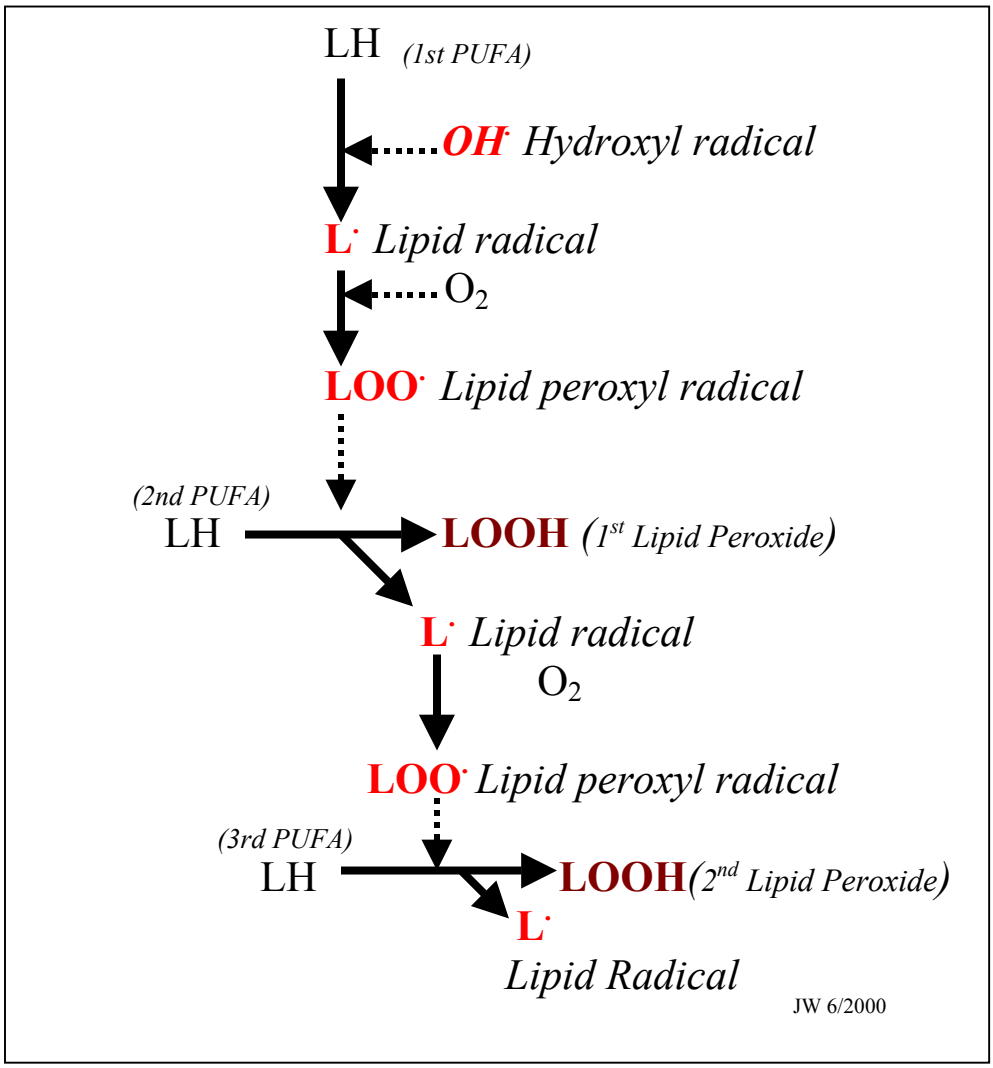

Figure 8 The Lipid Peroxidation Chain Reaction

As shown in figure 8, through this chain reaction, a single initiating radical can result in the conversion of hundreds of fatty acid side chains into

lipid peroxides that alter the integrity and biochemical function of cell membranes (Yoshikawa et al. 1997).

Another fate of the oxidized fatty acid is the formation of a cyclic peroxide that continues to be oxidized to malondialdehyde, F_2-isoprostanes, or other oxidation products (Roberts and Morrow, 2000).

Cancer

The idea that diet may be related to cancer risk has existed for decades with the first area of inquiry being the existence of dietary carcinogens. However, results from epidemiological studies conducted in the 1960's and 1970's shifted interest to the idea that diet may provide some potential protection from cancer (American Cancer Society 1996). Hundreds of studies conducted around the world since that time question whether populations whose diets are rich in certain micronutrients have lower cancer risks. There is evidence that micronutrients such as the antioxidants β-carotene, vitamin E , vitamin C and selenium are associated with reduced cancer risks (McLarty 1997). For example, out of 21 lung cancer studies, 15 found a significant inverse association with β–carotene while 4 found inconsistent results and 4 found no association.

It is estimated that 88-90% of human cancers are environmentally induced, approximately 35% by diet (Watson et al. 1986). Much experimental

data indicate that free radicals have a role in the initiation and promotion of cancer (Cross et al. 1987). Initiation and promotion of tumors involve changes in DNA either as a result of an inherited genetic anomaly or damage to the DNA strand. In view of the association between DNA damage and carcinogenesis, it is likely that any agent capable of modifying DNA could be carcinogenic. Free radicals fall into this category.

Hydroxyl radical (OH·) attack on DNA generates a series of modified purine and pyrimidine bases. It is estimated that oxidative lesions in DNA in normal cells average around 1 per 10^6 bases, a value that is even higher than the levels of adducts of known carcinogens that are detected in carcinogen-exposed cells. This implies that endogenous damage to DNA by free radicals is an important contributor to the age-related development of cancer. (Totter 1980, Loft and Poulsen 1996)

An important factor in cancer development may be related to mutations in the p^{53} tumor-suppressing gene, a transcription factor that acts to block cell division. If p^{53} genes are inactivated, then cells can enter the cell cycle with damaged DNA. Mutations of p^{53} are found in 50% of cancer lesions. For example, about 75% of colorectal cancers and 90% of squamous cell skin cancers have mutations on the p^{53} gene. Oxidative damage may account for some of the C to T and G to A changes often seen in the p^{53} gene in human cancer (Cerutti, 1994).

The most prevalent product of oxidative DNA damage is 8-hydroxy-2'-deoxyguanosine (8-OHdG). It is considered to be a reliable biological marker for oxidative stress (Sigenaga et al. 1990). The role of oxidation in DNA damage has been established by several in vitro and in vivo animal and human studies in which 8-OHdG, 5-hydroxymethyluracil, and other DNA oxidation products were measured (Fisher-Nielsen et al. 1993, Haegele et al. 1994, Djuric et al. 1991).

Other than direct damage to DNA by free radicals, oxidative damage to lipids and to proteins such as DNA repair enzymes could also lead to DNA mutations as outlined in Figure 9.

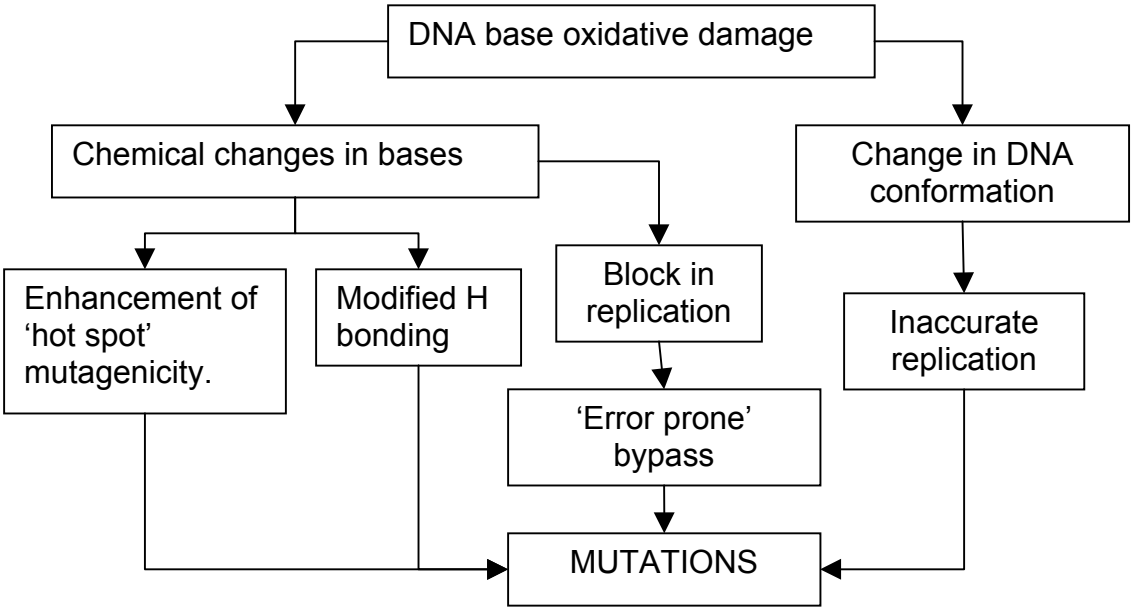

Figure 9 How Structural Changes in DNA can Cause Mutations. *Adapted from Halliwell and Gutteridge, 1999.*

In one study of humans (Haegele and Thompson, 1999) subjects were fed a high fruit/vegetable diet (12.5 servings/day) for 14 days. Serum levels of

α and β carotenoids as well as lutein increased significantly. Tocopherol levels did not increase. Urinary and lymphocyte 8-OHdG decreased significantly with intervention. Urinary 8-EPG (a prostanoid epimer and marker of lipid peroxidation) decreased by 33%. Change in urinary 8-EPG was significantly correlated to change in 8-OHdG, supporting the concept that lipid peroxidation and DNA damage are related and that overall oxidative stress is reduced by high fruit/vegetable intake. The results of this small study, although significant, should be regarded as preliminary. What remains to be determined in a clinical trial is whether there is a direct correlation between incidence of cancer, DNA oxidation, serum levels of antioxidant nutrients.

Diabetes

People with diabetes have an increased risk of atherosclerosis which is partially explained by an increased oxidizability of LDL (Lyons 1991, Baynes 1991). This increased oxidative stress is accompanied by a decreased antioxidant capacity. There is disagreement as to whether serum α–tocopherol is lower, but general agreement that vitamin C levels are below normal in diabetic patients. Diabetic patients tend to have higher plasma lipid peroxides and higher markers of oxidation, such as F_2-Isoprostanes (Mezzetti et al. 2000).

Diabetic complications may also be explained by increases in DNA damage due to oxidative stress. DNA damage was measured in 10 normal and 10 diabetic patients. A significant elevation in DNA strand breaks and

oxidized pyrimidines was seen in patients with Type I diabetes compared with normal subjects. Altered purines showed a strong positive correlation with blood glucose level (Collins et al. 1998).

Glycation of proteins leading to the accumulation of advanced glycation end products (AGEs) is known to be one of the sources of free radicals and has been strongly linked to the presence of diabetic complications (McCance et al. 1993) as seen in figure 10.

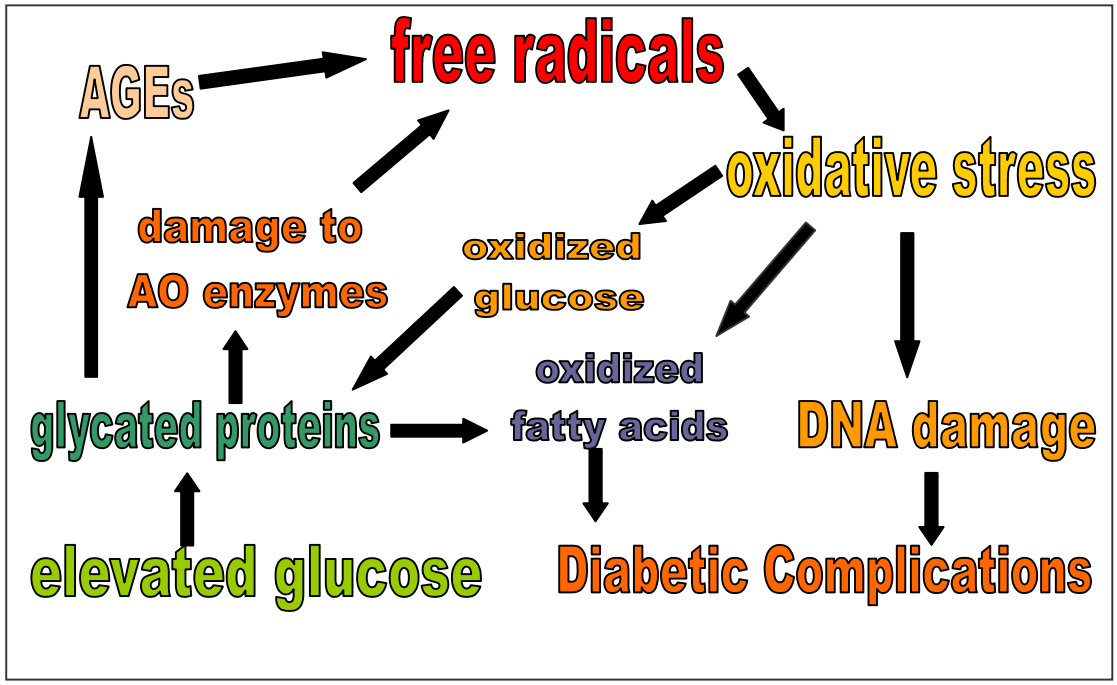

Figure 10. Oxidative Stress and Diabetic Pathology

These glycation products may directly release superoxide radical and H_2O_2, activate phagocytes, and reduce glutathione levels (Gutteridge and Halliwell, 1999).

Neurological

Alzheimer's Disease

A hypothesis implicating free radicals could explain both the heterogeneous presentation of Alzheimer's Disease (AD) and the fact that aging is a significant risk factor for its development. Three key facts support this hypothesis: (Christen 2000)

1. Neurons are extremely sensitive to free radical attack because
    - their glutathione content is very low.
    - their membranes are high in PUFAs.
    - brain metabolism requires a lot of oxygen.
2. AD brain lesions are associated with typical free radical damage, i.e. DNA damage, oxidized protein, oxidized lipids, glycosylated end products.
    - Oxidized DNA is observed in the parietal cortex of AD patients.
    - Many studies have shown increased lipid peroxidation in the AD brain.
    - Protein oxidation is more marked in AD patients in the regions showing the most pathophysiologic changes.
    - Several studies have identified elevated concentrations of oxidation end products in AD brains, including malondialdehyde, peroxynitrite, carbonyls, advanced glycosylated end products (AGEs), superoxide dismutase –1 and heme oxygenase (an enzyme up-regulated in response to oxidative stress)

- Two hallmarks of AD associated with AGEs are senile plaques consisting of long β-amyloid peptides and neurofibrillary tangles, mainly composed of tau proteins. The AGE receptor is also a β-amyloid receptor.
- Apo E genotype, E4 allele has been linked to AD and the oxidation status of the brain.

3. Metals capable of catalyzing free radical production are present, e.g. iron, copper, zinc, aluminum.
   - The concentration of iron is higher in the brains of AD patients. Recall that iron is involved in formation of the potent hydroxyl radical.
   - Lower concentrations of copper were found in the brains of AD patients. Copper is an important part of antioxidant enzymes, Cu/Zn SOD and cytochrome –c oxidase.
   - There are conflicting reports about aluminum concentrations in AD brains.
   - Zinc induces amyloid formation in humans and there is increasing evidence that high zinc concentrations can mediate neuronal death associated with other brain injuries.

Many in vitro studies have confirmed a direct toxic effect of β-amyloid on cultures of neurons. This β-amyloid toxicity is prevented by vitamin E and

other antioxidants and mediated by hydrogen peroxide. (Behl et al, 1992, 1994)

Immune Diseases

Oxidation and generation of free radicals is an essential component of cell-mediated immunity. At physiological levels, free radicals are vital for antigen presentation and cell proliferation. However, at high levels they can decrease immune function (Hughes, 1999). Oxidative stress is detrimental to lymphocytes probably due to lipid peroxidation of PUFAs in the cell membrane and oxidation of plasma LDL that is lymphotoxic. Loss of membrane fluidity in lymphocytes has been correlated to decreased response to immunological challenges (Douziech et al. 2002).

Antioxidants can improve immune response by controlling the production of free radicals in the cell. Neutrophils kill extracellular pathogens through oxidative bursts. Uptake of vitamin E prior to the oxidative burst protects the neutrophil from destruction by the free radicals generated. Vitamin C is believed to work with vitamin E in this protection. Supplementation with these two antioxidants has been found to normalize the reduced chemotactic and bactericidal activities of neutrophils in compromised individuals (Grimble, et al. 1998).

Manifestation of disease by chronic inflammation are seen in rheumatoid arthritis and inflammatory bowel diseases. Chronic immune induced

inflammation has been decreased in animals supplemented with antioxidant enzymes or vitamin E (Inserra et al. 1997). An example is seen in the animal model of rheumatoid arthritis in which local lipid peroxidation is correlated to inflammation. Administration of antioxidants directly into the joint decreases inflammation (Sies 1984).

Thirty elderly patients, hospitalized for more than three months, were supplemented with daily antioxidant vitamins: 8000 I.U. vitamin A, 100 mg vitamin C, and 50 mg vitamin E or a placebo. Their cell-mediated immune function was assessed before and after supplementation and improvement was found only in the supplemented group (Penn et al. 1991). Another study found significant improvement in the delayed-type hypersensitivity test response in healthy adults supplemented for 30 days with 800 mg/day vitamin E (Meydani et al. 1990).

AIDS

Oxidative stress is believed to contribute to the decline of CD4+ lymphocyte counts and play a significant role in the progression of HIV infection to AIDS. This is understandable since the functioning of lymphocytes is closely linked to their redox potential (Zhang et al. 1997). It is known that lymphocytes of patients with AIDS are deficient in glutathione which makes them sensitive to oxidative stress. Recently the drug N-acetylcysteine (NAC), a

precursor of GSH, has been proposed for use in AIDS (Roberford and Calderon, 1995).

Plasma levels of vitamin E are known to be lower in HIV-infected patients than in controls. Supplementary vitamin E is significantly associated with slower progression from HIV infection to AIDS and increases CD4+ counts (Abrams et al. 1993). In AIDS patients plasma levels of zinc, magnesium, selenium, vitamin B_{12}, β-carotene and other carotenoids are low. The flavonoid, quercetin, inhibits the protein kinase C (PKC)-induced phosphorylation of I-κB that can liberate NF-κB to play a role in viral replication (Zhang 1997). Much more work needs to be done to determine which antioxidants can be used effectively in treating HIV infections.

Eye Diseases

Several epidemiological studies suggested an association between cataract incidence, macular degeneration, and oxidative stress (Franks, 1999). Nine of eleven studies show an inverse association between at least one type of cataract and at least one antioxidant nutrient. Three of five studies show inverse associations with macular degeneration and at least one antioxidant. However, there are many inconsistencies in the results. One of the biggest problems is the measurement of long-term nutrient intake and status. To date, only studies that involve nutrient supplements have been able to assess intake over an extended period of time. All these studies all indicate little or no

benefit from vitamin supplementation until used for 5 to 9 years. Significant reduction in cataract risk was observed after 10 or more years of supplementation (Jacques 1997). This finding suggests that we re-evaluate the validity of short term studies of antioxidants that reported minimal or no effects.

Goldberg and co-workers reported an inverse association between Age-Related Macular Degeneration (AMD) and consumption of fruits and vegetables high in vitamin A and C from the HANES-I survey.

Most of the experimental research on the etiology of cataract and AMD has focused on vitamins C, E, and carotenoids. Animal studies have shown that feeding increased amounts of vitamin C prevented and delayed progress of cataract in guinea pigs and rats. Animal studies have also shown that vitamin E is capable of delaying cataract development in rats and rabbits (Jacques 1997).

The Age Related Eye Disease Study reported a moderate beneficial effect of antioxidants, vitamins, and zinc supplementation in reducing progression to severe age related macular degeneration (Hyman and Neborsky, 2002). There are other trials currently underway to evaluate the effect of antioxidant vitamins on cataracts and AMD, including Women's Health Study, Women's Antioxidant Cardiovascular Study, and Physicians Health Study II.

Oxidative Stress and the Aging Process

Life extension can be realized by modulating two major pathways:

- biological process
- pathological process

Modulating both of these pathways as summarized in Table 11 results in the maximum benefit in terms of increasing lifespan.

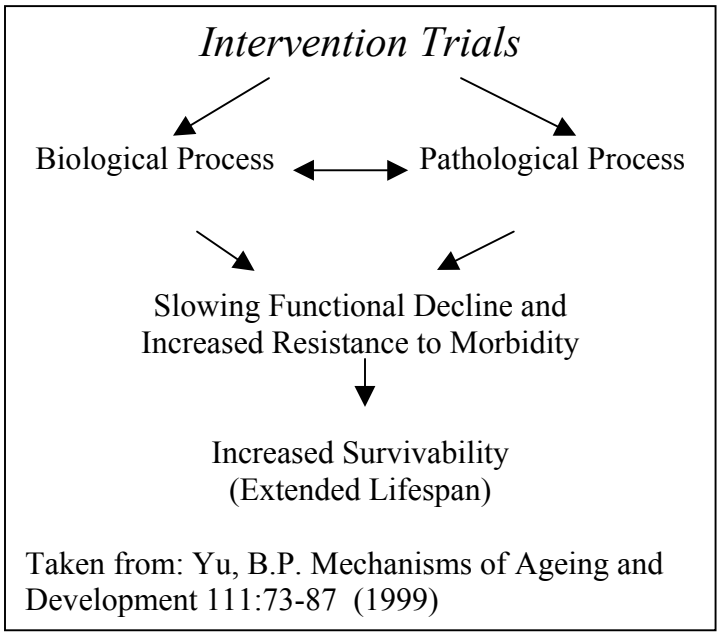

Figure 11. Modulation of Oxidative Stress to Extend Lifespan

Longevity is determined by the net effect of these two pathways including how they affect each other (crosstalk). Currently the only paradigm shown to affect both average and maximum lifespan is dietary restriction. Studies have shown that calorie-restricted rats exhibit increased resistance to oxidative

stresses. Perhaps one of the mechanisms by which calorie restriction may increase life span is by increasing resistance to oxidative stresses and enhancing antioxidant defenses. Antioxidant intervention is the focus of one of three general categories of hypotheses based on current understanding of aging: (Yu 1999)

- Genetically programmed mechanisms

  Although genetics play a role in longevity, research shows that it is not the whole story since external environmental factors play major roles in determining the course of aging.

- Neuronal-endocrine failures

  Hormonal intervention includes among others, the use of growth hormone, estrogen, testosterone, melatonin, and DHEA to treat a variety of age-related debilitating conditions.

