FOOD AND FOOD-DERIVED BIOACTIVE COMPOUNDS: RELEVANCE TO HOMEOSTASIS OF THE ANTIOXIDANT, CARDIOVASCULAR, AND IMMUNE SYSTEMS

by

Mercedes C. Argüelles

A dissertation submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN NUTRITIONAL SCIENCES

In partial fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2005

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation

prepared by Argüelles, Mercedes C.

entitled "Food and Food-Derived Bioactive Compounds: Relevance to Homeostasis of the Antioxidant, Cardiovascular, and Immune Systems"

and recommend that it be accepted as fulfilling the dissertation requirement for the

Degree of Doctor of Philosophy

_____ Date: April 12, 2005

Watson, Ronald R.

_____ Date: April 12, 2005

Thomson, Cyndi

_____ Date: April 12, 2005

Goll, Darrel

_____ Date:

_____ Date:

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: April 12, 2005

Dissertation Director: Watson, Ronald R.

STATEMENT BY AUTHOR

This dissertation has been submitted impartial fulfillment of requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

SIGNED: _____

Mercedes C. Argüelles

DEDICATION

I dedicate this dissertation to my mother, Carmen Celia, to “la Mater”, Mother Thrice Admirabilis of Schönstatt, and to my spiritual father, Fr. Josef Kentenich. Their unconditional love, guidance, and support at all time, were the lifeline through this long journey. Finally, I owe special thanks to all the *ANGELS* la Mater sent to walk at my side on the road to Emmaus were they nourished my body and my spirit.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Recent studies support the hypothesis that beyond meeting nutritional needs, diet may modulate various functions in the body, playing detrimental or beneficial roles in some diseases. Concepts in nutrition are expanding from emphasis on preventing adverse nutrient deficiencies to actually increasing survival, to the use of foods to promote a state of well-being and better health by reducing the risk of pathogenic consequences. These concepts are particularly important in light of the increasing cost of health care, the steady increase in life expectancy, and the desire of older people for improved quality of life in their later years. Such changes in emphasis have stimulated and supported research on functional foods to address the physiological effects and health benefits of foods and their bioactive compounds. Among the most promising targets for functional foods' *in vivo* modulation are: redox and antioxidant, cardiovascular, and immune systems. In addition, intense research interest has focused on identifying and characterizing biologically active components in foods from both plants and animals that potentially could reduce risks from a variety of chronic diseases or optimize health.

Our hypothesis is that dietary consumption of food-derived bioactive compounds (protein-peptides, nutritional supplements, and plant extracts) will have significant *in vivo* effects on homeostasis within the cardiovascular, immune, and redox-antioxidant systems. To address this hypothesis, the following specific aims are proposed: 1) to determine whether bioactive protein-hydrolysates will lower systolic blood pressure in the spontaneous hypertensive rat (SHR) model; 2) to evaluate the immunomodulatory effect of different doses of six nutritional compounds on an aged murine model with

impaired immunological system; and 3) to evaluate the effect of natural antioxidants derived from popular plant extracts (hop and borage) on oxidative stress and tissue α-tocopherol activity.

Antioxidant defense system and oxidative status methodology were optimized to facilitate these goals. Also we conducted the validation and start-up of the tail-cuff plethysmography for the hypertension study. We found that all protein-hydrolysates showed a tendency to lower systolic blood pressure over time in the SHR model. However, there was no significant difference between any of the protein hydrolysates tested and the control group. Consumption of the six nutritional compounds tested gave no indication of effectiveness in preventing immune dysfunction caused by aging. On the other hand, the Th1 and Th2 cytokine responses of the aging mice did show a significant difference from that of the young mice, consistent with previous immunological findings. The plant extracts from hop and borage, given as a dietary supplementation neither prevented oxidation nor enhanced the activity of tissue α-tocopherol in a murine retroviral infection model.

CHAPTER 1: INTRODUCTION

In recent years, researchers have become interested in food-derived active compounds for the development of physiologically functional foods as important modulators of cellular homeostasis.