- Oxidatively stressed modifications of cellular lipids, proteins, and DNA.

The Free Radical Hypothesis of Aging (also called the Oxidative Stress Theory of Aging) suggests that age related changes are manifestations of the body's inability to cope with oxidative stress that occurs throughout the lifespan. Normal and pathological aging has been associated with increased sensitivity to free radicals, probably as a result of increases in pro-oxidant mediators and a decrease in antioxidant defense. How this oxidative stress

causes the aging process is not known, but it is believed to involve lipid and protein peroxidation, increases in DNA oxidation products, and deficits in calcium regulatory mechanisms that eventually lead to cell death.

Studies conducted over the past years have determined the following changes in mitochondria associated with aging: (Vina et al 1999)

1. Some mitochondrial membrane carriers are impaired.

   Metabolic studies using whole cells, (isolated hepatocytes) show that aging affects mitochondrial function by impairing specific inner mitochondrial membrane processes such as malate transport. (Gluconeogenesis from lactate and pyruvate, but not from glycerol or fructose decline with aging, the former involving mitochondrial membrane transport of malate using the dicarboxylate carrier.) Ketogenesis from oleate, also dependent on mitochondrial performance, likewise decreased in hepatocytes from old animals. Other mitochondrial functions, such as urea synthesis in hepatocytes, does not decline with age. Since gene expression of the mitochondrial carrier is not changed with aging, post transcriptional modifications appear to be involved in the age related loss of these carriers and is likely to be in response to chronic oxidative stress.

2. Mitochondrial membrane potential declines with age.

   Because this is the driving force for ATP synthesis, it likely reduces the energy supply from the mitochondria which in turn may affect protein synthesis. Studies in isolated mitochondria have shown that oxidative

stress causes inhibition of respiration, affecting mitochondrial membrane potential.

3. Mitochondria also enlarge with aging.

   Acute oxidative stress is known to cause mitochondrial swelling.

4. Oxidation of mitochondrial glutathione increases with aging. (GSSG:GSH ratio is increased with aging)

5. Age-related mitochondrial DNA damage correlates with glutathione oxidation.

6. Administration of thiol containing antioxidants protects against age-related glutathione depletion and partially prevents age-related decline in neuromuscular coordination. Administration of other antioxidants, such as Gingko Biloba extract Egb 761, also prevents mitochondrial DNA damage (Droy-Lefaix and Packer 1999).

Normally, more than 80% of cellular ATP is derived from oxidative phosphorylation, and the remaining 20% from anaerobic glycolysis. Aged animal tissues have higher glycolytic activity and lower mitochondrial oxidative phosphorylation activity which is apparently associated with alterations of the structure and function of the mitochondrial F_0F_1-ATP synthase complex. Mitochondria from senescent rats show that mitochondria have a decreased F_1 content which appears to be associated with a decrease in intramitochondrial

GSH. Both of these phenomena seem to be related to oxidative damage of the mitochondrial proteins (Guerrieri et al. 1998).

A large amount of evidence has accumulated over the years implicating oxidative stress as being intimately involved in the physiological changes seen in aging and age-related and neurodegenerative diseases. Consequently, many studies have been conducted to examine the effects of diet components and specific nutrients in preventing or delaying the onset of these changes (Cantuti-Castelvetri et al. 2000).

Antioxidant Defenses

An antioxidant has been defined by Halliwell and Gutheridge as 'any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate'. When free radicals are generated in vivo many antioxidants come to play in defending the organism from oxidative damage as shown in Figure 12.

As a first line defense, the preventive antioxidants such as peroxidases and metal chelating proteins suppress the generation of free radials. Next, the radical-scavenging antioxidants such as vitamin C and vitamin E scavenge radicals to inhibit the oxidation chain initiation and prevent chain propagation as a second line of defense. This may also include the termination of a chain reaction by the reaction of two radicals. The repair and de novo enzymes act as the third line of defense by repairing damage and reconstituting membranes.

These include lipases, proteases, DNA repair enzymes and transferases (Niki 1997).

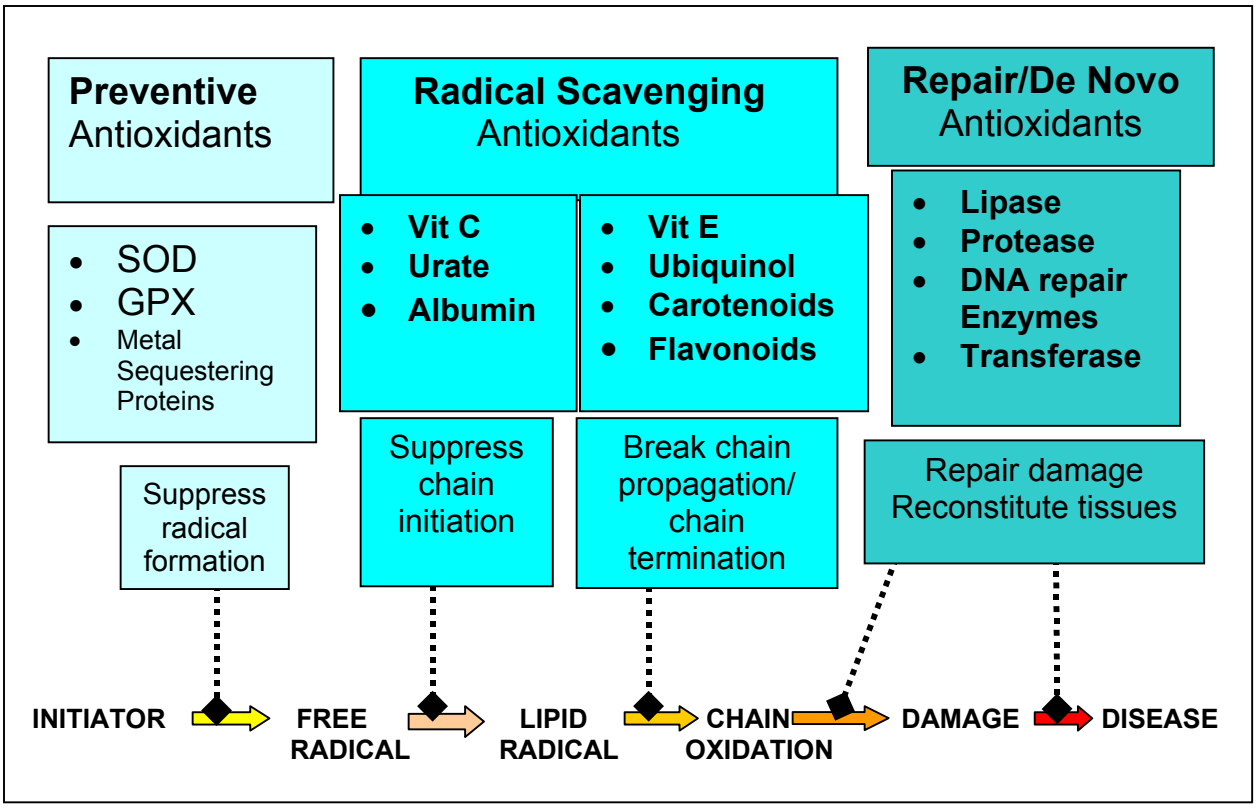

Figure 12 Antioxidant Groups and Actions
SOD Superoxide Dismutase, GPX Glutathione Peroxidase

Endogenous antioxidants

There is a vast network of intracellular and extracellular antioxidants with diverse roles within each area of defense (Figure 13). For example, vitamin C acts as a cytosolic antioxidant while vitamin E protects the cell membrane and glutathione acts to protect both. Glutathione (GSH) is required by three separate enzymes, a peroxidase, a reductase, and a transferase.

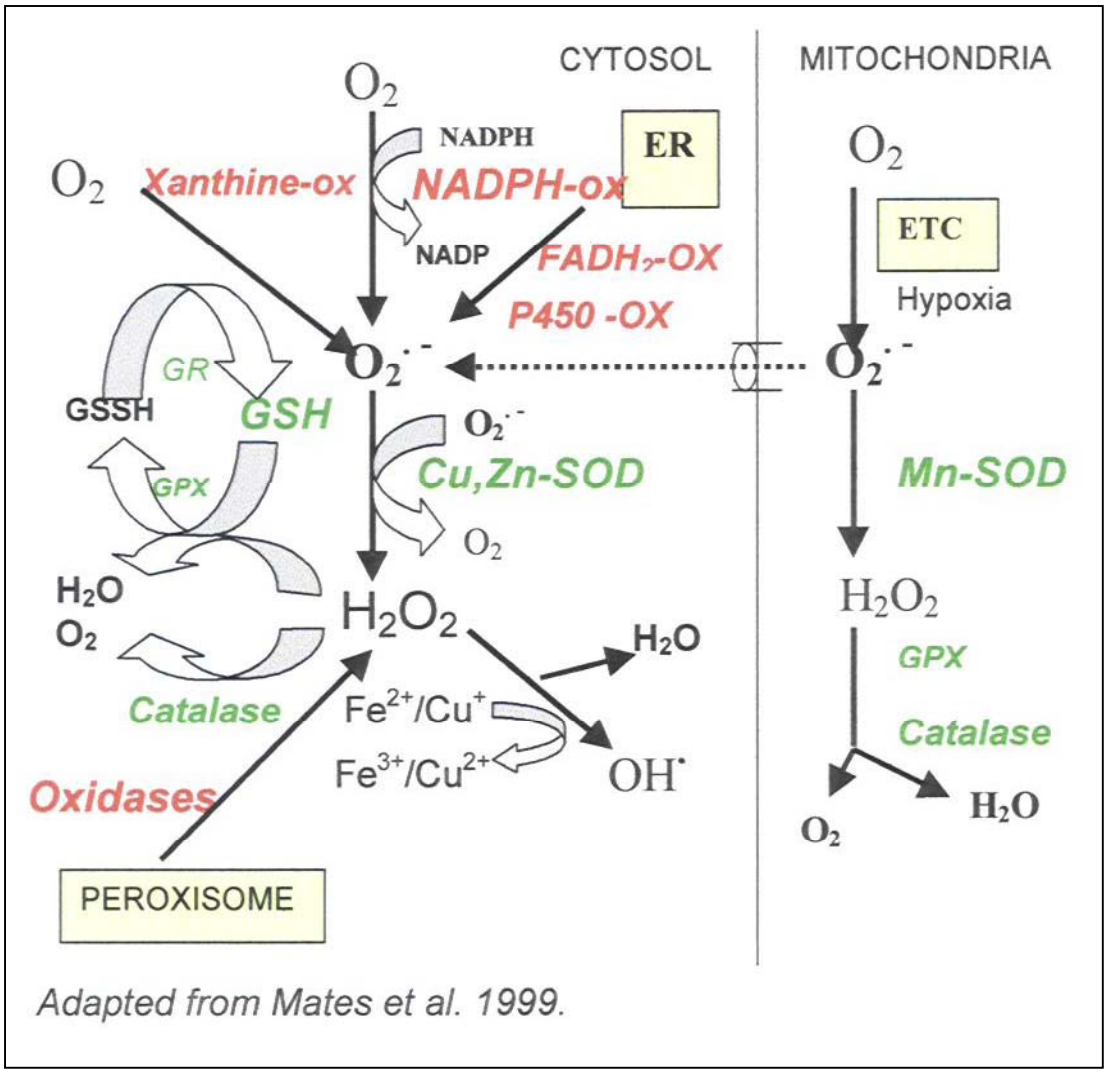

Figure 13 Generation of Reactive Oxygen Species and Endogenous Mechanisms Against Damage by Active Oxygen. ***Adapted from Mates et al. 1999.***

Catalase converts H_2O_2 to O_2 and H_2O. The enzyme superoxide dismutase (SOD) converts the superoxide radical to H_2O_2. Some of the antioxidant enzymes exist in several forms. For example, membrane, cytosolic, and plasma forms of glutathione peroxidase have been isolated. SOD has membrane, cytosolic, and extracellular forms. The levels and locations of these antioxidants must be tightly regulated for cell survival. The antioxidant enzymes, SOD, glutathione peroxidase (GPX), and catalase (CAT) work within the cells to remove most superoxides and peroxides before they react with metal ions to form more reactive free radicals. Peroxidative chain reactions initiated by free radicals that escaped the antioxidant defenses are terminated by chain- breaking water or lipid soluble antioxidants (Mates et al. 1999).

Exogenous antioxidants

Diet plays a vital role in the production of the antioxidant defense system because some antioxidants such as vitamin E, C, and, β-carotene are essential nutrients and other antioxidant enzymes are dependent on essential minerals (Figure 14). For example, SOD contains zinc and glutathione peroxidase contains selenium (Punchard and Kelly 1997).

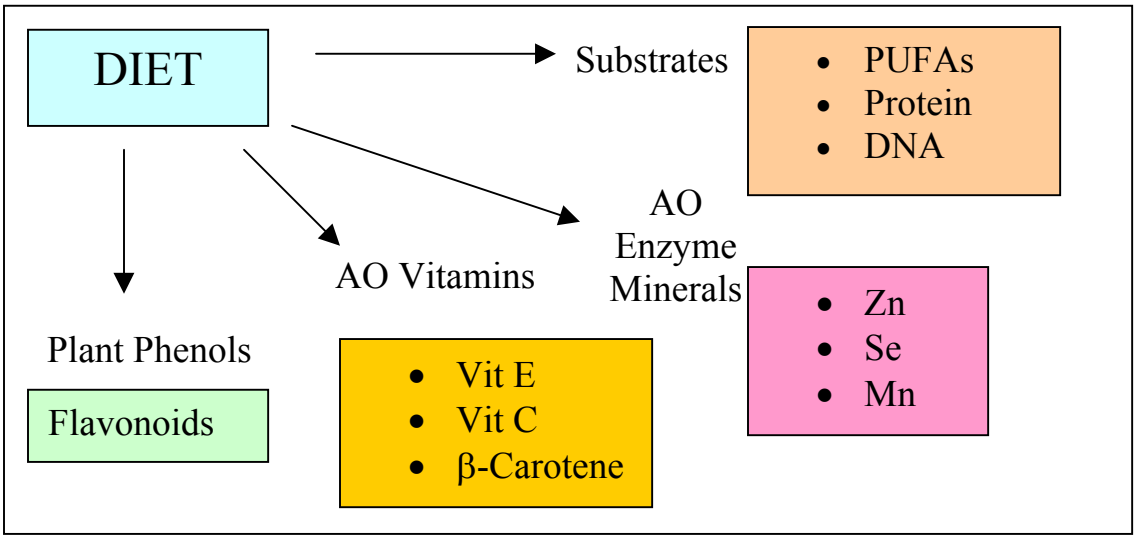

Figure 14. Contribution of Diet to Antioxidant Defenses.

Diet also plays an important role in the oxidation process by affecting the substrates that are subject to oxidation. The best example is the oxidation of lipids. Polyunsaturated fatty acids (PUFA) having two or more double bonds, are increasingly susceptible to free radical attack as the number of double

bonds increase. As shown in Figure 15, antioxidants available at the site of radical attack break the chain of oxidation by being preferentially oxidized by the attacking radical, thereby preventing oxidation of the adjacent fatty acid.

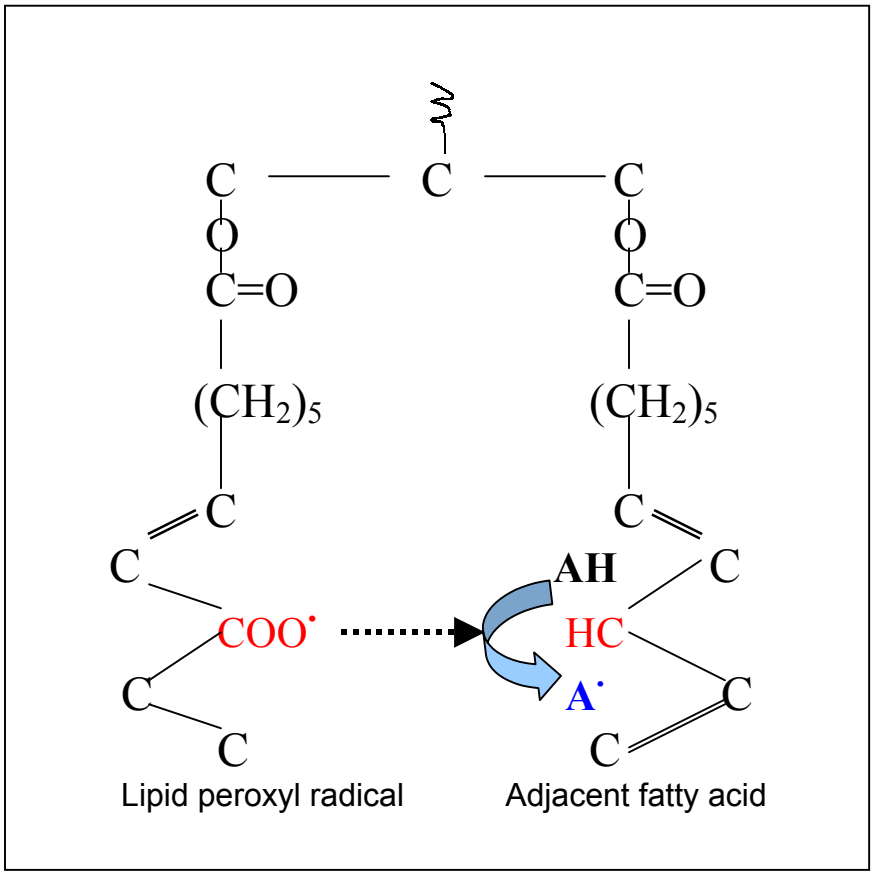

Figure 15 Antioxidant Protection of Triglyceride.
AH: antioxidant

Membrane and lipoprotein fatty acid composition is determined primarily by diet. Consumption of meat yields more arachidonic acid (20:4) while fish yields more eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). On average, the vegetarian diet has less total fat and consequently, less total

polyunsaturated fatty acids (Niki 1997). This could help to explain health benefits sometimes seen with the vegetarian diet.

The following is a listing of the most well known components of the in vivo antioxidant defense system:

Enzymes:

- Superoxide Dismutase (SOD)removal of superoxide radical
- Catalase reduction of H_2O_2 to water
- Glutathione Peroxidase reduction of H_2O_2 to water
- Thioredoxin reduction of peroxides

Metal ion sequestration:

- Metallothionein chelates Zn, Ag, Cu, Cd, Hg
- Phytochelatins chelates Cd, Zn, Cu
- Transferrin chelates Fe
- Albumin chelates Fe and Cu

Low molecular mass (endogenous)

- Urate scavenges $NO_2\cdot$, $OH\cdot$, chelates metal ions

Note: The following have been shown to be antioxidants in vitro, but uncertain in vivo:

- Bilirubin, α-keto acids (pyruvate and α–ketoglutarate), sex hormones, melatonin, coenzyme Q, lipoic acid, carnosine, anserine, melanins

Low molecular mass (derived from diet)

- Ascorbic Acid spares tocopherol, scavenges free radicals
- Vitamin E scavenges peroxyl radicals, most important chain breaking inhibitor of lipid peroxidation.
- (Carotenoids) in vivo antioxidant role is uncertain
- Plant Phenols suggested but not proven to inhibit LDL oxidation in vivo

Adapted from: Halliwell and Gutteridge *Free Radicals in Biology and Medicine, 1999.*

Evidence of Antioxidant Effectiveness

Animal Studies

Several animal studies suggest that feeding antioxidants can inhibit the progression of atherosclerotic disease. Carew et al., using the Watanabe heritable hyperlipidemic rabbit, did one of the most cited studies in this area in 1987. The cholesterol-lowering drug Lovastatin was compared with the drug Probucol to determine differences in treatment effect. Probucol is structurally very similar to butylated hydroxytoluene (BHT), a widely used antioxidant. Uptake and degradation of LDL was measured and confirmed to occur predominantly in the intimal foam cells of monocyte-macrophage origin. Rabbits treated with the antioxidant drug showed half the rates of degradation compared to those treated with Lovastatin although LDL cholesterol levels were similar. More importantly, the study showed that Probucol significantly reduced the rate of development of fatty-streak lesions compared to Lovastatin treated rabbits even though plasma cholesterol levels were similar.

In later experiments using this model, rabbits fed synthetic vitamin E exhibited similarly reduced ex vivo LDL oxidation and a significant 32% reduction in atherosclerotic lesion involvement in the aortic arch region (Williams et al 1992). In 1990, Verlangieri and Bush fed male monkeys 108 IU of d-α–tocopherol/day and monitored vascular changes by ultrasound for three

years. Vitamin E significantly inhibited the progression of atherosclerotic disease.

Vitamin E has been shown to increase the average life span in the rotifer, fruit fly, nematode, and rat (Yu 1999). Studies using the mammal model have shown mixed results, however. Early studies investigated the effect of chronic vitamin E deficiency in accelerating aging and lipofuscin (an aging pigment) in rats fed either standard rodent chow or a vitamin E deficient diet for 14 months. The control group included 45-60 day old rats on control diet. The effect of vitamin E deficiency was greater than the effect of aging alone on behavioral and histological parameters (Lal et al. 1973). Similar findings were seen in a study by Sarter and van der Linde, 1987, in terms of finding a higher content of lipofuscin in the hippocampus.

These studies and many others indicate that vitamin E has some protective effects against free radical injuries and age-related degenerative processes. However, the studies that show an effect use weanling animals because the deficiency cannot be invoked in adult animals. Therefore, the neuromotor/behavior outcomes may be a developmental phenomena rather than being indicative of an accelerated aging process.

Alpha-lipoic acid is a coenzyme in the oxidative decarboxylation of α-keto acids and has been shown to have antioxidant action. It scavenges free radicals and chelates transition metals. α-Lipoic acid (100 mg/Kg body weight) has been shown to improve memory in aged mice (Stoll et al. 1994).

Epidemiological Studies

The **WHO/MONICA Study**, reported by Gey et al in 1991 provided some of the first supportive evidence of the antioxidant hypothesis for prevention of cardiovascular disease. In this cross-cultural study (Figure 16) of 16 countries, both the blood levels of α–tocopherol and the tocopherol/cholesterol ratio inversely correlated with mortality rates. However, vitamin C and β–carotene were found not to be protective.

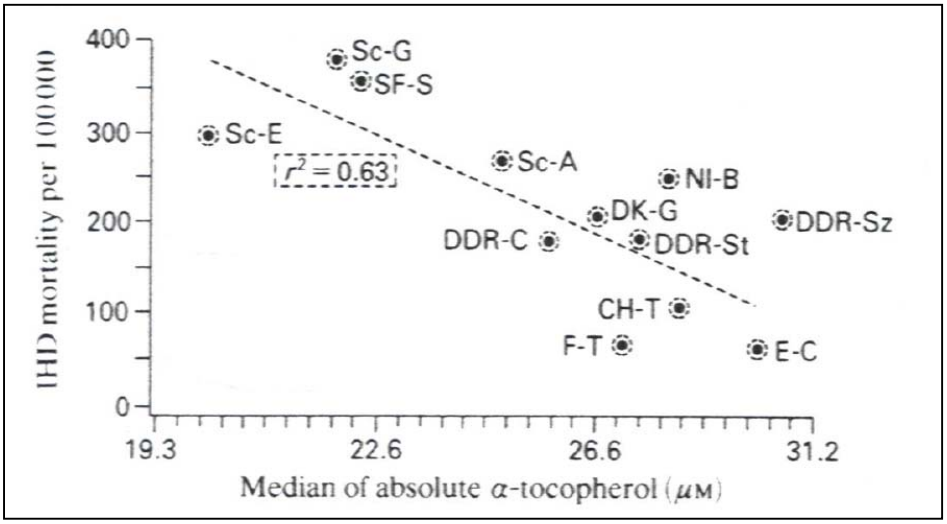

Figure 16 Inverse correlation between Ischemic Heart Disease and serum α–tocopherol levels .

The **Health Professionals Study** studied 39,910 male health professionals, 40-75 years of age and free of obvious coronary disease, to assess their intake of vitamin C, β-carotene, and vitamin E (Rimm et al. 1993).

During 4 years follow up, 667 cases of coronary disease were observed. After controlling for several risk factors, there was a 39% reduction in major coronary events in the highest intake category for vitamin E as compared to the lowest. Men who took at least 100 IU of vitamin E per day for at least 2 years had a relative risk of 0.47-0.84 compared to those who did not take a supplement. Of the 4814 men who reported previous history of CVD, there was a modest, but non-significant inverse association between intake of flavonoids and subsequent coronary mortality rates (Rimm et al. 1996). β-Carotene intake was not associated with a lower risk among non-smokers, but it was associated with lower risk in smokers. A high intake of vitamin C was not associated with a lower risk of coronary disease.

The **Nurses Health Study** involved 87,245 nurses aged 34-59, free of obvious cardiovascular disease (Stampfer et al.1993). Again, detailed dietary questionnaires were used to assess intake of antioxidant nutrients, including vitamin E. During the 8 year follow-up period, 437 non-fatal heart attacks occurred as well as 115 deaths due to coronary disease. The participants in the top quintile of dietary vitamin E intake showed 23-50% less heart disease after correction for age and smoking. Participants who took supplements of vitamin E for at least 2 years had a relative risk of major CVD of 0.38-0.91 (mean: 0.59) after adjustment for age, smoking, other risk factors and intake of other antioxidant nutrients. There was no correlation between reduced risk and intake of vitamin C or β–carotene.

A National Institute of Aging study, Established Populations for Epidemiological Studies of the Elderly (**EPESE**), involved four communities in the eastern part of the United States. In this study, 11,178 subjects 67-105 years of age were interviewed twice, three years apart, and then followed for about six years. During the follow-up period, there were 3490 deaths. The use of vitamin E supplements was associated with a significant reduced relative risk for all causes of mortality of 0.66 and risk of death from heart disease was 0.53. Persons reporting the simultaneous use of vitamin E and vitamin C supplements had a relative risk all-cause mortality of 0.58 and for coronary heart disease mortality of 0.47, but there was no significant interaction between the two antioxidant vitamins (Losoncszy et al. 1996).

Another American study, the **Iowa Women's Health Study**, involved 35,000 post-menopausal women followed for an average of 7 years (Kushi 1996). A 62% reduction in CVD deaths was found in those in the highest quintile of vitamin E intake from food. Oddly, no significant association was seen when the intake of vitamin E from supplements were evaluated. However, no information was collected on the length of time supplements were used. There was no reduced risk of cardiovascular disease seen with vitamin C or β-carotene intake.

In a Finnish prospective study, 2748 men and 2385 women were followed for an average of 14 years (Knekt et al. 1994). A comparison was made between risk of heart disease among those in the highest tertile of

vitamin E intake versus those in the lowest. Men in the highest category had a 34% (non-significant) reduction of risk of CHD death while women had a 65% (significant) reduction. A reduction in risk was also seen with intake of β-Carotene.

The **Zutphen Elderly Study** was a longitudinal investigation of risk factors for chronic disease in elderly men using a cohort of 805 men aged 65-84 living in Zutphen, in eastern Netherlands (Hertog et al. 1993). A dietary history was conducted and the subjects were followed for five years. Flavonoid intake was significantly inversely associated with mortality from cardiovascular disease and showed an inverse but non-significant relation with incidence of myocardial infarction. The relative risk of CVD mortality in the highest versus lowest tertile of flavonoid intake was 0.42. After adjustment for intake of other antioxidants, and significant variables, the risk was still significant.

Flavonoid intake was also inversely associated with mortality from CVD in the **Seven Countries Study** (Hertog et al. 1995) and in a Finnish study, conducted between 1972 and 1992 (Knekt et al. 1996).

A 1990 study conducted in France found that people who drink wine daily have a lower incidence of heart disease than their counterparts in the U.S. and other Western countries despite a high fat diet (Renaud et al. 1992). This observation, termed the "French Paradox" prompted much further study which indicated that aside from the cardioprotective effect of ethanol,

antioxidant plant phenols may explain the lower incidence due to reduced LDL oxidation (Frankel et al. 1995).

Epidemiological data show a very strong inverse relationship between consumption of fruits and vegetables and the risk of cancer with an overall risk reduction between 30 and 50%. (Block 1992). Of 156 retrospective and prospective studies, 128 found significant protective effects of fruit and vegetable intake. In most of the cancer sites studied, and after controlling for confounding factors, those with the lowest consumption of fruits and vegetables experience approximately twice the risk for cancer compared to those with high intake. This protection was assumed to be related to β-carotene intake until intervention trials revealed conflicting findings. (G. Block 1999)

Fruits and vegetables contain numerous substances with antioxidant activity including carotenoids, vitamin C, and polyphenols. Many studies have examined the association of these nutrients with various cancers. For example, lung cancer is the most prevalent cancer world wide and in the United States. Many have studied an association between intake and blood levels of β–carotene, vitamin C, vitamin E, and selenium. Individual studies suggest protective associations for each, but the totality of the evidence at present is not convincing for any one of these antioxidant micronutrients (Ziegler et al. 1996).

Several studies link vitamin C intake with reduced risk of several cancers, including oral, esophageal, stomach, colon and lung (Block 1991). Nine of eleven investigators studying the role of vitamin C in lung disease found a significant reduction with high intake even after controlling for smoking. Eight studies reporting on vitamin C intake and cancers of the esophagus and oral cavity found significant reduced risk with higher intake of the vitamin. Similar results were seen in seven studies regarding stomach cancer. A meta-analysis (Howe et al. 1990) of twelve major breast cancer studies found a strong and significant protective association with vitamin C intake.

Other studies have linked the intake of β-carotene with reduced risk of several cancers, especially lung and stomach(Van Poppel 1995). A strong inverse relationship has been shown between lycopene with reduced risk of several cancers, especially prostate, lung, and stomach (Giovannucci 1999).

Several epidemiological studies also support the hypothesis that selenium status is inversely related to the risk of some kinds of cancer (Combs 1997).

Clinical Trials

Several clinical studies indicate that supplementation with antioxidants such as Probucol, vitamin C, and vitamin E, but not β-carotene, increases the resistance of LDL to oxidation ex vivo. However, it is not clear whether they

also reduce the severity of atherosclerosis. A few randomized, double blind, placebo-controlled human trials have given inconsistent results.

The Cambridge Heart Antioxidant Study (**CHAOS**) trial in England evaluated the effect of vitamin E supplementation (400 or 800 IU/day) or placebo on the risk of myocardial infarction in 2,000 male and female patients with evidence of coronary atherosclerosis (Stephens et al. 1996). Results demonstrated that vitamin E supplementation significantly decreased the risk of nonfatal MI and cardiovascular related mortality by 47% after 200 days. Shortcomings: This trail had a short duration and an imbalance of smokers in the randomized groups at baseline. Also, there was an unclear demonstration of follow-up completeness and a low number of cardiac events recorded (14 vs. 41 events out of 2000 patients).

A randomized intervention trial involving almost 30,000 adults from Linxian, China evaluated the effects of supplementing with specific combinations of antioxidant vitamins over a five year period (Blot et al. 1993). The combination of beta-carotene, selenium and vitamin E (30 mg/day) had a marginally significant effect in reducing total mortality and a trend towards a reduction of CVD mortality (-9%). No effect was seen with vitamin C. Shortcomings: This was a complex study design with an undernourished population.

Another randomized double-blind study aimed at investigating the effects of antioxidant supplements on the incidence of lung cancer in Finland (**ATBC**)

enrolled 29,133 male smokers 50 to 69 years of age who had smoked an average of 36 years (Alpha Tocopherol Beta-Carotene Group 1994). The study participants received daily supplements of 20 mg synthetic beta-carotene, 50 mg synthetic vitamin E, both or a placebo and were followed for five to eight years. Analysis of heart disease in the ATBC Study involved 27,271 participants with no prior history of MI. After six years, coronary events were reduced by 4% in those supplemented with vitamin E and were increased 1% in those receiving β-carotene. Vitamin E decreased CVD deaths by 8% while β-carotene had no effect.

Another intervention trial, Carotene and Retinol Efficacy Trial (**CARET**), designed to study the risk of lung cancer in smokers, ex-smokers, and workers exposed to asbestos were supplemented with 30 mg β-Carotene + 25,000 IU vitamin A or placebo over 4 years (Omenn et al. 1996). The relative risk of death from CVD was non-significantly increased in the supplemented group compared with placebo. The trial was terminated early because of an increased incidence of lung cancer in the treatment group.

The Gruppo Italiano per lo Studio Della Sopravvivenza nell'Infarto Miocardico Study **(GISSI)** is a secondary prevention trial recently completed that involved 11,324 patients who had survived an MI within the 3 month period prior to enrollment in the study (Marchioli et al. 1999). There were four treatment groups: 300 mg/d of synthetic vitamin E, 0.9 g/d of a mixture of n-3 PUFA consisting of a 2:1 ratio of docosahexanoic acid and eicosapentanoic acid

esters, or both, or neither. The n-3 PUFA supplemented group realized a significant reduction in the endpoints of death, nonfatal MI, and stroke over the 3.5 year follow-up period which was not further improved with the addition of vitamin E. However, vitamin E supplementation alone resulted in 20% reduction in cardiovascular deaths and 35% reduction in sudden death.

Another study reported in the New England Journal of Medicine (Yusuf et al. 2000) enrolled 2545 women and 6996 men 55 years of age or older who were at high risk for CVD. These patients were randomly assigned to treatment groups receiving either 400 IU (natural) vitamin E or placebo and either an angiotensin-converting enzyme (Ramipril), or placebo. Over an average of 4.5 years there were no significant differences in deaths from cardiovascular disease, unstable angina, congestive heart failure, or other secondary outcomes for those assigned the vitamin E supplement compared to the placebo.

The recently reported Vitamin E Atherosclerosis Prevention Study (VEAPS) found that supplemented vitamin E 400 IU/day increased plasma levels of the vitamin and reduced oxidation parameters, but did not reduce intima-media thickness (Hodis et al. 2002).

Also recently reported was the a randomized placebo-controlled study of 20,536 high risk individuals supplemented with 600 mg vitamin E, 250 mg vitamin C, and 20 mg β-carotene daily. Although plasma levels of each supplemented vitamin increased substantially, there were no significant reductions in the five year incidence or mortality from any type of vascular

disease, cancer, or other major outcome (Heart Promotion Study Collaborative Group, 2002).

Antioxidant supplementation for cancer prevention has been studied in a few large scale double blind intervention studies. The Linxian trial (Blot et al. 1993) was conducted in relatively poorly nourished residents from the general population of Linxian county, China. The study was designed to investigate the effect of feeding four different nutrient combinations on the development of esophageal and gastric cancers. As shown above, the combination of antioxidant nutrients had a reduction in cancer deaths, especially in regards to gastric cancer. The relative risk of 0.55 for lung cancer has been questioned because of limited statistical power in that only 31 total lung cancer deaths occurred.

In the ATBC Study, an unexpected finding was that participants receiving β-carotene alone or in combination with vitamin E had a significantly higher incidence of lung cancer than those receiving the placebo (ATBC Group 1994). β-Carotene supplementation had no significant effect on the incidence of other major cancers, i.e. prostate, bladder, colon/rectum, and stomach. Within the placebo group, higher dietary β-carotene intake and serum concentrations at baseline were both associated with a lower subsequent lung cancer incidence, consistent with observational data. Those receiving 50 mg/day synthetic vitamin E had a 32% reduction in prostate cancer risk.

This finding of increased incidence of lung cancer with β-carotene supplementation was also seen in the Carotene and Retinol Efficacy Trial (CARET), described earlier in the CVD section (Omenn et al. 1996). This trial was stopped after two years when it was determined that the incidence of lung cancer was increased by 28% in the supplemented group. This was seen only in the smokers while a non-significant protective effect was seen in non-smokers. The CARET data also show that baseline serum β-carotene levels were inversely correlated with the subsequent incidence of lung cancer in both the placebo and supplemented groups.

An additional trial, a follow up to the Physicians' Health Study examined the effect of supplemental β-carotene vs. placebo in 22,071 male U.S. physicians. There was no significant effect on cancer or total mortality, positive or negative with 12 years of supplementation with 50 mg β-carotene every other day. It is interesting to note that an interim analysis of data was done at the 5-9 year period which revealed a significant reduction in cancer incidence in the antioxidant supplemented group which was no longer significant at the conclusion of the trial.

A 10 year double-blind, placebo-controlled clinical intervention with over 1300 older Americans was recently reported. Participants were given a daily oral supplement of selenium enriched yeast (200 μg Se/day). Results showed a significant reduction in all cancers as well as cancer mortality rate(Combs et al. 1997).

In a dietary intervention study, 10 stable type II diabetic patients were placed on a high flavonol diet (quercetin) for two weeks, after following a low flavonol diet for the preceding two weeks. Lymphocytes were subjected to an oxidative challenge ex vivo and DNA damage was measured. DNA damage was significantly reduced following consumption of the high flavonol diet, compared to the low flavonol diet (Lean et al. 1999).

Studies in humans have demonstrated beneficial effects of various antioxidants in one of the complications, diabetic neuropathy. One such study supplemented Type I and II diabetics with α–lipoic acid, vitamin E or selenium for 12 weeks. Results showed improvement in neurological symptoms and decreased lipid peroxidation (Ziegler et al. 1996).

A randomized, double-blind, placebo-controlled clinical trial supplemented 28 Type I diabetic patients with a flavonoid based antioxidant medication while following a standardized 1800-2000 calorie diet (Keenoy et al. 1999). Baseline measurements were made at the beginning of the trial, and reassessed after 3 months. Glycated hemoglobin (HbA_{1c}) values decreased slightly but significantly whereas the decline was non-significant in the placebo group. However, the difference in the two groups was believed to be confounded by differences in study participants such as sex and initial HBA_{1c} levels. There were significant differences in the supplemented group as seen by increased glutathione reductase activity, increased plasma protein thiol

content, and increased lag time of ex vivo copper induced LDL oxidation. Other antioxidant enzymes and oxidation products (TBARS) were not significantly changed with treatment.

Earlier work has shown that vitamin C and vitamin E inhibit hemoglobin glycation and vitamin E inhibits LDL oxidation. It was suggested that the improvement in HBA_{1c} and increase in oxidation lag time effects may be seen due to the flavonoid's ability to spare one of both of these two vitamins in vivo.

A study of normally aging subjects, with no diagnosis of Alzheimer's Disease or Parkinson's Disease, showed that daily oral supplementation of 300 mg of vitamin E with 1000mg vitamin C for 12 consecutive months improved short term memory, psychomotor performance, and overall mood of both males and females, and verbal memory only in females (Sram et al. 1993). Another study supplemented diets of healthy aging people with approximately 30 mg/day of vitamin E for six years. These supplemented individuals also scored better than controls on four of the cognitive measures analyzed in the New Mexico Aging process Study (La Rue et al. 1997). Phytochemicals, specifically Gingko Biloba extract Egb 761 have also been studied in humans with Alzheimer's Type Dementia (DAT) and elderly patients with memory impairments. All scores improved with supplementation compared to placebo groups.

Conclusions

Oxidative stress is clearly associated with a wide range of chronic and acute disease processes. From the standpoint of oxidative stress mechanisms, the hypotheses on etiology of heart disease, cancer, neuro-degenerative, and other diseases are certainly plausible. They are also "proven" to work theoretically in vitro. However, the real test of efficacy is in the living system. The epidemiological data generally indicate a benefit of consuming diets that are higher in the antioxidant nutrients. In some studies, it is not clear whether the benefit is derived from the specific nutrients under study, or another food component with health benefits yet to be discovered. Or, perhaps there is a particular combination of antioxidant nutrients that provide protection. It seems to be apparent that supplementation with natural vitamin E in 100-400 IU/day doses for more than two years is protective against heart disease. Some studies also show β-carotene, vitamin C and flavonoids to be protective. However, some of this enthusiasm must be diminished when reviewing the clinical trial data which give very mixed results ranging from 47% reduction MI with vitamin E supplementation to 28% increase in lung cancer with β-carotene supplementation.

Several questions remain unanswered despite years of intensive research in the area of antioxidants in disease prevention. One of the most elusive questions seems to be how well in vitro, ex vivo, and animal studies relate to human application. Are certain subgroups more receptive to treatment than

others? Is there a critical time during which antioxidants can make a significant difference in one's risk of developing a chronic disease such as cancer, diabetes, or heart disease? What are the interactions between various antioxidants? Has the delicate balance between oxidants and antioxidants been tipped by environmental stresses? If so, can the increased intake of dietary antioxidants correct the imbalance without causing other health concerns?

Fortunately, there are many more studies underway and many more in the planning stages. Until we know more about the possible benefits of consuming antioxidants in supplemental concentrations, the best advice is to strive to get more nutrients through the diet by selecting more antioxidant dense foods, such as fruits and vegetables, and to evaluate antioxidant supplements individually, in terms of efficacy and safety.

Clearly, there is a need for more long term, controlled trials in normal and specific chronic disease prone subgroups to study a dose response of each antioxidant nutrient, alone, and in combination with other antioxidant and non-antioxidant nutrients. This thesis research project focused on the interaction between specific flavonoids and a-tocopherol.

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Chapter 2

Methods for Assessing Antioxidant Stress and Antioxidant Quality in vivo.

If there is a "gold standard" for measuring oxidative stress and antioxidant quality in vivo, it would be to conduct a controlled clinical trial in which very large groups of homogeneous humans are fed an identical diet supplemented with the treatment compound of interest. Ideally, these subjects would be housed in a treatment facility to decrease confounding effects for a study period of several years. At the end of the study, samples of all biological tissues would be sampled and analyzed to determine oxidative damage and differences in pathology caused by the study compound. This scenario is obviously out of the realm of possibility because of ethical, legal, and logistical reasons. We are therefore left with less than ideal ways to discover what compounds or lifestyle factors will prevent oxidative stress and physiological damage associated with disease and mortality.

Epidemiological studies, both descriptive (relating the incidence of a disease to a particular dietary component) and experimental (supplementing the diet with a study compound and examining what happens) have provided much of the evidence to date for the beneficial effects of antioxidants including vitamin E and flavonoids.

Whenever possible, epidemiological studies should be accompanied by measurement of biomarkers of oxidative damage to determine if the antioxidant effect was actually achieved. For example, if dietary surveys reveal an association between intake of an antioxidant and decreased morbidity from heart disease, then measurement of LDL peroxidation coupled with a measurement of that antioxidant in LDL would be more meaningful than merely associating the diet with the disease.

Measures of antioxidant effect are most commonly used in animal studies and in vitro studies. A recent review (Bray, 2000) described five general categories of methods used to assess oxidative stress.

Chemical approach

This approach involves measuring the chemical end products of oxidative damage in biological samples. These biomarkers, sometimes called "footprints", are markedly increased when free radicals react with cellular macromolecules including membrane fatty acids, structural proteins, enzymes, RNA, and DNA.

Lipid peroxidation is the most frequently measured biomarker and involves two basic methods:

Measurement of primary end products, hydroperoxides.

Peroxide assays are further categorized by measuring:

- Total peroxide:

Iodine liberation- can be applied to extracts of biological samples if other oxidizing agents are absent (Ando, 1990) .

FOX Assay-(ferrous oxidation xylenol orange)- peroxides oxidize FeII to FeIII, detected by xylenol orange with absorbance at 540 nm (Nourooz-Zadeh, 1999).

Glutathione Peroxidase (GPX)- GPX reacts with H_2O_2 and organic peroxides oxidizing GSH to GSSG. Addition of glutathione reductase and NADPH results in stoichiometric consumption of NADPH or GSSG either of which can be measured using HPLC (Whanger et al., 1988) or spectrophotometric methods.

Cyclooxygenase (COX)- stimulation of COX activity is usually measured as oxygen uptake (Williams, et al. 1998)

- Separation of products: separate various classes of lipid peroxides by HPLC equipped with a chemiluminescence detector (Frei et al. 1988).

Measurement of secondary end products, MDA, volatile hydrocarbons such as pentane and ethane, and F_2-Isoprostanes.

- Malondialdehyde (MDA) is most often measured by the thiobarbituric acid reactive substances (TBARS) assay. The most sensitive assays are with HPLC detection (Templar, et al., 1999).

TBARS works well in defined systems, such as liposomes and microsomes, but its application to body fluids and tissue samples is unreliable because:

Aldehydes other than MDA can form chromogens that absorb at 532nm.

TBARS rarely measure free MDA content of the lipid system but rather measure MDA generated by decomposition of lipid peroxides during the acid heating stage of the test.

Several sugars, amino acids and bilirubin are reactive to TBA

TBARS have been reported to overestimate MDA levels by more than 10 fold (Halliwell and Gutteridge, 1999).