1.1 IMPORTANCE OF FOOD-DERIVED BIOACTIVE COMPOUNDS

The primary role of diet is to provide enough nutrients to meet metabolic requirements while giving the individual a feeling of satisfaction and well-being. Recent knowledge, however, supports the hypothesis that, beyond meeting nutrition needs, diet may modulate various functions in the body and may play beneficial or detrimental roles in some diseases. Concepts in nutrition are expanding from emphasis on preventing adverse effects and survival to an emphasis in the use of foods to promote a state of better health and to help reduce the risk of pathogenic consequences. These concepts are particularly important in light of the increasing cost of health care, the steady increase in life expectancy, and the desire of older people for improved quality of their later years. These changes in nutrition emphasis have stimulated and supported research on functional foods or food-derived bioactive compounds to try to understand the role of food components in modulating body functions, maintaining and improving health, and reducing the risk of major diseases.

As a working definition for the present investigation, a food is said to be functional if it contains a component that benefits certain physiological functions in the body. That component must be relevant to either the state of health or even to the

reduction of disease risk. The initial step in research of a functional food or food-derived bioactive compound is the identification of a specific link between one or a few components of this food and a biological function, (i.e. genomic cellular, biochemical, or physiological) that is potentially beneficial for health. In our investigation of potential functional foods, we designed three studies to evaluate the functions that are susceptible to modulation by select food components (i.e. cardiovascular, immune, and antioxidant). These components are pivotal to maintain a healthy state and when altered, may be linked to a change in the risk of a disease. Some of the most promising targets for functional foods are the following: 1) *Cardiovascular system*: Many functional foods have been found to be potentially beneficial in the prevention and treatment of cardiovascular diseases, the leading cause of mortality in the United States. An accumulating body of evidence suggests that consumption of certain foods or their associated physiologically active components, e.g. bioactive peptides, may be linked to decreased risk of cardiovascular disease by several potential mechanisms, among which is reducing the risk of high blood pressure. 2) *Antioxidant defense systems*: These systems require a balanced and satisfactory intake of antioxidant vitamins, as well as non-vitamin antioxidant ifood components such as polyphenols. Redox activities and antioxidant protection are important for almost every cell and tissue, and their imbalance is thought to be involved in pathogenic processes of numerous diseases. A sufficient supply of antioxidants from diet might help prevent or delay the occurrence of pathological changes associated with oxidative stress.

3) *Immune system*: Its modulation by non-nutritive dietary components, such as some biologically active food components, e.g. polyphenols, and/or animal derived components, may have important implications for improvement in quality of life of the elderly population and for general defense functions. For this dissertation the bioactive food components chosen to be investigated comes from plant and animal sources. The rationale for their selection follows.

1.2 BIOACTIVE MILK-PEPTIDES AS INHIBITORS OF ACE

Angiotensin-I-converting enzyme (ACE) is a key protein in the regulation of blood pressure. ACE, a dipeptide liberating carboxypeptidase (peptidyldipeptide hydrolase, EC 3.4.15.1), classically associated with the renin-angiotensin system, converts angiotensin I into angiotensin II, a highly potent vasoconstrictor (1). Also ACE plays a key physiological role in the regulation of local levels of several endogenous bioactive peptides such as bradykinin, a vasodilatory molecule. Exogenous ACE inhibitors having an antihypertensive effect *in vivo* were first discovered in snake venom (2). Several food protein sources including fish, gelatin, and maize protein contain ACE-inhibitory peptides (3). Milk proteins are also precursors for a range of peptides which inhibit ACE (4). Casein-derived inhibitors of ACE are known as casokinins, whereas whey-derived inhibitors are know as lactokinins.

1.2.1 PHYSIOLOGICAL EFFECTS

ACE is widely distributed in mammalian tissues. It is present in plasma, lung, kidney, heart, skeletal muscle, pancreas, spleen, placenta, arteries, testes, uterus, and brain. Numerous studies in spontaneously hypertensive rats (SHR) have demonstrated an antihypertensive effect following intravenous and oral ingestion of casein-derived ACE inhibitory peptides. These peptides correspond to tryptic and *Lactobacillus helveticus* protease (5). Oral ingestion of a tryptic digests of whole casein produced an antihypertensive effect in SHR (6). A placebo controlled study in hypertensive humans definitively demonstrated a significant reduction in blood pressure following daily ingestion of 95 ml of Calpis sour milk which contains highly potent tripeptide inhibitors of ACE, i.e. β-casein f(84-86) and β-casein f(74-76) and κ-casein f(108-110) (7).