- Ethane and pentane- Measurement of these volatile hydrocarbons is non-invasive. Oxidation of w-3 PUFAs generates ethane, whereas oxidation of w-6 PUFAs generates pentane gas. Increased alkane exhalation in rodents has been demonstrated in deficiencies of vitamin E and exposure to ethanol, carbon tetrachloride, and excessive iron. It has also been measured in a wide array of clinical conditions associated with oxidative stress such as myocardial infarction, rheumatoid arthritis, and respiratory distress syndrome. There are some practical limitations due to background levels of pentane and isoprene in the human breath which are difficult to separate for analysis. Also, hydrocarbon gases are produced by

bacteria, are present as potential air pollutants, and may be confounded by oxygen concentrations in vivo (Springfield et al. 1994).

- F2-Isoprostanes are considered to be the most valuable currently available biomarker of lipid peroxidation in the human body (Roberts and Morrow, 1997).

Isoprostanes are chemically stable and can be measured with great sensitivity and specificity by GC/MS (Morrow and Roberts, 1999). Isoprostanes from all four major categories can be simultaneously detected using tandem MS and homologous internal standards. Immunoassays are directed against iPF2a-III (or its metabolite) which quantitatively is a minor F2 isoprostane. It may also be formed in a COX dependent manner, but the amount is trivial (Meagher and Fitzgerald 2000). F2-Isoprostanes are formed in membranes from arachidonyl-containing lipids during free radical-catalyzed lipid peroxidation. (Klein et al, 1997, Moore and Roberts, 1998) In studies using experimental animals, isoprostanes increased in plasma and tissues as a result of vitamin E deficiency (Awad et al, 1994). Furthermore, in an animal model of atherosclerosis (the apoE-deficient mouse), vitamin E supplementation not only suppressed F2-Isoprostane production, but also decreased atherosclerotic lesion formation (Reilly, 1998). Measurement of F2 isoprostanes in biological samples is difficult because they must be separated from

other isoprostanes, prostaglandins, and their metabolites. The methods used most often involve multiple chromatographic steps, including separation on silica and reverse-phase cartridges as well as TLC. The most reliable, sensitive, and specific method for the recovery of F2 isoprostanes involves a stable isotope dilution assay utilizing capillary gas chromatography/electron capture negative ionization mass spectrometry (GC-ECNI-MS). This method uses a combination of silica and reverse-phase cartridges, HPLC, and GC-ECNI-MS [Mori et al. 1999]

Protein oxidative modification is largely dependent on their amino-acid composition. CYS, PHE, and TYR are most susceptible to oxidation. Most often measured are:

1. Thiol oxidation of sulfur amino acids.
2. Carbonyl content of aromatic amino acids.

Oxidative modification of proteins has been reported in a variety of pathologic conditions (Stadtman 1991). There is considerable variability in baseline carbonyl concentrations in certain tissues, depending on how the assay is performed. Results may be confounded by oxidized proteins and amino acids in the diet.

DNA oxidative damage is most commonly measured by the primary oxidation products: 8-hydroxyquanosine and 8-hydroxyadenine.

Oxidative damage may cause DNA-protein crosslinking, damage to the deoxyribose-phosphate backbone and chemical modifications of purine and pyrimidine bases. These oxidative changes may result in mutations and strand breaks and are associated with cancer and aging as discussed in Chapter 1. A summary of the major products of radical damage is presented in Figure 1.

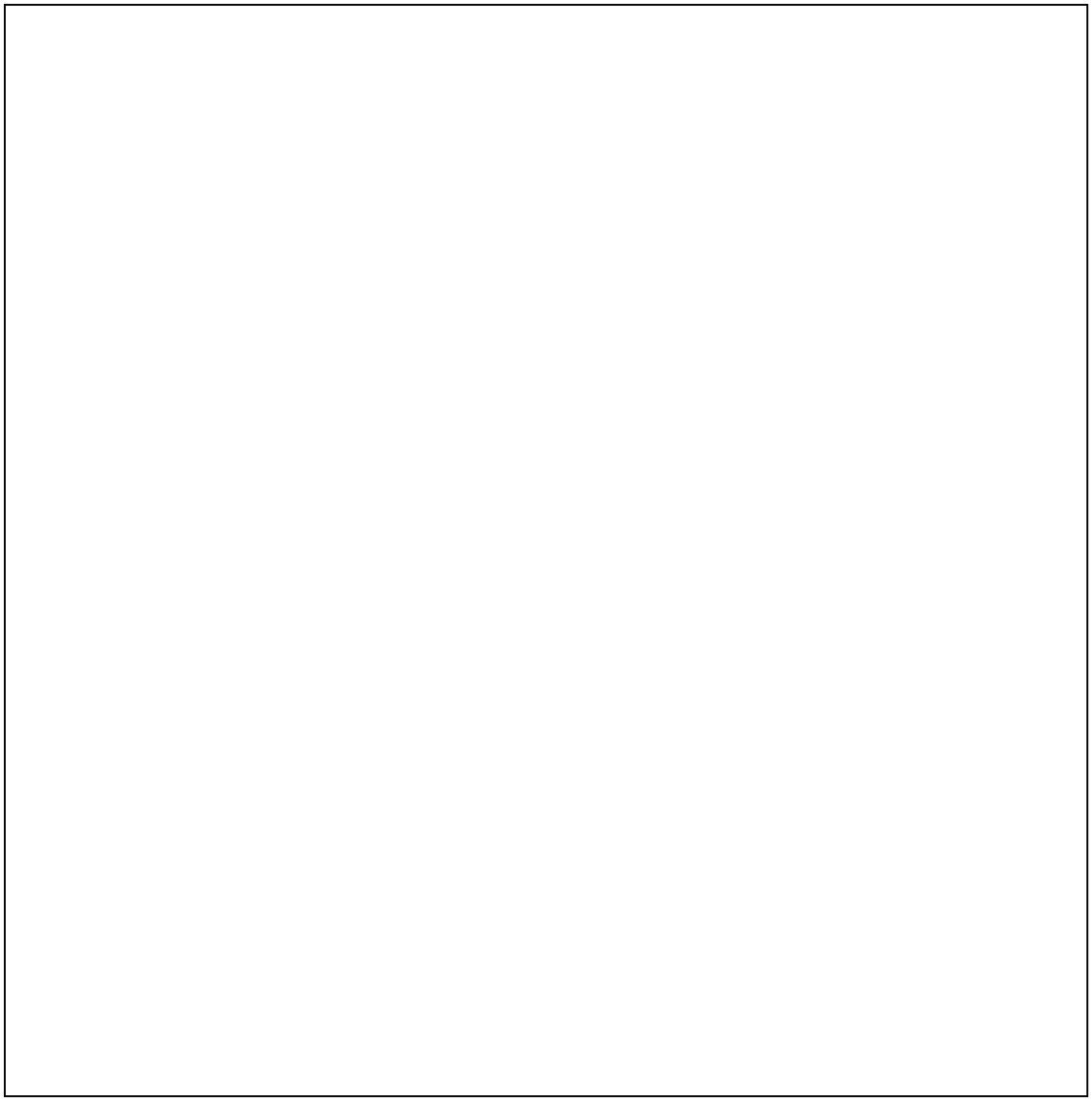

Figure 1. Major products of free radical damage. Taken from De Zwart et al. 1999.

2. Balanced approach-consists of evaluating the balance between pro-oxidant and antioxidant (redox balance) by measuring ROS (reactive oxygen species) generation and antioxidant defenses in the system.

ROS generation:

- Direct detection of ROS generation in vivo is very difficult (Electron spin resonance) because of the short half life and low concentrations of ROS.
- Indirect detection involves using a compound (spin trap) that reacts with the ROS to make a more stable adduct that can be detected by an ESR spectrometer.

Antioxidant defenses:

- Comprehensive analysis of enzymatic and non-enzymatic aspects of the antioxidant defense system, or
- Selective analysis of a specific aspect of the defense system, e.g. lipophilic or hydrophilic components.
- Interpretation of the data is a major challenge.

1. Potential approach-involves comparing the tendency of biological samples to oxidize after the addition of an external pro-oxidant.

- LDL oxidation is measured by isolating LDL from plasma and subjected to oxidation by copper or other oxidants. The lag phase is measured

between oxidant entry and onset of detectable quantities of lipid peroxidation products.

- Other examples:
  - ORAC (oxygen radical absorbing capacity)
  - TEAC (trolox equivalent antioxidant capacity)
  - TRAP (total peroxyl radical-trapping potential)
  - FRAP (ferric-reducing antioxidant power)
  - TOSC (total oxyradical scavenging capacity)
  - PE (phycoerythrin) based assays
  - Microdialysis sampling with chemiluminescence detection

- Applicability of these assays is limited because they don't provide information about the mechanism of specific components of the antioxidant-defense system.

2. **Molecular approach**- involves the detection of early molecular events such as signal transduction and transcription-factor activation.
   - Two transcription factors are activated by oxidative stress:
     - Nuclear factor κB (NF-κB)-is normally found in an inactive form, but is activated by ROS, toxins, bacterial lipopolysaccharide and various cytokines. Once activated NF-κB migrates to the nucleus where it

induces transcription of genes involved in the defense response. Therefore, the detection of NF-κB activation is a marker for initial stages of ROS production.

- AP-1 is also activated by ROS leading to the increased expression of ROS metabolizing enzymes such as glutathione-S-transferase, NADP:quinone reductase, and glucuronyltransferases. Studies have shown that superoxide radical and hydrogen peroxide induce expression of jun and fos protein, major components of AP-1.
- Measurement of these activated forms require isolation and incubation of nuclear extracts with specific probes, such as the electrophoretic mobility shift assay.

3. **Clinical approach**- involves using non-evasive methods such as MRI and NMR to diagnose the early clinical manifestations of oxidative stress.
    - Currently there are no methods available that directly detect ROS non-invasively. Proton MRI has been developed for animal studies to measure tissue damage and may have application soon for measurement in humans.

There is much debate over which method is the best indicator of oxidative stress or antioxidant effectiveness. Probably the best answer is that no one method is best because they all have advantages and disadvantages. An evaluation of the most popular methods is included in Table 1.

Requirement	**Alkanes**	**Aldehydes**	**Isoprostanes**	**Oxidized DNA bases**	**Oxidized Amino Acids**
Speed	–	±	–	–	–
Ease of analysis	–	±	–	–	±
Sensitivity	±	–	±	±	±
Specificity	±	–	+	±	±
Confounding factors	–	–	±	±	±
Correlation with damage	+	+	+	±	±
Non-invasiveness	+	+	–	±	–

Table 1 Comparison of Major Methods Used to Measure Antioxidant Effectiveness.

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Chapter 3

Tocopherol-Flavonoid Interactions: Summary of Current Research

Vitamin E

Proposed antioxidant mechanisms of vitamin E

Antioxidants may intervene in preventing the initial oxidation by quenching the original free radical, by stopping the oxidation chain, or by repairing the oxidized damage. Evidence of this relationship has been the subject of several studies.

Vitamin E, especially in the most active form as a-tocopherol (a-TOH) is considered an essential chain-breaking antioxidant.

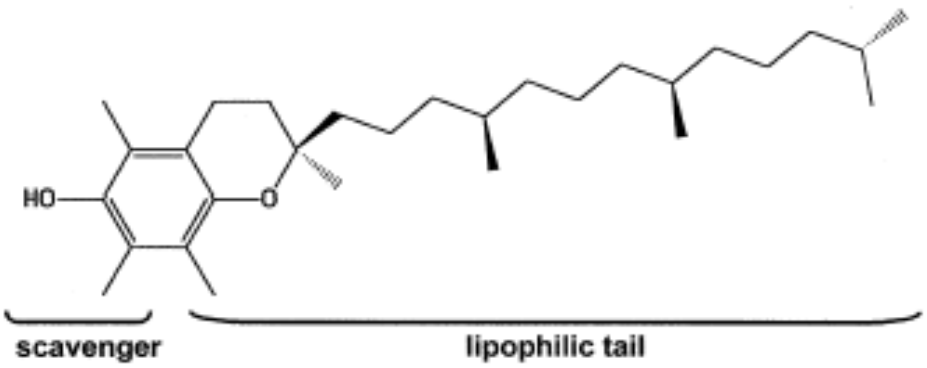

Figure 1. α-Tocopherol structure.

When affiliated with LDL, it provides protection against oxidative modification (Neuzil et al. 2001). There are approximately 10 molecules of a-TOH and 400 polyunsaturated, oxidizable lipid groups in the lipoprotein phospholipid monolayer (Esterbauer et al. 1992).

The rate constant for the interaction of a-TOH with a free radical is three or four orders of magnitude greater than that for the reaction of an oxidizable lipid group with the radical. In this way, α-TOH is able to divert free radicals away from vulnerable lipids thus breaking the chain of oxidation.

The tocopheryl radical (TO·) is less reactive than the radical it quenches because of a lower reduction potential and chemical reactivity. It is normally reduced back to its original form by another antioxidant, or by reacting with another free radical, thereby ending the oxidation chain. α–Tocopherol is known to react with a variety of free radicals at high rates (Niki et al. 1993).

$$\alpha\text{–TOH} + \text{ROO}^{\bullet} \xrightarrow{2.6 \times 10^{6}\ M^{-1} s^{-1}} \alpha\text{-TO}^{\bullet} + \text{ROOH}$$

However, the tocopherol radical reacts with lipid hydroperoxides of oxidized LDL at a much slower rate:

$$\alpha\text{–TO}^{\bullet} + \text{LOOH} \xrightarrow{5 \times 10^{3}\ M^{-1} s^{-1}} \alpha\text{TOH} + \text{LOO}\cdot$$

The tocopherol radical is much more likely to react with another free radical to be oxidized to tocopherol quinone, or reduced to tocopherol by ascorbate.

$$\text{TO}^{\bullet} + \text{Asc} \rightarrow \text{TOH} + \text{Asc}\cdot$$

$$2.0 \times 10^5\ M^{-1}s^{-1}$$

The ascorbate radical is a weak oxidant and is normally reduced to ascorbate by monodehydroascorbate reductase and dehydroascorbate reductase at the expense of NADH and GSH. Antioxidant radicals are also known to neutralize each other by forming non-radical dimers.

$$TO^{\bullet} + TO^{\bullet} \xrightarrow[10^2\ M^{-1}\ s^{-1}]{} TO\text{-}TO$$

Other dietary components may work in cooperation with tocopherol. This review will focus on flavonoids with emphasis on quercetin.

Flavonoids

For structure of flavonoids, see Figure 1, Chapter 5.

Since the report of in vitro flavonoid inhibition of LDL oxidation by Rankin et al. in 1988, there has been increasing interest in the interactions of flavonoids with lipoproteins. The reduction rate constant of flavonoids is very high for superoxide radicals (10^2 to 10^6 M^{-1} s^{-1}) with quercetin and catechin in the 10^4 M^{-1} s^{-1} range.

Quercetin, one of the most abundant flavonoids in the human diet, has been the focus of much research regarding its cardio protective effects including inhibition of low-density lipoprotein oxidation (Yamamoto et al.

1999), inhibition of platelet aggregation (Tzeng et al. 1991), increased smooth muscle relaxation (Duarte et al. 1993), inhibition of proliferation and migration of aortic smooth muscle cells (Alcocer et al. 2002), and modification of ischemia-reperfusion injury (Huk et al. 1998). Quercetin is a less lipophilic compound than tocopherol and therefore probably resides at the lipid/aqueous interface.

The catechins (+)-catechin and (-) epicatechin are trans and cis stereoisomers, respectively. The biological activities are affected by these structural differences (Rice-Evans et al. 1995). Catechins are reported to have significant antioxidant function in vivo despite not have all of the structure-activity relationships (SAR) that are most often associated with optimal antioxidant effectiveness:

- Hydroxylation of the B ring, preferably in the catechol moiety
- C2-C3 double bond in combination with
- Hydroxyl group at C3

The research on possible interactions between tocopherol and quercetin and catechin or other flavonoids is significant, but limited and will be reviewed here.

Antioxidant Interactions

In vitro

A limited number of in vitro assays have investigated interactions between flavonoids and alpha tocopherol. Quercetin and three other flavonoids, kempferol, morin, and myricetin, were added to human LDL in concentrations ranging from 1.0 to 20.0 μM. Plasma quercetin has been measured at 0.6μM after eating a food containing 64 mg of quercetin (Hollman et al. 1996). LDL was incubated in sodium phosphate buffer (pH 7.4, 10 mM) with oxidation initiated by adding either 5.0 μM $CuSO_4$ at 37C or 1.0 mM 2.2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) at 40C. All flavonoids showed a dose dependent protection of alpha tocopherol with quercetin being most effective. There was significant regeneration of alpha tocopherol in LDL (Zhu et al 2000).

Another in vitro study reported ten years earlier investigated the effect of quercetin, morin, fisetin and gossypetin in protecting ^{125}I-labelled LDL from oxidation initiated by macrophages. 1.0 μM quercetin delayed depletion of α-tocopherol up to 24 hours as compared to the flavonoid-free control in which α-tocopherol was depleted within 4 hours. Lipid hydroperoxides were not generated in the quercetin treated sample until the ten hour point whereas the control had steadily increased production of lipid hydroperoxides to 500nmol/mgLDL protein at that point. However, lipid hydroperoxides were not significantly different by the end of the study at 24 hours. Degradation of the

labeled LDL was also similarly delayed 10 hours by quercetin compared to four to six hours in the control sample. Interestingly, the antioxidant protection exhibited by the individual flavonoids in inhibiting degradation of LDL did not correlate with earlier measurements of their ability to inhibit lipoxygenase and oxygenase in intact rat peritoneal leucocytes (DeWhalley et al. 1990)

The combination of quercetin and alpha tocopherol has also been studied in the metmyoglobin oxidation of a fish oil-bile salt emulsion. This medium with average particle sizes of 2.0 μm was used as a model for the digestive tract. It was concluded that the synergistic antioxidant effect was unlikely due to regeneration of α-tocopherol by quercetin because they were consumed simultaneously during oxidation, but more likely the synergism was due to the different location of each antioxidant in the emulsion(Hoshino et al. 1997).

The mixture of the quercetin glucoside, rutin, in combination with ascorbic acid and α-tocopherol in a ratio of 4:4:1 was studied in human LDL mildly oxidized by copper ions or ultra violet radiation. A supra-additive antioxidant effect was observed and determined to result from both an inhibition of LDL oxidation and, at the cellular level, protection from the cytotoxic effects of oxidized LDL (Negre-Salvayre et al. 1995).

Catechin is a powerful inhibitor of copper induced oxidation of LDL in vitro (Mangiapane et al. 1992). It was also shown to prevent or delay the formation of TBARS in human plasma subjected to oxidation by water and lipid soluble radical generators. MDA, α-tocopherol and β-carotene were measured

in samples exposed to the aqueous phase initiator, AAPH, and the lipid phase initiator, AMVN. Catechin exhibited a higher antioxidant capacity when radicals were initiated in the aqueous phase where it inhibited TBARS formation by 88-96% compared to 40-60% inhibition in AMVN-exposed plasma. Catechin increased the lag time of α-tocopherol and β-carotene depletion in plasma exposed to each radical initiator in a concentration dependent manner. When oxidation was initiated by the aqueous phase antioxidant AAPH, β-carotene was protected longer. The explanation is that this lipophilic antioxidant is embedded deeper in the lipoprotein than α-tocopherol thereby being less susceptible to radicals generated in the aqueous phase. The lag phase for depletion of both lipid soluble antioxidants was less apparent but considerable in AMVN-exposed plasma supplemented with catechin. This indicted that catechin could scavenge lipid phase generated oxidants that had leaked out into the aqueous phase. Further conclusions from this study indicate that catechin was not oxidizing α-tocopherol at any concentration used (0.01-1mM). Nor was it shown to recycle α-tocopheryl quinone back to α-tocopherol (Lotito and Fraga 1998).

In vivo interactions:

Catechin has been shown to exhibit antioxidant properties in various biological systems. Two early studies demonstrated this by measuring its ability to prevent the increase in liver chemluminescence in ethanol- (Videla et al. 1983) and carbon tetrachloride-treated mice (Fraga et al. 1987).

The effect of 1% tea catechins on lipid peroxidation and conservation of α-tocopherol was studied in rats fed 30% palm oil or perilla oil diets for 31 days. The diets contained 60 mg/kg α-tocopherol. 1% tea catechins is equivalent to humans consuming 4.5 g/day. As expected, the levels of α-tocopherol and lipid peroxidation were significantly affected by the saturation of the dietary fat. α-Tocopherol decreased and lipid peroxidation increased by feeding the highly unsaturated perilla oil diet. The addition of tea catechins prevented this decrease of tocopherol in plasma and erythrocytes and suppressed lipid peroxidation as measured through TBARS in plasma. The authors suggested that tea catechins may prevent an increase in lipid peroxidation in the plasma by maintaining α-tocopherol levels (Nanjo et al. 1993).

A later study administered 10 and 50 mg/200 g. body weight epicatechin by intragastric intubation to fasted Wistar rats and then collected plasma at one and six hours after administration. Copper oxidized plasma from rats dosed with 10 mg epicatechin had significantly less cholesterol ester

hydroperoxide (CE-OOH) accumulation and α-tocopherol consumption compared to the control rats. Interestingly, this effect was not further significantly improved in rats dosed with 50 mg epicatechin despite substantially increased plasma concentrations of the flavonoid and its metabolites. It is noteworthy that in vitro experiments using the same concentrations of epicatechin and oxidation conditions revealed decreased CE-OOH accumulation and inhibition of α-tocopherol consumption in a concentration-dependent manner from 8-80μM. The in vivo study, measured total plasma epicatechin concentrations ranging from 29.2μM for the 10mg dose to 65.4 μM for the 50 mg dose at one hour. At the six hour post EC administration point, the values were 0.9 μM and 23.5 μM respectively (DaSilva et al. 1998a). Quercetin was also administered intragastrically to fasting rats at 2 or 10 mg/200 g body weight. Blood was collected at one and six hours after administration and plasma was separated and assayed. Plasma was oxidized over a six hour period using $CuSO_4$ with production of CE-OOH and consumption of α-tocopherol monitored. Plasma analysis measured free and conjugated quercetin and its 3'-O-methylated form (isorhamnetin). When 2 mg quercetin was administered, no free quercetin or free isohamnetin could be detected in the plasma at one or six hour samples. However, conjugated metabolites were present with 70% of those non-methylated. After six hours total quercetin metabolites decreased by 65%, but interestingly the concentration of methylated metabolites remained unchanged. Administration

of 10 mg quercetin increased metabolite levels 6.4 fold after 1 hour and 3.9 fold after 6 hours, compared to the 2 mg dose. The ex vivo oxidation of plasma, control plasma, and control plasma supplemented with equivalent concentrations of flavonoids were compared. A dose dependent inhibition of oxidation was observed with the plasma drawn one hour after administration of quercetin. However, plasma drawn six hours after quercetin administration revealed an increased level of oxidation compared to the one hour sample. This may be explained by the additional 5 hours of fasting between the one and six hour collections, during which ketone bodies and free fatty acids may have increased, thereby increasing plasma oxidation.

A follow up in vitro assay in which 4 to 80 μM quercetin was added to control plasma collected six hours after administration of the blank carrier propylene glycol resulted in inhibition of copper-induced oxidation in a dose dependent manner. α-Tocopherol consumption was inhibited by quercetin in a dose dependent manner in plasma drawn at 1 and 6 hours after administration following in vitro oxidation. This work concluded that quercetin provides protection against copper ion-induced oxidation of plasma lipids after absorption and metabolic conversion.(DaSilva et al. 1998b)

The influence of quercetin and catechin (2:1) was investigated in rats fed diets enriched in either PUFA or MUFA. Rats were fed diets supplemented with this flavonoid combination at 8 mg/kg diet for four weeks. Microsomal α-tocopherol levels were higher in flavonoid supplemented groups compared to

non-supplemented groups in both fat enriched diets. A significant increase in plasma α-tocopherol was observed only in rats consuming the diet enriched with MUFA. The production of conjugated dienes and TBARS after 12 hours of copper-catalyzed oxidation was significantly decreased with flavonoid supplementation only in the PUFA enriched diet (Fremont et al. 1998).

Another study compared the effects of diets containing 20 g/kg green tea powder, 250 mg/kg β-carotene, low isoflavone soy protein isolate, high isoflavone (genistein) soy protein isolate, or 4000 mg/kg vitamin E to the control diet devoid of vitamin E. The LDL/VLDL fraction was isolated and oxidized using 5 μM copper. They measured lag phase, conjugated dienes, lipid peroxides, and TBARS. The vitamin E and genistein enriched diets significantly improved all oxidation parameters. The green tea enriched diet significantly improved lag phase only and β-carotene had no significant effect on oxidation. It is noted that vitamin E and genistein were fed at pharmachological doses while green tea was fed at a level equivalent to that consumed by frequent tea drinkers. Consumption of α-tocopherol was not measured.(Anderson et al. 1998)

The antioxidant effectiveness of proanthocyanidins as grape seed extract was studied in the rat. These polyphenolic compounds are metabolized in vivo to monomers including gallic acid, catechin, and epicatechin. Plasma obtained one hour after intragastric administration of grape seed extract was oxidized ex vivo with $CuSO_4$ or AAPH while oxidation lag time and formation of CE-OOH

as well as consumption of α-tocopherol were measured. The plasma from treated animals was significantly more resistant to both $CuSO_4$ and AAPH induced oxidation compared to control animals. α-Tocopherol consumption during in vitro oxidation of plasma was also decreased in the proanthocyanidin-fed rats compared to the controls (Koga et al. 1999).

The rat model was studied to determine differences in oxidative parameters and in vitro oxidative loss of vitamin E following consumption of red wine compared to an equivalent alcohol water solution for 45 and 180 days. Red wine consumption resulted in higher hepatic SOD and GSH-PX activities after 45 days of treatment. Both treatments lowered MDA and enhanced hepatic catalase activity in both periods studied. In plasma MDA was lower with both treatments after six months, but plasma vitamin E was maintained at a higher concentration after wine compared to ethanol consumption (Roig et al. 1999).

Rats were fed a normal, vitamin E deficient, or vitamin E deficient supplemented with 0.25, 0.50, or 1% cacao liquor for 7 weeks. Cacao liquor, one of the main ingredients of cocoa and chocolate contains as its major antioxidative components epicatechin, catechin, and its oligomers as procyanidins. In this study, vitamin E levels were not affected by cacao liquor supplementation, but TBARS formation as a result of vitamin E deficiency was suppressed in a dose dependent manner up to the 0.5% level (Yamagishi et al. 2001).

Human Studies

In a cross-over study, six healthy men were fed identical high fat, fried meals with either 400 mL red wine or an isocaloric hydroalcoholic beverage to determine the extent of plasma and LDL oxidative stress and the capacity of red wine to counteract it. The meal served with wine provoked a significant increase in plasma total antioxidant capacity and α-tocopherol level. Postprandial LDL from the red wine fed group was more resistant to metal catalyzed oxidation while the hydroalcoholic beverage fed group showed increased susceptibility (Natella et al. 2001). An earlier human study, however, showed no effect on LDL ex vivo oxidizability or plasma tocopherol levels (De Rijke et al. 1995). One cross-over study reported decreased plasma F_2-isoprostanes in smokers administered de-alcoholized red wine with no significant change in serum tocopherol (Caccetta et al. 2001).

Conclusions

In vitro studies demonstrate an interactive relationship between various flavonoids and α-tocopherol. Reduced oxidation parameters and depletion of α-tocopherol are reported in LDL and various emulsions with more than an additive effect when both antioxidants are present.

In vivo animal studies using tea catechins, grape seed extract, red wine and cacao liquor have reported decreased tocopherol depletion and plasma

resistance to oxidation. However, administration of individual flavonoids are only reported with bolus feeding.

Human studies measuring the effect of feeding flavonoids on tocopherol status are very limited and also involve feeding red wine or tea as sources of flavonoids. One study suggested a protective effect of red wine on plasma tocopherol while two studies report no significant effect. Only one human study has reported a decrease in F_2-isoprostanes with wine consumption.

In conclusion, no human or animal studies have measured tocopherol status and the oxidation end-product F_2-isoprostanes to evaluate the effect of feeding a specific flavonoid with tocopherol. It is therefore the intent of this doctoral research to investigate this effect using the rat model.

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Chapter 4

QUERCETIN AND α-TOCOPHEROL INTERACT IN A LIPID SYSTEM

Joye K. Willcox and George L. Catignani*

Department of Food Science, North Carolina State University
Raleigh, NC 27695, USA Campus Box 7624

*Correspondence: George L. Catignani, Box 7624, North Carolina State University, Raleigh, NC 27695-7624. e-mail: George_Catignani@ncsu.edu

Paper No. FSR _____ of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

ABSTRACT

Interaction of quercetin and α-tocopherol was evaluated using the Oxidative Stability Instrument. The Oxidative Stability Index (OSI) offers a simple, reproducible measurement of antioxidant activities in a lipid environment. Both quercetin and α–tocopherol have been linked to a wide array of health benefits through in vitro, in vivo and epidemiological studies. The interaction between these two antioxidants has been measured in a variety of lipid systems representing specific and non-specific cellular environments. In this system, utilizing tocopherol stripped corn oil (TSCO), quercetin at 5-20 mM concentrations, provided superior antioxidant protection against lipid peroxidation compared to d-α-tocopherol. When the two antioxidants were combined, the antioxidant effect as measured by the OSI decreased indicating antagonism. More focused evaluations are needed to further elucidate this potential interaction in this pure lipid environment under these conditions.

KEYWORDS: Flavonoid, Antioxidant, OSI, Interaction, α-tocopherol

INTRODUCTION

Lipid peroxidation is a degradative process associated with the progression and possibly the initiating events of atherosclerosis. Isolated polyunsaturated fatty acids (PUFAs) and those incorporated into lipids are readily attacked by free radicals, thus becoming lipid peroxides which are toxic and capable of damaging most body cells[1]. This oxidative attack will continue through a chain reaction unless the generated radicals are neutralized by co-localized antioxidants. Because the biological environment is a mixed milieu of antioxidants, it is important to understand how various antioxidants interact to provide antioxidant protection.

α-Tocopherol (a-TOH) is quantitatively the most important low molecular weight lipophilic redox active, component in human circulation and is regarded as a possible modulator of atherogenesis[2]. Many epidemiological, in vitro, and in vivo studies have confirmed this.

Epidemiological results also relate the intake of flavonoids in the diet with a decreased risk of cardiovascular disease[3]. Quercetin, one of the most abundant flavonoids in the human diet, has been the focus of much research regarding its cardio protective effects including inhibition of low-density lipoprotein oxidation[4]. This flavonoid is a hydroxyl radical ($OH\cdot$) scavenger[5] and a potent antioxidant against lipid peroxidation in phospholipids bilayers[6] and low density lipoproteins[7]. Quercetin is reported to scavenge radicals in an

aqueous environment, while a-TOH is most commonly described as a peroxyl radical scavenger.

Interactions between these two polyphenolic antioxidants have been reported for the inhibition of lipid peroxidation in various in vitro models[8, 9]. Hoshino et al. [10] reported a synergistic effect between quercetin and tocopherol in fish oil-bile salt emulsion with quercetin demonstrating an antioxidant effect superior to that of α-tocopherol. An interaction between quercetin and α-tocopherol involving the regeneration of α-TOH has been demonstrated in human low-density lipoprotein (LDL)[11]. The study reported here was conducted to investigate the relative and interactive behaviors of quercetin and α-TOH in a pure lipid system under oxidative conditions.

MATERIALS AND METHODS

Apparatus.

The Oxidative Stability Instrument (Omnion, Inc., Rockland, MA) was used to oxidize samples in this study. The Oxidative Stability Instrument oxidizes oil samples using heat and aeration while continuously monitoring changes in electrical conductivity that result from the formation of volatile secondary oxidation products. A slope change algorithm defines the Oxidative Stability Index value (OSI), as the number of hours required to reach an induction point in electrical conductivity (indicative of a sharp rise in the

formation of volatile oxidation products). See Figure 1. An increase in OSI values relative to a control is indicative of an increase in the amount of time required to oxidize a sample, thus indicating an increase in the antioxidant capacity of the sample. The use of the OSI value for the determining the oxidative stability of oils has been accepted as an AOCS Official Method Cd 12B-92 [12].

Materials. Tocopherol stripped corn oil (TSCO) was purchased from Dyets (Bethehem, PA). dl-α-Tocopherol and quercetin dihydrate were purchased from USB (Arlington Height, IL). TSCO and dl-α-tocopherol were stored under nitrogen at 4°C prior to use.

Sample preparation. Stock solutions of quercetin and α-TOH were prepared in acetone, verified spectrophotometrically, and stored under nitrogen at 4C until used. Appropriate aliquots for 0, 5, 10, and 20 mM concentrations of each compound were added to 5 g (5.4348 mL) TSCO,and evaporated under nitrogen prior to being placed in the OSI apparatus.

Reaction Conditions. Assays were run in triplicate over the course of several days under identical conditions: temperature was set at 100°C, with dried compressed air supplied at 8.8 L/hour. To reduce the possibility of changes in the rates of oxidation due to changes in the humidity of the incoming air, an

in-line air drier was used. As a control TSCO with no additions was included in all experiments.

RESULTS AND DISCUSSION

Concentration versus OSI time in hours were plotted to compare quercetin and α-TOH and are shown in Figure 1.

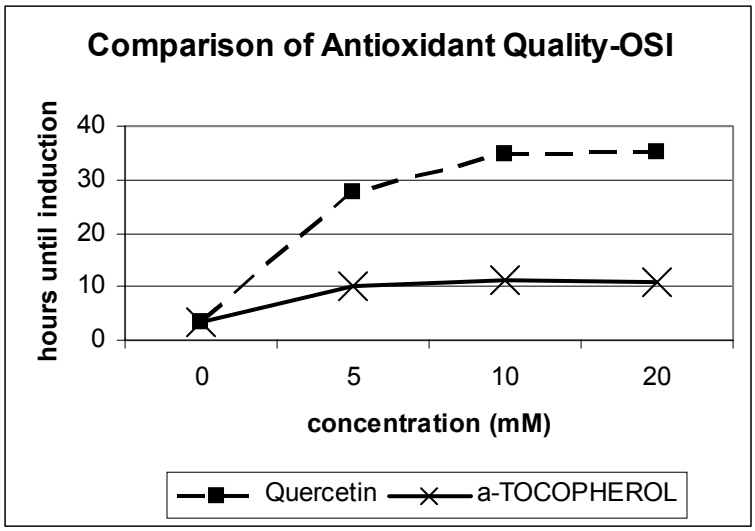

Figure 1. Comparison of Antioxidant Quality of Quercetin and α-Tocopherol in tocopherol stripped corn oil (TSCO).

Quercetin showed superior antioxidant effect compared to α-TOH at all concentrations tested as indicated by increased OSI values. The dose effect leveled off for α-TOH at 5mM and for quercetin at 10mM. Quercetin, a flavonoid aglycone, is somewhat lipophilic compared to other plant antioxidants, but it is certainly less lipophilic than a-TOH[13].

Tocopherol is thought to reside within lipid membranes and LDL bilayers with its chromanol ring stabilized by hydrogen bonding with the ester carbonyl groups of phospholipids. Under our experimental conditions of accelerated oxygenation of a highly polyunsaturated fat oil (61 % PUFAs), quercetin showed a 2.8 to 3.5 fold increase in antioxidant protection over α-TOH at the concentrations tested.

This superiority of quercetin is unexpected when considering the five fold greater ratio of inhibition to production of peroxyl radical rate constants reported for these two compounds in the methyl linoleate model [14]. However, the results of our study are in agreement with those of Schwartz et al. who reported that α-tocopherol exhibited inferior antioxidant activity compared to its water-soluble analog, Trolox, and other more polar hydrophilic phenolic compounds in a bulk oil system[15].

The difference we report may be explained by a difference in stability of the phenoxyl radical produced in this model. Planarity of the flavonoid molecule is considered an important factor for optimal antioxidant activity by producing a more stable phenoxyl radical[16].

Interestingly, when both compounds were added, a less than additive, or from the perspective of quercetin, an antagonistic effect was measured as shown in Figure 2.

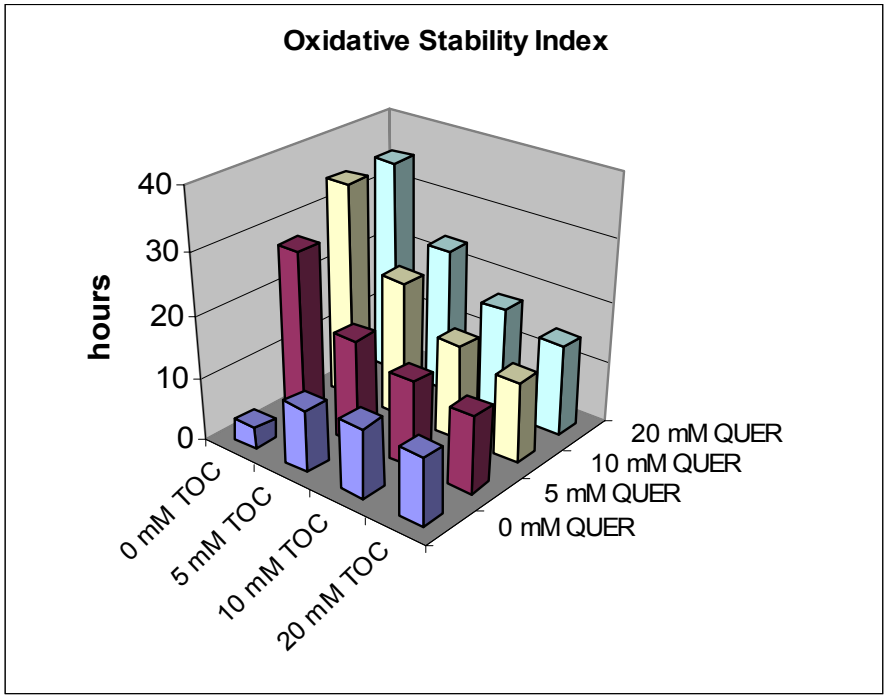

Figure 2. Oxidative Stability Index (OSI) Values for 0-20mM Concentration Combinations of Quercetin (QUER) and α-Tocopherol (TOC).

We may speculate that at these concentrations the combination of quercetin and tocopherol may have reduced antioxidant effectiveness due to pro-oxidant activity of one or both phenoxyl radicals. Considering the reducing potentials (E_7) (Quercetin, E7 = 0.22v; Tocopherol, E7=0.5v), tocopherol is more likely to be a pro-oxidant. At each concentration of quercetin, the addition of tocopherol reduced oxidative protection.

To understand this in vitro antagonism further study is needed using lower concentrations of quercetin with measurements of residual tocopherol, quercetin, and their oxidized metabolites. These results showing antagonism

in a lipid environment support the assumption that quercetin is more likely to provide positive interaction with and sparing of tocopherol by residing at the lipid/aqueous interface of the cell membrane or LDL bilayer. However, further study using these concentrations of quercetin and tocopherol in a mixed lipid/aqueous environment under these experimental conditions would be needed to confirm this assumption.

ABBREVIATIONS USED: OSI - Oxidative Stability Index ; TSCO- Tocopherol Stripped Corn Oil; α-TOH- alpha tocopherol, PUFA-polyunsaturated fatty acid; OH• - hydroxyl radical; LDL – low density lipoprotein.

ACKNOWLEDGMENT

We would like to thank Dr. Tim Sanders, USDA/ARS Market Quality and Handling Research Unit for the use of the Oxidative Stability Instrument.

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Chapter 5

APPLICATION OF THE OXIDATIVE STABILITY INDEX FOR ASSESSING THE ANTIOXIDANT PROPERTIES OF FLAVONOIDS

Gabriel K. Harris, Joye K. Willcox and George L. Catignani*

Department of Food Science, North Carolina State University
Raleigh, NC 27695, USA Campus Box 7624

*Correspondence: George L. Catignani, Box 7624, North Carolina State University, Raleigh, NC 27695-7624. e-mail: George_Catignani@ncsu.edu

Paper No. FSR _____ of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

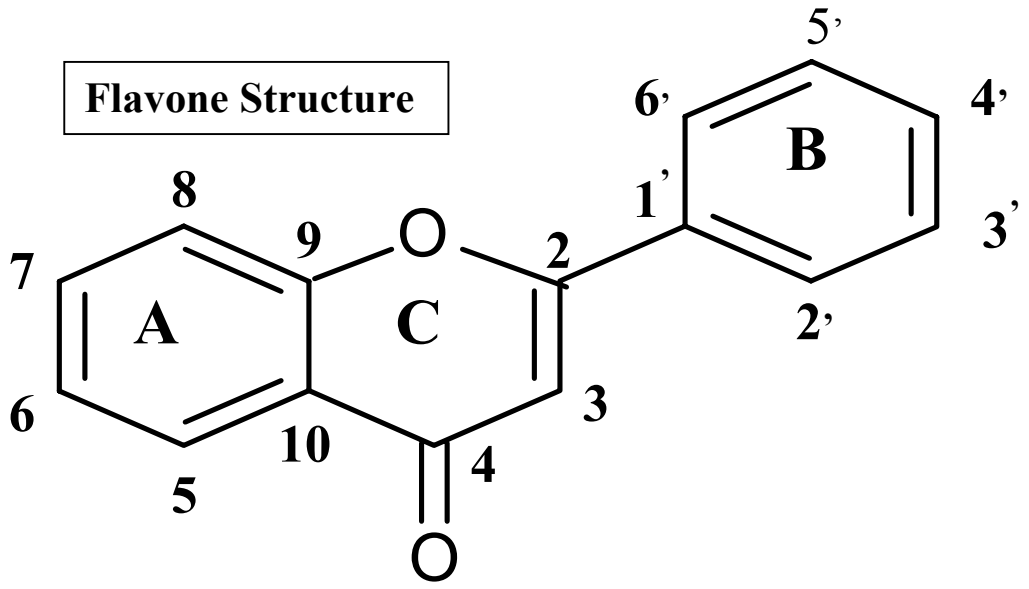
ABSTRACT

The antioxidant activities of representative flavonoid classes (flavonols, flavones, flavanones, isoflavones and flavanols) relative to dl-α-tocopherol were evaluated using the Oxidative Stability Index (OSI) value. At 5 mM concentrations in tocopherol stripped corn oil (TSCO), the relative antioxidant activity was determined as follows: (+) catechin > quercetin > (+,-) taxifolin > dl-α-tocopherol > THI (3', 4', 7-trihydroxyisoflavone) > luteolin. The values were measured as 245%, 201%, 132%, 100%, 64%, and 61% of dl-α-tocopherol OSI values, respectively. Intra-assay and inter-assay coefficients of variation were 1.43% and 2.73% . Peroxide Induction (PI) values were utilized as a comparison method using compounds and conditions identical to those in OSI experiments. Relative values for OSI and PI of the flavonoids tested showed a 0.98 correlation. This method also revealed differences in antioxidant activity of catechins due to stereochemistry. At 5 mM, (-) epicatechin and (+,-) catechin demonstrated 17% and 58%, respectively, of the activity of (+) catechin. The OSI offers a simple, reproducible method for the evaluation of flavonoid antioxidant activities in a lipid environment.

KEYWORDS: Flavonoid, Antioxidant, OSI, Stereochemistry

INTRODUCTION

Flavonoids are polyphenolic compounds produced as secondary metabolites by plants and are among the most plentiful of the naturally occurring phenols. Over 4,000 flavonoid compounds have been identified (Hollman and Katan, 1999). All are based on the flavone molecule which contains 15 carbon atoms made up of a pair of phenolic rings, designated A and B, and a third heterocyclic "C" ring (Cook and Samman, 1996). The major flavonoid classes are distinguished by variations in the level of oxidation and pattern of substitution of the six-membered C ring **(Figure 1)**.



Flavonoid	Class	C ring substituents	B ring			A ring OH
			Attached To C ring	Substituent C3'	Substituent C4'	
Quercetin	Flavonol	*C2=C3, C4=O, C3-OH*	C2	OH	OH	C5,C7
Rutin	Flavonol	C2=C3, C4=O, C3-Rham	C2	OH	OH	C5,C7
Luteolin	Flavone	C2=C3, C4=O, C3-H	C2	OH	OH	C5,C7
Chrysin	Flavone	C2=C3, C4=O, C3-H	C2	H	H	C5,C7
THI	Isoflavone	C2=C3, C4=O, C2-H	C3	OH	OH	C7
Genistein	Isoflavone	C2=C3, C4=O, C2-H	C3	H	OH	C5,C7
(+)Catechin	Flavanol	C2-C3, C4-2H, C3-OH*[a]	C2	OH	OH	C5,C7
(-)Catechin	Flavanol	C2-C3, C4-2H, C3-OH*[b]	C2	OH	OH	C5,C7
(-) Epicatechin	Flavanol	C2-C3, C4-2H, C3-OH**	C2	OH	OH	C5,C7
(+,-) Taxifolin	Flavanone	*C2-C3, C4=O, C3-OH*	C2	OH	OH	C5,C7

Figure 1. Study flavonoids, class, and distinguishing substituents.
THI: 3'4'7 (OH)-isoflavone; C2=C3: double bond; C2-C3: single bond; C4=O: carbonyl group; C3-H: hydrogen; C3-OH: hydroxyl; Rham: (sugar) Rhaminose; Flavanols-(+,-) Catechin: racemic mixture of (+) and (-) catechin; *[a] :trans to B-ring (2R,3S); *[b] :trans to B-ring (2S,3R); **: cis to B-ring.