The antihypertensive potential of milk protein-derived peptides is dependent on the ability of these peptides to reach their target site without being degraded and as a consequence inactivated by the action of intestinal or plasma peptidases. Resistance to peptidase degradation may be a prerequisite for an antihypertensive effect during the oral ingestion and the intravenous infusion of ACE inhibitory hydrolysates/peptides. For example, α_{s1}-casein f(104-109), a potent ACE inhibitor *in vitro*, was shown to have no hypotensive effect *in vivo* (5). On the other hand, peptide degradation or fragmentation may result in more potent ACE inhibitory activities. For example, removal of C-terminal glutamine from β-casein f(169-175) increased the *in vitro* ACE inhibitory potency; however, both β-casein f(169-174) and f(169-175) had strong antihypertensive activities

in SHRs (5). These results emphasize the necessity of performing *in vivo* studies in all cases.

The majority of milk protein-derived peptides reported to date (for example: α_{s1}-casein f (104-109) IC_{50} = 22 μmol/l; β-casein f (169-175) IC_{50} = 1000 μmol/l) do not have ACE inhibitory potencies approaching that of captopril (IC_{50} = 0.006μmol/l). However, as naturally derived peptides, they would not be expected to have the side-effects associated with synthetically produced drugs used in the control of hypertension, i.e. cough and alterations in serum lipid metabolism.

Milk protein-derived peptides represent a group of bioactive peptides that have significant potential as naturally-derived agents for the prevention/control of blood pressure and related diseases. The use of these peptides in functional foods requires ongoing studies, including extended clinical trials, to demonstrate their long-term efficacy and safety.

1.3 BIOACTIVE FOOD COMPONENTS AND THE IMMUNE SYSTEM

The immune system acts to protect the host from infectious agents that exist in the environment (viruses, bacteria, fungi, parasites) and from other noxious insults. The system is a complex and highly interactive network of cells and their products. The immune system has two functional divisions: the innate and the acquired. Both components involve various blood-borne factors (complement, antibodies, and cytokines) and cells. The immune system regulates itself by means of helper and suppressor cells

and soluble products. Nutrients and other food and food-derived components of the diet have the potential to affect almost all aspects of the immune system (8).

The relation of single nutrients to immune function (9), as well as the effect of nutrient-nutrient interactions (10) in humans and in animal models, has been reviewed comprehensively. Several of the vitamins associated with diets high in fruit and vegetables have been shown to improve immune status, particularly in older individuals. In a placebo-controlled, double-blind trial, 12 mo of supplementation with an over-the counter multivitamin- and –mineral supplement improved delayed-type hypersensitivity skin responses in subjects aged 59-85 y, an effect that was not detected after 6 mo of supplementation (11). This effect w as associated with significant increases in serum concentration of ascorbic acid, β-carotene, folate, vitamin B-6, and α-tocopherol at 6 mo, 12 mo, or both. Vitamin supplementation alone also improves response to delayed-type hypersensitivity tests in healthy individuals >65 y of age (12). Subjects received a placebo or 60, 200, or 800 mg *all-rac*-α-tocopherol daily for 235 days. The group given 200 mg/day had the greatest increase in delayed –type hypersensitivity and antibody titer to hepatitis B compared with the placebo and the other 2 α-tocopherol doses.