The structures of flavonoids within these major classes vary in the pattern of substitution of the A and B rings. Further variations are due to glycosylation with differing sugar moieties (Pietta, 2000). These structural variations influence the ability of flavonoids to react with free radicals in providing antioxidant protection.

The antioxidant activity of flavonoids may have important implications for human health. Recent studies have indicated that flavonoids and other phenolic antioxidants in the diet may act to prevent heart disease and cancer (Prior and Cao, 2000; Ahmad and Mukhtar, 1999; Avarim and Fuhrman, 1998).

The antioxidant activity of flavonoids in lipids has also spurred interest in their use as preservatives of high fat foods. Patents have been issued for the use of green tea extracts, which contain high concentrations of catechins, as lipid antioxidants (U.S. patents 4,891,231 and 4,839,187).

Flavonoid antioxidant properties have been demonstrated in a variety of systems (Ollila et al. 2002; Sawai and Moon, 2000; Nanjo et al., 1999; Rice-Evans et al., 1995; Shahidi and Wanasundara, 1994; Fumio, et al., 1993). Much research is focused on antioxidants that can prevent lipid peroxidation because of the extensive health consequences of this pathological process. Many techniques are available for measuring the effectiveness of antioxidants in decreasing the rate of peroxidation of membrane lipids, lipoproteins, or fatty acids. The most specific assays of lipid peroxidation are also the most difficult

and time-consuming, such as HPLC analysis of conjugated dienes (Holley and Slater, 1991) and the measurement of F_2-isoprostanes by GC-MS (Morrow and Roberts, 1999). Because the oxidation of individual polyunsaturated fatty acids is paramount to this degradative process, it is important to have a standard method of rapidly screening antioxidant effectiveness in a lipid system. This method may also have application for evaluation of interactions between flavonoids and other antioxidants such as α-tocopherol.

The Oxidative Stability Instrument oxidizes oil samples using heat and aeration while continuously monitoring changes in electrical conductivity which result from the formation of volatile secondary oxidation products. A slope change algorithm gives the Oxidative Stability Index value (OSI) defined as the number of hours required to reach an inflection point in electrical conductivity (indicative of a sharp rise in the formation of volatile oxidation products). An increase in OSI values relative to a control is indicative of an increase in the amount of time required to oxidize a sample, thus indicating an increase in the antioxidant capacity of the sample. The use of the OSI value for the determining the oxidative stability of oils has been accepted as an AOCS Official Method Cd 12B-92 (Firestone, 1992) as a replacement for the Active Oxygen Method (AOM), AOCS Official Method Cd 12-57 (Firestone, 1989).

The purpose of this study was to examine the usefulness of the OSI method in providing a simple, reproducible assay for measuring the antioxidant activity of selected flavonoids in a lipid system.

MATERIALS AND METHODS

Apparatus. The Oxidative Stability Instrument (Omnion, Inc., Rockland, MA) used to oxidize samples in this study is represented in **Figure 2.**

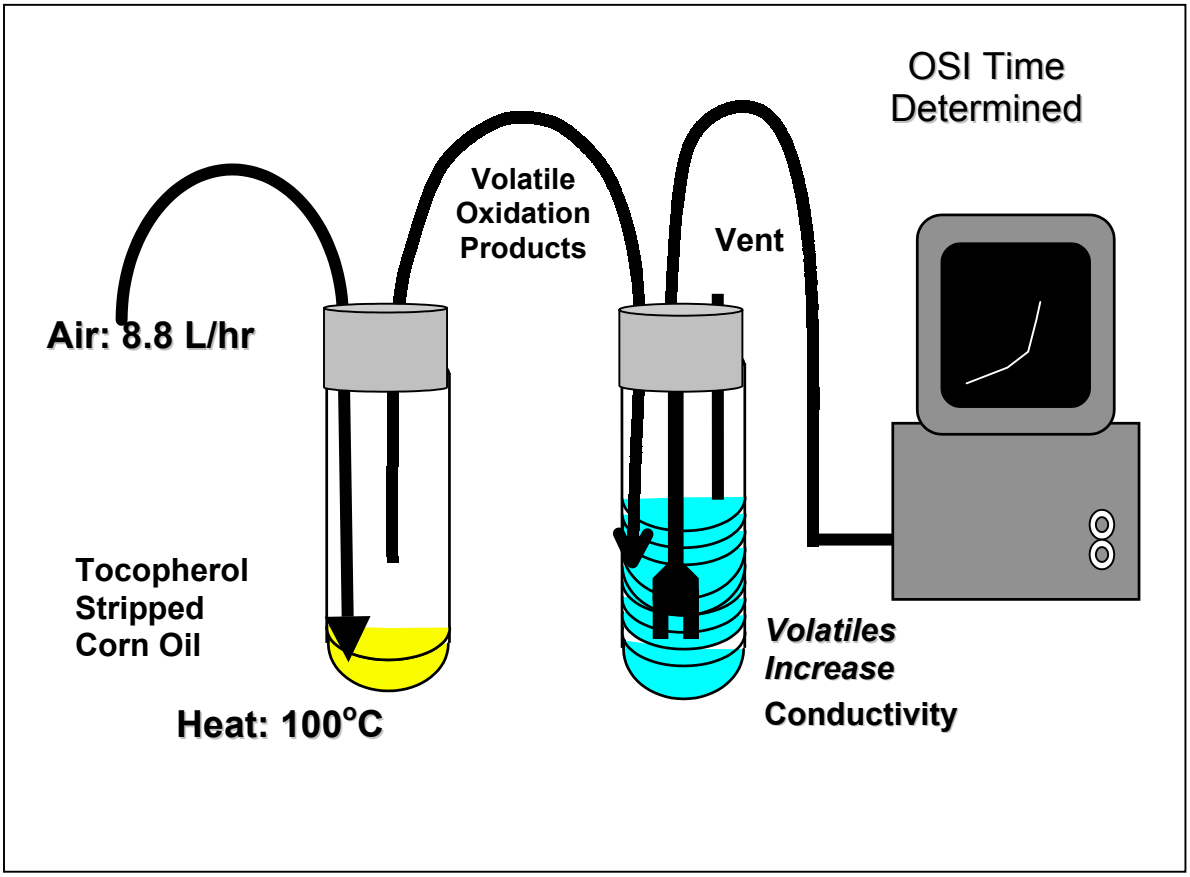


Figure 2. Oxidative Stability Instrument (OSI) Schematic. L/hr: Liters of air per hour. Air is bubbled into heated oil, producing volatile secondary oxidation products that move to second tube where they are trapped in deionized water. A conductivity sensor transmits this information to the OSI machine that calculates the OSI time in hours.

Materials. Tocopherol stripped corn oil (TSCO) was purchased from Orion Chemicals (Pittsburgh, PA). dl-α-Tocopherol was purchased from Roche

Biochemicals (Nutley, NJ). Quercetin monohydrate, rutin hydrate, chrysin, genistein, (+)catechin, (-)epicatechin, (+, -)catechin, and (+,-) taxifolin were purchased from Sigma (St. Louis, MO). Luteolin and 3',4',7 trihydroxyisoflavone (THI) were purchased from Indofine (Somerville, NJ). TSCO was stored under nitrogen at 4°C prior to use. Taxifolin, genistein, and dl-α-tocopherol were maintained at 0°C. All other compounds were kept at room temperature, as recommended by the supplier.

Preparation of Suspensions. Because these flavonoids were dispersed as dry powders into TSCO, it was important to establish a uniform particle size and dispersion. A uniform maximum particle size was assured by sifting all flavonoids through a 40 mesh sieve prior to suspension. Sonication and homogenization were tested as methods to assure uniform particle dispersion. Neither resulted in higher OSI values than stirring alone, so these methods were not used in subsequent experiments. Initially, flavonoids were dissolved in methanol prior to additional to oil. Because flavonoids rapidly fell out of solution after addition to oil and sufficient quantities of alcohol remained in the resulting suspensions, dramatically reducing OSI induction times, this practice was discarded.

Sample preparation. Flavonoids were added to TSCO and stirred for 30 minutes at room temperature on a magnetic stir plate. The resulting suspensions were pipetted into reaction tubes in 5 g quantities.

Reaction Conditions. All experiments were run under identical conditions: temperature was set at 100°C, with dried compressed air supplied at 8.8 L/hour. To reduce the possibility of changes in the rates of oxidation due to changes in the humidity of the incoming air, an in-line air drier was used. As references, TSCO, with and without dl-α-tocopherol, were included in all experiments.

Preliminary evaluations. In order to establish the appropriate concentrations for overall comparisons, the effects of concentration on OSI values were determined for the following flavonoids at the concentrations indicated: quercetin, rutin, (+)-catechin, (-)-epicatechin, chrysin (0, 1, 5, 10, 100 mM); (+,-)-catechin, luteolin, (+,-)-taxifolin (0, 1, 5, 10 mM); THI and genistein (0, 1, 5mM).

BHT (butylated hydroxytoluene) and dl-α-tocopherol were evaluated at all concentrations listed above as reference antioxidants. BHT was included in preliminary evaluations because it is a commonly used lipid antioxidant and dl-α- tocopherol was selected because it is considered the most biologically significant lipophilic antioxidant. The use of BHT and dl-α-tocopherol provided a first approximation of the relative antioxidant activities of the flavonoids used in this study. Each antioxidant was run in triplicate at each concentration.

Comparative assays. Flavonoids displaying the highest antioxidant activities within their respective classes: luteolin (flavones), THI (isoflavones),

(+,-) taxifolin (flavanones), quercetin (flavonols) and (+)catechin (flavanols) were compared on the OSI at 5 mM. This level was chosen because dl-α-tocopherol failed to demonstrate significant increases in antioxidant activity above 6 mM . Additionally, all of the flavonoids tested, with the exclusion of (+) catechin, failed to demonstrate increased antioxidant activity with increased concentration above 10 mM. This experiment was conducted in 3 blocks, with all samples run in triplicate each time.

As a comparison to OSI evaluation, these selected flavonoids at 5 mM concentrations, were assessed for antioxidant activity using peroxide value. Under reaction conditions identical to those for previous experiments, flavonoid suspensions were oxidized using the OSI. Triplicate samples were removed at 6 regularly spaced time intervals, based on the mean OSI value of each flavonoid, to insure that samples were taken close to the induction time. These time periods ranged from 40 to 1680 minutes. Peroxide values were determined using AOCS method Cd 8-53. Peroxide values at each time point were plotted to generate a curve of time versus peroxide values for three replicates of each compound. In order to mimic the algorithm used to determine OSI values, inflection points for these curves were determined mathematically for time versus peroxide value curves using the tangent method. The intersection of two lines, one tangent to the initial flat portion of the curve and the other tangent to the steep portion of the curve was considered an inflection point. These values were termed peroxide induction

(PI) values. PI values, indicative of the onset of rapid primary oxidation product formation, were then used to assess the antioxidant activity of flavonoids. With the exclusion of THI, which was unavailable in sufficient quantities at the time of the experiment, compounds were identical to those used for the 5 mM OSI experiment.

Comparison of catechin isomers. The effects of stereochemical configuration on antioxidant activity were tested in an additional experiment using catechin isomers. Samples of (+)catechin, (-)epicatechin, and (+,-) catechin were run in triplicate at 5 mM in a 3 block experiment. Samples were processed and OSI values were determined as described above.

Statistical Analysis. Data were analyzed using the General Linear Models (GLM) procedure of SAS Institute (Cary, NC). Duncan's Test was used to test for significant differences among treatments. Significance of effect was set at $P \leq 0.05$ for all comparisons. A correlation coefficient was determined for PI versus OSI values.

RESULTS AND DISCUSSION

Screening Assay. A total of 10 different flavonoid suspensions were screened for antioxidant activity during preliminary evaluations. A plot of OSI values for 0-10 mM concentrations of these flavonoids, as well as BHT and dl-α-tocopherol, is shown in **Figure 3.**

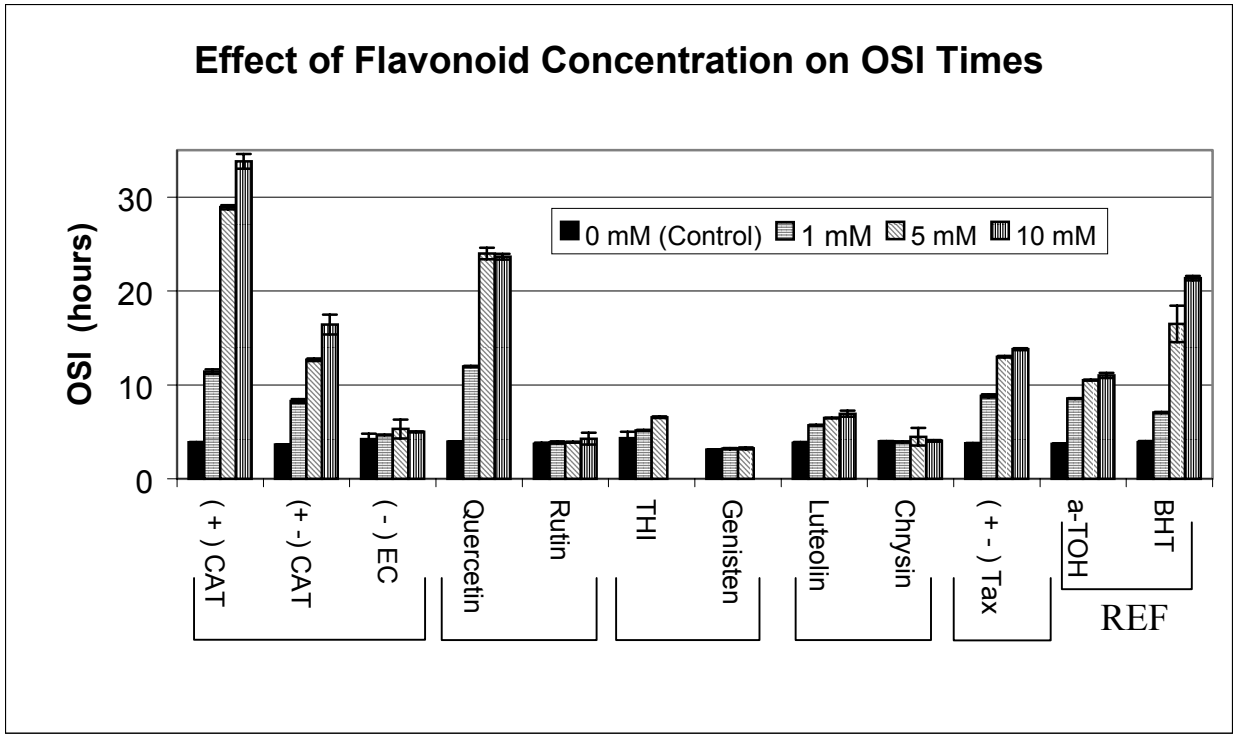


Figure 3. Effect of concentration on antioxidant activity of selected flavonoids by class and reference antioxidants. 0 mM Control: Tocopherol Stripped Corn Oil, no flavonoid or other antioxidant added; 1mM, 5mM and 10 mM indicates molar concentration of flavonoid added; REF: Reference antioxidants

Because epicatechin, (+,-) catechin, rutin, chrysin and genistein demonstrated lower antioxidant activity than other flavonoids in their respective structural classes, (flavanols, flavonols, flavones, and isoflavones) they were not included in the comparative assays.

Concentration versus OSI value curves for flavonoids tested in preliminary evaluations showed sharp initial increases in OSI values, which decreased in slope or plateaued at high concentrations. With the exclusion of (+) catechin, all flavonoids tested failed to demonstrate additional antioxidant activity with increasing concentration above 10 mM (data not shown). This indicated that a maximum effective concentration had been reached.

Comparative Assay. At a concentration of 5 mM, antioxidant activities as determined by the OSI values were as follows: (+) catechin > quercetin > (+,-) taxifolin > dl-α-tocopherol > THI > luteolin**.** These values equate to 245%, 201%, 132%, 100%, 64%, and 61% of dl-α-tocopherol OSI value. Duncan's procedure found all OSI values, except THI and luteolin to be significantly different ($P < 0.05$).

Comparison of Peroxide Induction and OSI Times. The PI values were qualitatively similar to those determined from the 5 mM OSI experiments. All curves based on peroxide determinations showed an induction period typical of lipid oxidation followed by a sharp increase in peroxide values. The fact that OSI values for each compound were greater than PI values reflects the increased time period required to form secondary oxidation products. This is in keeping with classic free radical mechanisms since OSI values measure secondary oxidation product formation while peroxide values measure primary products. There was 98% correlation between the relative values of the OSI and PI for these antioxidant compounds **(Figure 4).**

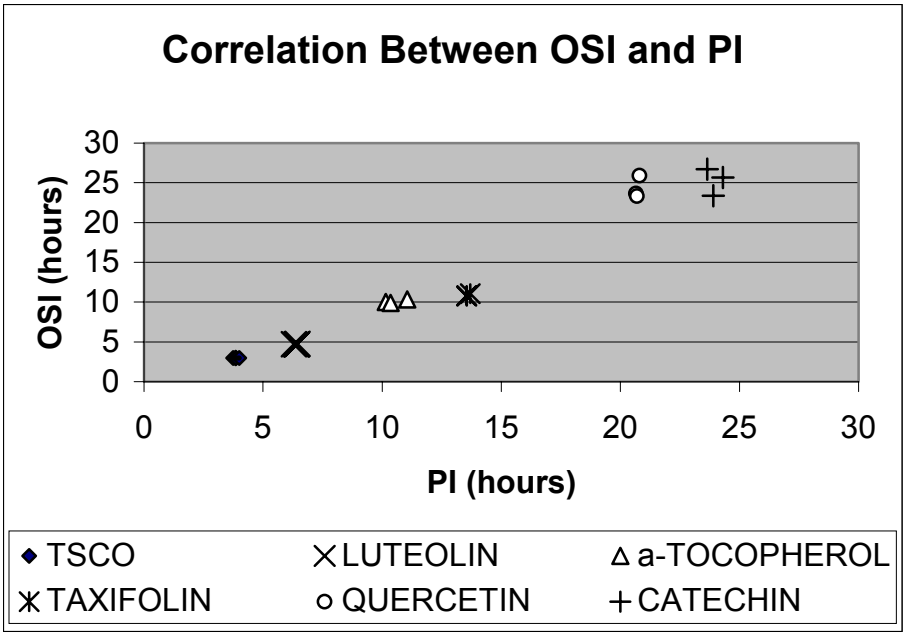


Figure 4. Correlation between Oxidative Stability Index (OSI) and Peroxide Induction (PI) value for compounds at 5mM. TOC= α-tocopherol, TSCO= tocopherol stripped corn oil (control). Triplicate values are shown for each control and test compound.
Correlation Coefficient: 0.98

The average intra-and inter-assay coefficients of variation (CV) for the OSI experiments were 1.43% and 2.73%, respectively.

Due to the significant ($P < 0.05$) differences in OSI values observed at several concentrations between (+)catechin (2R, 3S- pentahydroxyflavan-3-ol) and (-)epicatechin (2S, 3S- pentahydroxyflavan-3-ol) in preliminary evaluations, an additional experiment comparing catechin isomers was conducted in order

to determine the validity of the original findings. At 5 mM concentrations, (-)epicatechin and (+,-) catechin displayed 17% and 58% of the antioxidant activity of (+)catechin **(Figure 5).**

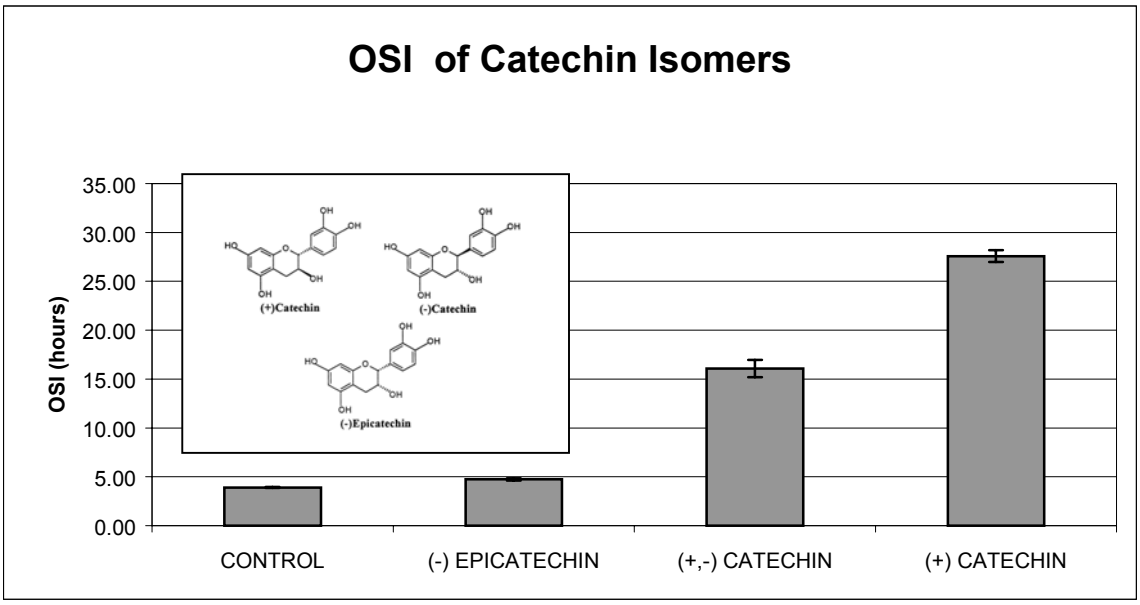


Figure 5. Effects of stereochemical configuration on the antioxidant activity of catechin isomers in tocopherol stripped corn oil.

A number of structure-activity studies have identified moieties important to flavonoid antioxidant function (Heijnen, et al. 2002; Awad et al. 2001; Aurora et al., 1998; Chen, 1996; and Hu, 1995). The number and position of hydroxyl groups is paramount among them. The results of this study support the above assertion with the caveat that factors such as stereochemistry may be important to antioxidant function as well (Guo et al, 1999). Planarity of the flavonoid molecule is considered an important factor for optimal antioxidant activity by producing a more stable phenoxyl radical (Dugas et al. 2000).

Structural planarity can be accomplished by C3 hydroxylation and trans as opposed to cis configurations. These assertions were supported by our data that also show increased antioxidant protection with catechin as compared to its cis epimer, epicatechin . Catechin was shown to be an effective antioxidant in a peanut oil system with an OSI value similar to that measured herein (Chu and Hsu, 1999). We measured similar OSI values for quercetin and dl-α-tocopherol in follow-up studies using tocopherol stripped safflower oil and corn oil (data not shown).

It is apparent from this study and others that the insolubility of flavonoids in oil does not prevent them from acting as antioxidants. Taxifolin, a lipid insoluble flavonoid which occurs naturally in foods such as peanuts and oregano, has been shown to exhibit antioxidant activity in several oil systems (Vekiari, 1993; Pratt and Miller, 1984). In agreement with our findings, α-tocopherol exhibited inferior antioxidant activity as compared to its water soluble analog, Trolox, and other more polar hydrophilic phenolic compounds in a bulk oil system (Schwarz, et al. 1996). The antioxidant quality of α-tocopherol increased when an emulsifier was added to the bulk oil system. These results are in accordance with earlier studies involving the addition of phospholipids to tocopherol enriched oils (Koga and Teroa, 1995). Other lipid-insoluble agents have been shown to inhibit oxidation in oil systems. Lignin was shown to be an effective antioxidant in TSCO under conditions of heating and aeration similar to those used in this study (Catignani and Carter,

1982). Vitamin C has been used in combination with the emulsifier lecithin and α-tocopherol to delay the onset of oxidation in marine oils stored at 80°C (Loliger, 1989).

In previous studies, glycosylation was shown to have a negative effect on the antioxidant activity of flavonoids (Pulido et al., 2000; Dugas et al., 2000; Saija et al., 1995; Das and Pereira, 1992). The inhibitory effects of glycosylation were observed in this study as well. Note the disparate antioxidant activities of rutin (3',4',3,5,7-pentahydroxyflavone-3-rutinoside) and its aglycone quercetin (3',4', 3,5,7-pentahydroxyflavone).

The stability of flavonoids to OSI reaction conditions was of some concern at the outset of this project because the decomposition of flavonoids may have produced volatile products thereby erroneously lowering OSI values. The flavonoids used in this study have been demonstrated to be heat stable to at least 200°C, so that heating alone was not a concern (Budavari, 1989). The combined effects of heating and aeration on volatile flavonoid breakdown product formation are not known. The similarity of OSI and PI values negated this concern because PI values measure peroxides which are non-volatile oxidation products.

The work presented herein demonstrates that the OSI provides a simple, reproducible method for determining the antioxidant properties of various flavonoids. The OSI provides data highly correlated to the peroxide value in a fraction of the time. This methodology could prove useful as a testing

procedure for assessing the antioxidant activity of individual compounds, or combinations of compounds that include flavonoids. Eventually these techniques may serve as useful tools to screen antioxidant compounds in food science or clinical science settings.

ABBREVIATIONS USED: OSI - Oxidative Stability Index ; TSCO- Tocopherol Stripped Corn Oil; THI - 3', 4', 7-trihydroxyisoflavone; PI - peroxide induction value.

ACKNOWLEDGMENT

We would like to thank Dr. Tim Sanders, USDA/ARS Market Quality and Handling Research Unit for the use of the Oxidative Stability Instrument.

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Chapter 6

Quercetin and Catechin Spare Tocopherol in vivo

Joye K. Willcox & George L. Catignani*
Dept. of Food Science
North Carolina State University

*Corresponding Author, North Carolina State University, Box 7624, Raleigh, North Carolina 27695-7624, USA.

Paper No. FSR _____ of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

Abstract

We have investigated the effect of dietary supplementation with flavonoids and polyphenol containing compounds on tocopherol status in vivo. Seventy-two male Sprague-Dawley rats were divided into twelve treatment groups and for seven weeks were fed tocopherol sufficient or deficient AIN93-G diets alone or supplemented with one of five treatment compounds: 0.5% quercetin, 0.5% catechin, 0.5% epicatechin, 1% cocoa powder, or 1% lignin (all on a w/w basis of diet).

In tocopherol sufficient groups, all test compounds significantly increased liver tocopherol as compared to the control. The increase in tocopherol was also seen in plasma and adipose tissue with quercetin, catechin, and epicatechin. In the tocopherol deficient groups, adipose tissue tocopherol levels were significantly increased by all compounds.

Under these experimental conditions quercetin and catechin were sufficient to spare α-tocopherol in plasma, liver, and adipose tissue. Further investigations of the actions of these flavonoids in sparing tocopherol in a more oxidatively stressed animal model are warranted.

Introduction

α-Tocopherol, the most biologically active form of vitamin E, has been shown to scavenge lipid peroxyl radicals [1]. Although most well known for its role as a biological antioxidant, α-tocopherol also has non-antioxidant functions that explain its anti-atherosclerotic and anti-tumor effects and much research has and continues to investigate these functions[2].

Several animal studies[3,4,5] suggest that feeding vitamin E can inhibit the progression of atherosclerotic disease. While human observational studies have indicated positive antioxidant effects, randomized, double blind, placebo-controlled trials have given less consistent but overall positive indication of a protective effect of vitamin E for heart disease.[6, 7, 8].

Epidemiological studies [9],[10] also indicate a positive cardio-protective effect of dietary flavonoids. In vivo studies indicate reduced cardiovascular pathophysiology with the consumption of flavonoids rich foods[11,12] . However, there are few investigations on the effects of individual flavonoids[13].

Since both α-tocopherol and the flavonoids have been associated with a reduction in cardiovascular mortality, it would be beneficial to understand if the two act cooperatively in performing their biological roles. Studies of interaction between flavonoids and tocopherol are limited but significant. In 1990, De Whalley et al. showed that flavonoids reduced the oxidizability of LDL and depletion of α-tocopherol in vitro[14]. Likewise, Terao et al. in 1994, studied the interaction in vitro using egg yolk lecithin liposomes. They concluded that flavonoids are localized near the surface of the phospholipid bilayers where they scavenge aqueous oxygen radicals, thereby preventing the consumption of the lipophilic tocopherol [15]. The antioxidant interaction of catechin, epicatechin, quercetin, rutin, and myricetin has been studied in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems.

Results indicated that catechin and epicatechin spared α–tocopherol with a synergistic effect in delaying Fe^{2+} induced lipid oxidation[16].

Very few in vivo studies have investigated the combined effects of tocopherol and flavonoids. Nanjo et al. studied the effects of dietary tea catechins on the levels of alpha tocopherol and lipid peroxidation in both plasma and erythrocytes. The flavonoid prevented a decrease in plasma and erythrocyte α-tocopherol levels and suppressed lipid peroxidation [17]. The influence of dietary flavonoids on α-tocopherol status and LDL peroxidation in rats fed diets enriched with either polyunsaturated fatty acids (PUFA) or monounsaturated fatty acids (MUFA) was investigated in a 1998 series of studies. Plasma from rats fed PUFA diets supplemented with quercetin and catechin (2:1) at 0.8% (w/w)/kg diet had significantly decreased production of conjugated dienes during 12 hours of ex vivo oxidation. These studies concluded that this combination of quercetin and catechin is beneficial in diets rich in PUFA and MUFA because they contribute to antioxidant defense and reduce the consumption of α-tocopherol in membranes and lipoproteins[18].

A preliminary animal study (unpublished data) conducted in our lab investigated a possible interaction between alpha tocopherol and the flavonoid quercetin using the rat model. A non-significant increase in plasma and liver tocopherol coupled with a non-significant decrease in plasma malondialdehyde in animals fed both antioxidants, compared to feeding tocopherol alone, indicated that the flavonoid may have a positive effect on tocopherol's antioxidant action. One significant finding was the increase in plasma retinol used as a marker of oxidation status.

Other compounds containing phenolic groups as possible antioxidants are also being investigated. Rat model studies completed in our lab as a follow up to earlier in vitro studies[19] indicated that a fibrous compound, lignin, significantly increased plasma tocopherol[20]. More recently, researchers in Slovakia have shown that lignin, due to its antioxidant nature, provided a

protective effect on hamster and human cells exposed in vitro to H_2O_2 treatment[21]. Cocoa powder, rich in catechins, procyanidins and other polyphenols[22,23], has been shown to reduce parameters indicative of oxidation susceptibility in vitro [24] and in vivo [25,26].

The current project was designed to test the hypothesis that, in vivo, α-tocopherol is spared by dietary supplementation with three specific flavonoids, quercetin, catechin, and epicatechin, as well as a food source of flavonoids, cocoa, and the non absorbable polyphenolic compound, lignin.

Materials and Methods

Animals. Seventy-two weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, N.C.). They were fed a tocopherol sufficient or deficient control diet for one day while acclimating, then divided by equal total weights into twelve treatment groups for a seven week feeding trial. The protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee and complied in all respects to the *Guide for the Care and Use of Laboratory Animals* [27].

The study was conducted at the LAR (Laboratory Animal Research) facility at North Carolina State University School of Veterinary Medicine in Raleigh, North Carolina. The animals were housed in separate cages in a temperature and light (12:12) controlled room. Food and water were provided ad libitum. Initial and weekly body weights were taken until sacrifice with food intake measured daily. Fasted animals were sacrificed using CO_2 and exsanguination via cardiac puncture. Plasma, liver, and adipose samples were flash frozen in liquid nitrogen and held at –70 C in cryogenic vials until processed.

Diets. A basal vitamin E deficient diet, (Test Diet 01259), with oil and sugar excluded, was purchased from Harlan Teklad, Madison, WI. This diet was

otherwise comparable to AIN-93G [28] with the exception of vitamin A, which was added at 25150 IU/kg (220 μg/g) diet, vitamin D, added at 2800 IU/kg diet, and the exclusion of antioxidant (TBHQ). The fat source, later added to the diet, was tocopherol-stripped soybean oil, increased from the usual 7% (16.7% fat calories) to 11.05% (25% fat calories) fat. This was purchased from DYETS Inc., Bethlehem, PA. α-Tocopherol, purchased from Sigma, St. Louis, MO, was used instead of α-tocopheryl acetate to also more closely resemble the human diet. The α-tocopherol was mixed thoroughly into oil and then incorporated slowly into the basal diet mix. Similarly, added flavonoids, cocoa powder, and lignin were mixed into the sugar component of the diet. Diets were mixed under a chemical hood under reduced light conditions using a Hobart rotary mixer to insure proper dispersion of all additives into the basal mix. Twelve different treatment diets were mixed and stored at –20C until fed as listed in Table 1.

Alpha-tocopherol 75 IU/kg	**Control**	**Quercetin**	**Catechin**	**Epicatechin**	**Cocoa**	**Lignin**
	0.0	0.5% (w/w)	0.5% (w/w)	0.5% (w/w)	1% (w/w)	1% (w/w)
+	TO n=6	*TQ n=6*	TCa n=6	TEp n=5*	TCo n=6	TLi n=6
–	OO n=6	OQ n=6	OCa n=6	OEp n=6	OCo n=6	OLi n=6

* one animal died of a congenital defect at week three.

Table 1. Experimental design for addition of antioxidants to tocopherol sufficient and deficient AIN93G diets.

Analytical Procedures

Analysis of plasma tocopherol

Plasma tocopherol concentrations were determined using a modification of the HPLC method by Catignani and Bieri, 1983 [29]. Samples were concentrated for increased detection at lower tocopherol levels. An internal standard was used to calculate recovery. Briefly, 100 □L plasma and 50 □L of the internal standard tocopheryl acetate are vortexed intermittently and vigorously for 45 seconds. 200 □L hexane is added and vortexed for 45 seconds, then centrifuged at 800 g for 5 minutes. 150mL of the hexane layer is transferred to a disposable glass tube and evaporated under nitrogen at 30 C. The residue is dissolved in 10 □L methylene chloride, swirled to mix, then 40 □L MeOH is added and swirled gently to mix. The entire sample is injected onto the HPLC column (Phenomenex C18, 5 micron, 150 x 4.6 mm) using a Waters injector ,510 pump, and 996 photodiode array detector. The flow rate was set at 2.5 mL/minute with wavelength set at 292 nm. Output was processed with Millenium (v 2.10) software, Waters Inc.

Analysis of adipose tissue tocopherol

Supra scapular adipose tissue samples were prepared following a modification of the sample preparation method of Casal et al. 2001[30] with HPLC conditions of Barbas et al. 1998[31]. Briefly, approximately 50 mg sample is weighed into disposable glass tubes. 200 □L ethanol with 10 mg/mL ascorbic acid and 200 □L saturated NaCl solution is added and homogenized using a PRO Scientific PRO 200 homogenizer. 100 mL tocopheryl acetate (50 □g/mL) is added as the internal standard and vortexed. N-hexane (500 □L) with 2% 2-propanol and 0.01% TBHQ is added, vortexed and homogenized for 30 seconds. The sample is then centrifuged at 6000g for 5 minutes. 400 □L of the hexane layer is transferred to an amber vial. This extraction is repeated twice, pooling hexane extracts. The extracted samples are then dried under

nitrogen at 37 C with the residue dissolved in 100 □L chloroform, vortexed, and then further dissolved by adding 100 □L methanol with subsequent mixing by vortex. 50 □L followed by a 10 □L methanol flush are injected onto the Phenomenex C18 column, maintained at 40 C. HPLC conditions using the Waters 510 pump were 96.5% methanol , flow rate 2 mL/minute with linear detection at 292 nm and 0.1 AU scale using the UVIS 203 UV detector by Scientific Instruments, Inc. All procedures are performed under yellow light conditions. Peak area ratio of tocopherol/internal standard are used to calculate mg tocopherol/gm adipose tissue.

Analysis of Liver Tocopherol

Liver α-tocopherol was measured following the conditions described above for adipose tissue (Casal et al 2001, Barbas et al 1998). However, because of the differences in the expected concentrations, a sample volume of 50□L and detector sensitivity of 0.00001 AU were used.

Data analysis

Flavonoid compound and tocopherol amount were treated as fixed effects in the (6 x 2) crossed and randomized design. Since plots of the residuals versus the fitted values indicated that variability in concentration increased with the mean, variance-stabilizing log-transformations were used for the analysis of variance, carried out using the SAS statistical software package (SAS Institute, Cary, NC 2002). A post-hoc Dunnett's multiple comparison procedure was performed when flavonoid effect was significant.

Results

Physical and dietary data. There were no significant differences ($P<0.05$) observed among groups in body weight gain (338.86 $\pm$ 24.4 g), liver weight (14.33$\pm$ .38 g), liver weight as percentage of body weight (4.23 $\pm$ 0.14%), or dietary intake (16.2 $\pm$ 0.2 g/day).

Plasma tocopherol. Dietary supplementation with 0.5% quercetin and 0.5% catechin significantly increased plasma □-tocopherol in rats receiving a tocopherol sufficient diet (Table 2). There were no significant differences with 0.5% epicatechin, 1% cocoa powder, or 1% lignin in the normal tocopherol diet groups and no differences in any group receiving the tocopherol deficient diet (data not shown). Analysis of variance on logarithmically transformed data revealed a significant effect of flavonoid and a tocopherol/flavonoid interaction. (Table 2).

Liver tocopherol analysis. Dietary supplementation with 0.5% quercetin and catechin also increased liver concentrations of □-tocopherol, with a less significant effect seen with the addition of epicatechin, cocoa powder, or lignin. No significant effects were seen in supplementing the tocopherol deficient diet groups (data not shown). Analysis of variance on logarithmically transformed data revealed a significant effect of flavonoid and a tocopherol/flavonoid interaction. (Table 2).

Adipose tocopherol analysis. Quercetin (0.5% w/w), catechin (0.5% w/w), and epicatechin (0.5% w/w) significantly increased storage of vitamin E in supra-scapular adipose tissue stores of animals receiving the normal

tocopherol diets, while cocoa powder and lignin were non-significantly increased. No significant differences were measured in the tocopherol deficient diet groups (data not shown). Analysis of variance on logarithmically transformed data revealed a significant effect of tocopherol and flavonoid but the tocopherol/flavonoid interaction was not significant. (Table 2).

Treatment Group	Plasma mg/L	Total Liver μg	Adipose μg/gm
TO	6.62 ± 0.35 a	116.92 ± 15.60 a	32.24 ± 12.42 a
TQ	10.55 ± 0.64 b	266.80 ± 17.29 b	71.41 ± 32.54 b
TCa	10.94 ± 0.63 b	297.64 ± 21.22 b	78.48 ± 14.28 b
TEp	6.92 ± 1.15 a, b	196.55 ± 17.57 c	61.45 ± 8.60 b
TCo	8.66 ± 0.30 a, b	213.20 ± 25.00 c	49.88 ± 12.40 a, b
TLi	8.21 ± 0.73 a, b	201.24 ± 13.74 c	55.70 ± 28.82 a, b
Flavonoid	P = .004	P = 0.04	P = < 0.001
Vit E/ Flavonoid Interaction	P = 0.01	P = 0.02	NS

Table 2. Summary of plasma, liver, and adipose α-tocopherol.

Values in vitamin E sufficient treatment groups with phenolic antioxidant supplementation expressed as % of diet on a w/w basis: TO- Control (no flavonoids added), TQ- 0.5% quercetin , TCa- 0.5% catechin, TEp- 0.5% epicatechin, TCo- 1% cocoa powder, TLi- 1% lignin. Values are means ± S.E.M. Bottom rows summarize ANOVA analysis results for effect of flavonoids and α-tocopherol/flavonoid interaction. NS non significant at P < 0.05

Discussion

The objective of this project was to determine if the flavonoids, quercetin, catechin or epicatechin spare α-tocopherol in vivo. Alpha tocopherol is focused in this study because this vitamin is considered to be the most important inhibitor of the free radical chain reaction of lipid peroxidation in animals[32] . In addition to working as an antioxidant, α-tocopherol has many

functions related to cardioprotection including: inhibition of vascular smooth muscle cell proliferation[33,34], inhibition of protein kinase C activity[35] and regulation of phosphorylation cascades[36], up-regulation of α-tropomyosin gene expression[37], reduction of age-related increase in collagenase expression[38], downregulation of LDL scavenger receptors CD36 and SR-A[39], down-regulation of adhesion molecule expression[40], inhibition of platelet aggregation[41] and inhibition of intra-arterial thrombus formation[42].

We measured tocopherol in the plasma, liver, and supra-scapular white adipose tissue (WAT). Approximately 90% of the body's tocopherol pool is in WAT, stored in the lipid droplets in adipocytes[43]. It has been proposed that WAT tocopherol is a better indication of long term vitamin status than plasma tocopherol [44], but various locations of WAT show different affinities for long time storage of the vitamin. We chose to measure tocopherol in WAT taken from the back based on a previous study that reported it to be a site that accumulates tocopherol rapidly[45].

Quercetin is the most highly consumed flavonoid world wide, estimated to be 15 mg/day. Because catechin and epicatechin are the major flavonoids found in teas and cocoa and are consumed worldwide, they were selected for inclusion in the study. Both of these flavonoids have been shown to have antioxidant potential in vitro. However, there is very little information on in vivo activity. Lignin and cocoa powder were also selected for study due to their polyphenolic structure and potential as antioxidants[46 47].

Our results show that two flavonoids, quercetin and catechin, when fed at 0.5% (w/w) of diet, have a significant sparing effect on a-tocopherol in the plasma, liver, and adipose tissue. Epicatechin significantly increased tocopherol in adipose tissue. This interaction may be explained in at least two ways. First, the flavonoid may recycle the tocopheryl radical back to its reduced active state as reported for quercetin in in vitro micellar[48] and LDL studies[49].

TOH + LOO· → LOOH + TOC·

TOC· + FLAV-OH → TOH + FLAV·

Secondly, it may be due to the flavonoid's ability to function as an antioxidant at the cell membrane interface thus sparing the oxidation of α-tocopherol[50].

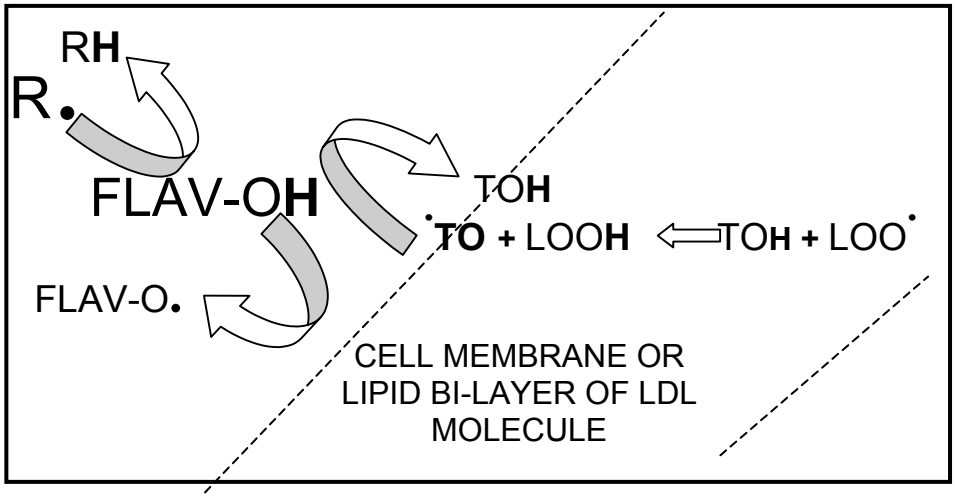

Figure 1. Proposed scenario of flavonoid sparing tocopherol at lipid/aqueous interface.

α-Tocopherol is present in the LDL molecule and cell membranes where it prevents oxidation of PUFAs by readily reducing the free radicals that are present[51]. The antioxidant function however depends on the ability of its radical to be reduced back to tocopherol by other antioxidants that are present, such as vitamin C[52], bilirubin[53], ubiquinol-10[54]. Since flavonoids are chemically suited to reside at the interface of hydrophobic and hydrophilic regions, they would theoretically be in an optimal strategic position to spare tocopherol[55].

The sparing of tocopherol by these flavonoids in vivo is a significant finding. Dietary intake of α-tocopherol is estimated to be 8 mg/day. If the sparing of tocopherol by quercetin and catechin can be confirmed in humans, recommendations may be warranted for co-consumption of food such as almonds (α-tocopherol source) and tea (flavonoid source). Dual supplementation of α-tocopherol and flavonoid for individuals at increased oxidative stress, for example, those with diabetes or chronic renal failure, or distance runners, may be more effective.