Peripheral blood mononuclear cells produce cytokines, e.g. ILs and TNF-α, that assist in the activation of T cells and enhance NK cell activity (10). Supplementation with α-tocopherol and ascorbic acid results in transient increase in cytokine production (13). Ascorbic acid (1 g), *all-rac*-α-tocopherol acetate (400 mg), ascorbic acid and α-tocopherol, or placebo were provided for 28 days as part of a randomized trial. On day 14, the combination of ascorbic acid and α-tocopherol increased IL-1β and TNF-α

production 1.8 and 1.5 fold, respectively; small increases were also detected with α-tocopherol, but ascorbic acid alone had no effect. However, by day 28, there was no difference in response among the supplement and placebo groups. Other cytokines not measured in this study may be influenced by sustained vitamin supplementation to modulate immune balance, resulting in the transient changes in IL-1β and TNF-α that were observed (13).

Natural killer (NK) cells, one of the types of immune cells, play an important function in immune surveillance, and NK cell activity is a component of the antitumor host defences, during tumor growth (14) and metastasis. It appears that nutrients and phytochemicals tend to affect NK cell activity without influencing cell number. Supplementation for 10-12 years with β-carotene (50 mg on alternate days) resulted in 1.6-fold greater NK cell activity in elderly men (aged 65-85 years) relative to placebo without an increase in the percentage of NK cells or an increase in interleukin 2 receptor expression or IL-2 production; no effects of supplementation were observed in middle-aged men (aged 51-64 years) (15). Similarly, in healthy male college-student volunteers aged 20-25 years, 9 mo of β-carotene supplementation (60 mg/d) did no alter NK cell numbers or virgin T cell, memory T cell, or cytotoxic T cell numbers (16).

1.3.1 DIETARY ANTIOXIDANTS

The immune system is highly reliant on accurate cell-cell communication for optimal function, and any damage to the signaling systems involved will result in an impaired immune responsiveness. Oxidant-mediated tissue injury is a particular hazard

to the immune system, since phagocyte cells produce ROS as part of the body's defense against infection. Adequate amounts of neutralizing antioxidants are required, therefore, to prevent damage to the immune cells themselves. Many antioxidants can be obtained directly from the diet. Numerous epidemiological studies have found strong associations between diets rich in antioxidant nutrients and a reduced incidence of cancer, and it has been suggested that a boost to the body's immune system by antioxidants might, at least in part, account for this (17). In addition, antioxidative factors such as tea polyphenols and flavonoids have been found to exert an inhibitory effect on chemical mediator release *in vitro* (18); hence, some of these immunoregulatory activities of bioactive antioxidant compounds could be expressed *in vivo*.

1.3.2 PROBIOTICS

Probiotics have several immune-enhancing effects that have been documented in different studies by different research groups (19). An enhancement of the circulating IgA secreting cell response has been observed in infants supplemented with *Lactobacillus casei*, and correlated with a shortened duration of diarrhea in the study group when compared with a placebo group (20). An improvement of the non-specific immune phagocytic activity of granulocytes has been shown in the blood of human volunteers after consumption of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (20). Ingestion of yogurt has been reported to stimulate cytokine production, including interferon-γ in human blood mononuclear cells (21). It has also been reported that the consumption of yogurt stimulates cytokine production by monocytes (22).

Perhaps the most intriguing aspect of probiotic modulation of immune response is through its effects on cytokine production. Cytokines and their regulation of the immune system have been studied intensively in the last several years in cell lines and primary cells of both rodents and humans (23-27). Several studies have shown that cytokine production by cells of the immune system can be altered by probiotic use. For example, the effects of four commercial strains of *Streptococcus thermophilus* found in yogurt on cytokine production were evaluated with a macrophage cell line and a T-helper cell line and compared with active strains of *L. bulgaricus*, *Bifidobacterium adolescentis*, and *B. bifidum* (24). All cytokines studied, TNF-α, IL-6, IL-2, and IL-5, were affected by heat-killed *S. thermophilus* in a strain- and dose-dependent fashion. All bacteria induced significant increases of IL-6 production in the macrophage cell line with *S. thermophilus*. The four *S. thermophilus* strains also strongly induced TNF-α production. IL-6 and, to a lesser extent, TNF-α production were also increased when the macrophages were costimulated with LPS and cells fthe three groups of lactic acid bacteria. After concurrent stimulation of a T cell line with phorbol 12-myristate-13-acetate, seven of the eight strains enhanced IL-2 and IL-5 production significantly (24).