Another interesting point is the difference in sparing of the tocopherol in plasma and liver by catechin and epicatechin. Our in vivo results indicate that catechin spares tocopherol significantly better than epicatechin in the liver. If we presume that the protection of tocopherol improves antioxidant capacity, this is in contrast to in vitro findings reported earlier by others that found epicatechin to have superior antioxidant function over catechin and quercetin in cell membrane and liposome systems. In those experiments, it was concluded that oil/water partition coefficients play an important role in determining their antioxidant activities both individually and through interactive association with α-tocopherol[56]. However, antioxidant quality is dependent on the ability of the compound to form a stable radical that is not prone to become a pro-oxidant. Our data support the concept that planar molecules form more stable radicals. The cis configuration of epicatechin may allow it to intercalate slightly into the lipid bilayer[57] , but a more stable phenoxyl radical is formed with the trans catechin structure. This also accentuates the point that in vitro antioxidant studies may not adequately represent what occurs in vivo.

Currently, flavonoid preparations, including quercetin, catechin, and epicatechin, are being sold as nutrition supplements. The efficacy, safety, or interactions with other nutrients, such as vitamin E or vitamin A have not been

adequately studied. This project is a first step in establishing that an interaction exists and can be measured in the rat model.

In summary, quercetin and catechin, when fed at 0.5% w/w of diet spares tocopherol in multiple tissues. Once interactions between various antioxidants are understood and an optimal level of each or a ratio of multiple antioxidants is established, a clinical trial will be needed to confirm the results in humans. Only then can definitive recommendations be made to the consumer regarding optimal levels or ratios of antioxidant intake.

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Chapter 7

Dietary Flavonoids Fail to Suppress F_2-Isoprostane Formation In Vivo

Willcox, Joye K.[a], Catignani, George L.[a], and Roberts, L. Jackson, 2nd[b]

[a]Dept of Food Science, North Carolina State University, Raleigh, N.C. 27695-7624
[b]Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6602

This publication was supported partially by funds from the Institute of Nutrition Research Grant Program of the University of North Carolina system, grant #30401 and NIH grants GM42056, GM15431, and DK26657 .

Correspondence: George L. Catignani, Box 7624, North Carolina State University, Raleigh, NC 27695-7624. e-mail: George_Catignani@ncsu.edu

Paper No. FSR 02-26 of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

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Abstract:

Dietary antioxidants, including α-tocopherol (α-TOH) and polyphenolic flavonoid compounds have been the subject of much research interest, but few studies have investigated interactions between these two antioxidants in vivo. We have conducted a feeding study to determine if supplementation with dietary flavonoids or polyphenol containing compounds will provide antioxidant protection in tocopherol deficient animals or exceed the antioxidant protection provided by α-TOH alone, using the sensitive and specific measure of lipid peroxidation, F_2-isoprostanes.

Seventy-two male Sprague Dawley rats were divided into twelve treatment groups to receive either α-TOH sufficient or deficient AIN93-G diet supplemented with one of five compounds: 0.5% quercetin, catechin, or epicatechin; or 1% cocoa powder or lignin. The fat source was polyunsaturated oil, increased from 7% to 11.05% (w/w with diet) to maximize lipid peroxidation while staying within a physiological range. After seven weeks of treatment, animals were sacrificed with plasma and hearts analyzed to determine differences in F_2-isoprostane levels.

None of the treatment compounds significantly decreased plasma or heart F2-isoprostanes compared to the control beyond the significant protection displayed by α-tocopherol. We conclude that under these experimental conditions, quercetin, catechin, and epicatechin do not suppress lipid peroxidation in vivo.

Key words: Antioxidants, lipid peroxidation, in vivo

Introduction

Lipid peroxidation is a degradative process that causes functional abnormalities and pathological changes leading to and exacerbating a myriad of chronic diseases. Oxidation is a natural component of the metabolic chain through which energy nutrients are converted into ATP. However, many physiological and environmental factors may cause overproduction of reactive oxygen species leading to levels of oxidation that cannot be ameliorated by endogenous antioxidants. Therefore, dietary intake of antioxidant nutrients becomes an important factor in controlling oxidative stress, lipid, protein, carbohydrate and nucleotide oxidation and the pathologies that ensue.

Much research in recent years has focused on methods to measure oxidative stress and identify specific nutrients that provide antioxidant protection in vivo. In vitro assays provide insight into the general chemical nature of antioxidant protection, but do not always provide answers that translate to the biological environment where oxidative damage is occurring.

The measurement of oxidative end products provides a meaningful estimation of the degree of oxidative stress. The majority of oxidation studies reported to date have employed measurements of lipid hydroperoxides, conjugated dienes, or malondialdehyde as TBARS (thiobarbituric acid reactive substances) to assess antioxidant quality. These methods have shortcomings in terms of specificity and sensitivity [1].

Antioxidant quality is also often reported as differences in levels of residual antioxidants and accumulation of oxidation products after a biological sample has been exposed to ex vivo oxidation by exposure to metal ions or lipid and water-soluble radical generators. This method may show treatment differences, but again, the environment of oxidation

does not necessarily mimic the biological environment of interest or the specific type of oxidation likely to occur in that particular tissue [2].

Much evidence indicates the quantification of F_2-isoprostanes provides a reliable and useful approach to assess oxidative stress and lipid peroxidation in vivo. F_2-Isoprostanes are specific end products of arachidonic acid peroxidation, formed in situ on phospholipids and then released in free form by phospholipases. Levels of F_2-isoprostanes increase dramatically in various animal models of free radical injury and correlated with measures of tissue damage. F_2-Isoprostanes have been shown to increase in several human disorders associated with enhanced oxidative stress [3] and with aging [4].

There is increasing evidence that flavonoids, vitamin E, and other antioxidant nutrients play a role in disease prevention. Many health benefits have been suggested for these antioxidants through epidemiological studies [5,6,7], in vitro assays[8], and a limited number of in vivo studies[9]. Cardiovascular disease [10], certain cancers [11], diabetes [12, 13], Alzheimer's disease [14], arthritis [15], and premature aging [16] are some of the more important disease processes associated with oxidative damage.

Other compounds having polyphenolic structures have also shown potential as biological antioxidants. Cocoa powder has been shown to reduce parameters of oxidation susceptibility in vitro [17] and in vivo [18]. Cocoa is rich in catechins, procyanidins and other polyphenols, the total polyphenol content estimated to be 6-8% by dry weight of the dry bean [19,20]. Lignin, a fibrous polyphenolic rich compound present in all plants, has also shown antioxidant potential in cell cultures treated with oxidants, as measured by DNA strand breaks [21].

There is general consensus that more in vivo research is needed in the area of antioxidant interactions [22]. Few in vivo studies have reported the antioxidant effects of individual flavonoids [23, 24, 25, 26] and none reported to date have investigated effects relative to vitamin E

status. The current project was designed to determine if dietary flavonoids interact with α-tocopherol in vivo to reduce parameters of oxidative status.

Materials and Methods

Animals. Seventy-two male weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, N.C.). They were fed the control diet for one day while acclimating, then divided by equal total weights into twelve treatment groups for a seven week feeding trial. The protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee and complied in all respects to the *Guide for the Care and Use of Laboratory Animals* [27].

The study was conducted at the LAR (Laboratory Animal Research) facility at North Carolina State University School of Veterinary Medicine in Raleigh, North Carolina. The animals were housed in separate cages in a temperature and light (12:12) controlled room. Food and water were provided ad libitum. Initial and weekly body weights were taken until sacrifice with food intake measured daily. Fasted animals were sacrificed using CO_2 and exsanguination via cardiac puncture. Plasma samples (pooled 3 rats per sample) and hearts were flash frozen in liquid nitrogen and held at –70 C in cryogenic vials until processed.

Diets. A basal vitamin E deficient diet, with oil and sugar excluded , was purchased from Harlan Teklad, Madison, WI. This diet was otherwise comparable to AIN-93G with the exception of vitamin A, which was added at 25150 IU/kg (220 □g/g) diet and vitamin D, added at 2800 IU/kg diet. The fat source, later added to the diet, was tocopherol stripped soybean oil, increased from the normal 7% (16.7% fat calories) to 11.05%

w/w (25% fat calories) to maximize oxidation potential while more closely resembling the percentage fat of the human diet. This was purchased from DYETS Inc., Bethlehem, PA. α-Tocopherol from Sigma, St. Louis, MO, was used instead of α-tocopheryl acetate to also more closely resemble the human diet. The α-tocopherol was mixed thoroughly into oil and then incorporated slowly into the diet mix. Similarly, added flavonoids, cocoa powder, and lignin were mixed into the sugar component of the diet. Quercetin dihydrate was purchased from Alfa Aesar, Ward Hill, MA, (+)-catechin and (-)-epicatechin from Sigma. Cocoa powder was a gift from Mars Inc., Hackettstown, N.J. and lignin (Indulin AT) a gift from Westvaco, Charleston, S.C. Diets were mixed under a chemical hood using a Hobart rotary mixer to insure proper dispersion of all additives to the basal mix. Twelve treatment diets were mixed as listed in Table 1. and stored at –20C until fed.

	Control	Quercetin	Catechin	Epicatechin	Cocoa	Lignin
75 IU/kg α-tocopherol	0.0	0.5% (w/w)	0.5% (w/w)	0.5% (w/w)	1% (w/w)	1% (w/w)
+	TO n=6	TQ n=6	TCa n=6	TEp n=5*	TCo n=6	TLi n=6
-	OO n=6	OQ n=6	OCa n=6	OEp n=6	OCo n=6	OLi n=6

Table 1. Addition of antioxidants to AIN93G diet. n=number of animals per group, *one animal died of a congenital defect.

Analytical Procedures

Analysis of plasma and heart F_2 Isoprostanes

Free and esterified F_2-Isoprostanes in plasma and heart samples were measured using GC-MS. Briefly, phospholipids were extracted from the tissue sample and subjected to alkaline hydrolysis. After adding a deuterated standard ([$^2H_4$] 15-F_{2t}-IsoP), the resulting free F_2-isoprostanes were then purified using C18 followed by silica solid phase extractions. The F_2-isoprostanes were then converted to pentafluorobenzyl esters and

then subjected to thin layer chromatography and then converted to trimethylsilyl ether derivatives. Samples were analyzed using a negative ion chemical ionization GC/MS [28].

Data analysis

Significance of treatment difference and interaction were determined according to the General Linear Models (GLM) using the statistical analysis system (SAS rel. 8.01 SAS Institute, Cary, NC., USA). Data were logarithmically transformed prior to analysis to stabilize the variance.

Results

Body weight gain, liver weight, dietary intake.

There were no significant differences ($P<0.05$) observed among groups in body weight gain (338.86 $\pm$ 24.4 g), liver weight (14.33$\pm$ .38 g), liver weight as percentage of body weight (4.23 $\pm$ 0.14%), or dietary intake (16.2 $\pm$ 0.2 g/day).

Isoprostane analysis of plasma and hearts.

In agreement with earlier studies [29,30] , plasma F_2-Isoprostanes were significantly decreased in all animals receiving tocopherol sufficient diets, as compared to those receiving tocopherol deficient diets. However, supplementation with flavonoid compounds did not significantly change F_2-Isoprostane concentrations in plasma or heart samples when compared to the control diets in both tocopherol deficient and sufficient diets (Table 2).

	Plasma (pmol/L)		**Heart** (pmol/g)	
Diet Treatment	**-TOH**	**+ TOH**	**-TOH**	**+ TOH**
Control	299±40 *a,1*	189±28 *b,2*	4.15±0.08 *a,1*	3.74±0.86 *b,1*
0.5% quercetin	253±22 *a,1*	175±4 *b,2*	6.23±1.12 *a,1*	3.86±0.56 *b,2*
0.5% catechin	264±18 *a,1*	178±0 *b,2*	5.01±0.02 *a,1*	3.98±1.40 *b,1*
0.5% epicatechin	320±54 *a,1*	164±8 *b,2*	3.61±1.20 *a,1*	3.64±1.00 *b,1*
1% cocoa powder	281±10 *a,1*	205±10 *b,2*	6.05±0.78 *a,1*	2.99±0.16 *b,2*
1% lignin	332± 6 *a,1*	195±8 *b,2*	4.25±0.66 *a,1*	4.36±0.10 *b,1*

Table 2 Effect of flavonoid compounds on F_2-Isoprostanes in plasma and hearts.
-TOH Tocopherol deficient diets, + TOH Tocopherol sufficient diets. Values are means ± SD. Similar sub-script letters in columns and numbers in rows indicate non-significant treatment differences compared to the controls in each tissue ($P<0.05$).

Analysis of variance (Table 3.) on logarithmically transformed data did not reveal a significant effect of flavonoid or a tocopherol/flavonoid interaction in the plasma or hearts.

	P-values		
	Tocopherol	Flavonoids	Interaction
Plasma F_2-Isoprostanes	< 0.0001	ns	ns
Heart F_2-Isoprostanes	<0.01	ns	ns

Table 3. ANOVA Summary of Results ns= non-significant at $P<0.05$.

Discussion

The primary goal of this project was to determine if an interactive relationship exists between α-tocopherol and the flavonoids, quercetin, catechin and epicatechin. Currently, flavonoid preparations, including quercetin and catechin, and epicatechin are sold as nutrition supplements. The efficacy, safety, or interactions with other nutrients, such as vitamin E have not been adequately studied. This study is a first step in establishing whether an interaction exists and can be measured in the rat model. Quercetin is the most highly consumed flavonoid world wide, estimated to be 16 gm/day [31]. Because catechin and epicatechin are major flavonoids found in teas and cocoa and are also highly consumed worldwide, they were selected for inclusion in the study. Each

of these flavonoids have been studied and shown to have antioxidant properties in vitro [32] but there is very limited in vivo information on their effects [33,34,35]. Lignin and cocoa powder were also selected for study due to their polyphenol content and potential as antioxidants [21, 18].

Our data show an expected significant decrease in plasma and heart F_2-isoprostanes in animals fed a tocopherol sufficient diet compared to those on a tocopherol deficient diet. In contrast, no significant decrease in F_2-isoprostanes was seen due to dietary supplementation with flavonoids (Table 3). Although some studies have shown decreased production of F_2-isoprostanes with flavonoid addition in vitro [36], their effect on levels of F_2-isoprostanes in vivo has not yet been determined [37, 38, 39], except a short lived postprandial effect [40]. The supplementation level selected for this project was an average of levels used in previous studies cited in the literature [9, 24, 41].

Many antioxidant studies that have shown a decrease in oxidation markers were conducted on animals or humans already oxidatively stressed. The flavonoids' antioxidant potential may not have been measurable at the oxidative stress level attained in this study. Although the fat source for this study was highly polyunsaturated and total fat content increased to promote lipid peroxidation [42,43], the oxidation level attained may have been insufficient to measure treatment differences. No studies reported to date have addressed the issue of defining study conditions for animals that would represent baseline or chronic disease levels of oxidation in humans.

In summary, supplementation with α-tocopherol did improve oxidative stress status in α-tocopherol deficient animals as confirmed by significant decreases in F_2-isoprostanes in the heart and plasma, but flavonoids, at the levels fed in this study did not improve oxidative status. More studies are needed, using different concentrations,

combinations, and forms of flavonoids, as well as providing a variety of stress levels and conditions.

Acknowledgment- This publication was supported partially by funds from the Institute of Nutrition Research Grant Program of the University of North Carolina system, grant #30401 and NIH grants GM42056, GM15431, and DK26657 .

Abbreviations: ATP adenosine triphosphate.

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Discussion and Conclusions

As reviewed in this thesis, oxidative degradation of lipids, protein, nucleotides, and carbohydrates have been linked to all of the chronic diseases that account for a majority of the morbidity and mortality of the western human population. Much research has been reported since the discovery of this link. There is confusion in the public sector regarding efficacy of dietary supplementation with antioxidant compounds due to the release of information from preliminary reports and less than adequately controlled studies. To intensify the problem further, well designed studies investigating specific groups of people for varying study periods have produced conflicting results. This is to be expected since antioxidants and all other nutrients may affect groups of people with different characteristics, e.g. age, adiposity, activity level, smoking status, etc. in different ways. The study period is also critical because antioxidants may present differing effects in the short term and long term. There may be a critical period of development in which the level of antioxidant may be pivotal in initiating or exacerbating a pathological change. Clinical presentation of oxidative damage of some tissues may not be measurable for several decades.

This research project began with a desire to understand how a lipophilic antioxidant, vitamin E, compares to and reacts with a less lipophilic flavonoid compound, quercetin. This was prompted by earlier

work in our lab conducted by Keith Harris and reported in Chapter 5. His data showed that dietary flavonoids, catechin and quercetin, were significantly better antioxidants than tocopherol in an accelerated oxidation lipid system. His data also showed that subtle stereochemical differences made profound differences in antioxidant effect as seen with (+)catechins, (+,-) catechins, and (-)epicatechin. Stearic hindrance was suggested to account for the differences in antioxidant effect by producing a more stable aroxyl radical. I began by my research project by evaluating quercetin and tocopherol under the same conditions using the OSI. I confirmed his data at each level tested. Since that time, a review of literature has revealed contrasting findings when comparing these two antioxidants.

Tocopherol has been shown in a methyl linoleate solution to be a better lipid peroxyl radical scavenger than quercetin[1]. However, quercetin has been found to be superior to α-tocopherol in a liposomal system exposed to AAPH which generates water soluble radicals[2]. This implied that localization of antioxidants and source of radical generation determines the effectiveness of antioxidants in a mixed lipid/aqueous environment.

Our results show a superior in vitro performance by quercetin in a corn oil system and an actual decrease in the antioxidant effect when tocopherol is present. Our presumption is that this decrease is due to hydrogen bonding between the hydroxyl groups of the chromanol ring of

tocopherol and the flavonoid. From the standpoint of physical attributes, the methyl linoleate model would be more representative of free fatty acids whereas the corn oil system, consisting of more triglycerides, would be more representative of the lipid bilayer.

Liposomes have a phopholipid bilayer structure similar to that of biological membranes. The phosphotidylcholine liposome models may be a good model of the interface of the cell membrane or lipid bilayer of LDL. At a pH of 5.4 which could be physiological in areas of anaerobic glycolysis such as in atherosclerotic lesions, quercetin has outperformed α-tocopherol at a concentration of 10^{-2} mol/mol phospholipid. As pH was increased to 7.2, however, α-tocopherol showed more protection against oxidation.

The fact that quercetin is a stronger radical scavenger than tocopherol in liposomes in contrast to the methyl linoleate solution is consistent with the proposal that quercetin is located near the surface of the phospholipids bilayer, whereas a-tocopherol resides further into the bilayer [2].

Some studies have reported that the interaction of antioxidants leads to enhancement or under other conditions a weakening of their individual actions. For example, L-cysteine was reported to have a synergistic effect with alpha tocopherol when mixed in soybean phosphotidylcholine liposomes. The addition of cysteine in the aqueous phase prolonged the inhibition period and reduced the rate of

consumption of vitamin E even when radicals were generated in the lipid bilayer[3]. Vitamin C has been shown to regenerate a-tocopherol from the tocopherol radical in several in vitro experiments in various models[4].

α-Tocopherol is considered to be mainly responsible for chain breaking lipid peroxidation in phospholipid bilayers. The observed improvement of α-tocopherol levels in red blood cell membranes in vivo upon prolonged green tea catechin consumption also supports this idea of antioxidant networks and the hypothesis that the levels of co-antioxidants are important for maintaining high levels of α-tocopherol. Our in vivo data support this concept as seen by the increased tocopherol levels in plasma, liver, and adipose tissue when quercetin and catechin were fed in tocopherol sufficient animals.

Although we were unable to measure treatment differences in oxidation products when feeding tocopherol with and without flavonoids, we did see that tocopherol outperformed the flavonoids as measured by the accumulation of F_2-isoprostanes. Since tocopherol is known and confirmed in this study to improve oxidative status, it would follow that protection or sparing of tocopherol by flavonoids should also improve oxidative status. We did not see improved oxidative status of plasma or heart when supplementing tocoherol deficient or sufficient diets with flavonoids. However, this may have been possible in a more oxidatively stressed model. If this is the case, catechin and quercetin should

provide oxidative protection and these two flavonoids should outperform the catechin stereoisomer, epicatechin.

This is in contrast to in vitro findings reported earlier by others that found epicatechin to have superior antioxidant function over catechin and quercetin in cell membrane and liposome systems. In those experiments, it was concluded that the oil/water partition coefficient plays an important role in determining their antioxidant activities, both individually and through interactive association with a-tocopherol[5].

Antioxidant quality is dependent on the ability of the compound to form a stable radical that is not prone to become a pro-oxidant. Our in vitro data support the concept that planar molecules form more stable radicals. If this concept is applied to the living system, although the cis configuration of epicatechin may allow it to intercalate slightly into the lipid bilayer[6], a more stable peroxyl radical is formed with the trans catechin structure.

The human organism has developed a comprehensive set of antioxidant defenses to prevent free radical formation and limit their damaging effects. These antioxidant mechanisms have been reviewed in Chapter 1 and discussed in various parts of this thesis.

Establishing the mechanisms by which free radicals are involved in pathogenesis of a disease is extremely difficult due to the short lifetimes of the radical species and the lack of sufficiently sensitive methodologies

to detect radical species or their products in vivo. Likewise, the evaluation of antioxidant treatment strategies is hindered by the lack of sensitive and specific measurement technologies. I chose to evaluate oxidation status by measuring F2-isoprostanes because currently it is the most sensitive and specific measurable end product. Through collaboration with Dr. L. Jackson Roberts, II of Vanderbilt University, we were able to detect the reduced production of this oxidative end product due to the presence of tocopherol, which was not further reduced with the addition of flavonoids.

Additional study is needed to determine if the sparing of tocopherol by flavonoids effects oxidative status and the pathologies that are believed to follow. A more oxidatively stressed model may provide this insight since more antioxidant effects have been reported in human studies involving the presence of chronic disease such as renal disease or oxidative stresses such as smoking.

The sparing of tocopherol alone in vivo is a significant finding because of the non-antioxidant functions of this vitamin that have been discussed earlier and reviewed recently by Azzi et al. Several important cellular reactions by a-tocopherol can be traced to its effect on PKC (protein kinase C) which may explain some of the anti-atherosclerotic and anti-tumor effects of this vitamin in vivo[7]

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