More recent studies have assessed the effects of probiotics on cytokine gene transcription. For example, there was no effect of repeated oral exposure to viable or nonviable *L. acidophilus, L. bulgaricus. L. casei* or *S. thermophilus* on basal cytokine mRNA expression in Peyer's patches, spleen or lymph nodes of mice, after 14 days of exposure (27). In another study, human peripheral blood mononuclear cells were stimulated with three nonpathogenic Lactobacillus strains and with one pathogenic

Streptococcus pyogenes strain. All bacteria strongly induced IL-1β, IL-6 and TNF-α m RNA expression and secretion of the cytokine protein. *S. pyogenes* was the most potent inducer of secretion of IL-2 and IFN-γ, and two of the *Lactobacillus* strains induced IL-12 and IFN-γ production. All strains induced IL-18 protein secretion (25). Additional effects of probiotics have been to reverse the age-related decline in the production of cytokines. For example, supplementing the diet of aging mice with several probiotic species restored IFN-γ levels compared with control mice (28). The mechanism of this reversal is unknown but may involve the ability of lactic acid bacteria to adhere selectively to M cells of Peyer's patches.

1.4 BIOACTIVE FOOD COMPONENTS AND ANTIOXIDANT DEFENSE

1.4.1 POLYPHENOLS

Phytochemicals or plant derived chemicals are to be found both among nutrients and non-nutrients. The non-nutrient phytochemicals refer to every naturally occurring chemical substance present in plants, usually in small amounts, and especially to those phytochemicals that are biologically active. Phytochemicals occur in all higher plants as an array of secondary plant substances, varying quantitatively and qualitatively with the plant anatomy. The major phytochemicals are polyphenolic compounds like flavonoids, phenolic acids, and phytoestrogens.

Polyphenols derived from various plant sources are the most abundant antioxidants in our diets (29). The varying biological sources are reflected by a diversity of structures: flavonoids, anthocyanins, coumestane, stilbenes and lignans; see Figure 1.1

and Figure 1.2 for chemical structures. Several thousand natural polyphenols with diverse chemical structures have been identified so far and they are classified into at least 10 chemical groups (30). Flavonoids account for about two-thirds of the total polyphenol intake, and phenolic acids make up the remaining one-third. Few single compounds, however, reach significant concentrations *in vivo*. The pharmacokinetics of the even fewer polyphenols so far assessed are as diverse as their chemical structures. Usually, less than 1 -26 % of ingested flavonoids are found in human urine (0.4 -1.4% for flavonols, 0.5 – 6% for catechins, 2.5 – 26% for isoflavones, and 1 – 7% for anthocyanins (31). This implies that the intestinal absorption of most polyphenols is rather limited and/or that polyphenols undergo substantial metabolism either in the body tissues or by the colonic flora (29). Polyphenols absorbed in the gut may also be secreted in the bile in metabolized and un-metabolized form and further metabolized by the colonic flora. Many polyphenols are glycosylated (31). Removal of the glycosides residues by intestinal glycosidase is expected to enhance intestinal uptake by diffusion. Some glycoside residues can only be cleaved by the colonic microflora. Metabolism and/or re-uptake in the colon may result in prolonged half-lives. The colonic microflora is also responsible for the cleavage of esterified polyphenolic acids. Extensive metabolism and antioxidant capacities of metabolites complicate estimates of dose-related antioxidant properties.

The research database indicates that polyphenols may have actions that include antioxidant, cancer prevention, anti-infection, and hormonal. Polyphenols may also induce our chemical defense enzymes, and act on blood clotting and on the vascular

system. However, solid evidence that they positively influence human health is lacking, and adverse effects have also been reported for certain polyphenols. A renewal of the interest in flavonoids arose from the work of Hertog and co-workers who in 1993 reported a preventive action of flavonoids against fatal outcomes of heart disease in humans (32). In the following years this observation has been repeated in other studies (33;34), but not uniformly (35). The research in recent years has focused on the antioxidant actions of flavonoids and other polyphenols. Their action on the vascular system is now being reviewed and investigated by modern techniques. The polyphenols are generally outstanding antioxidants, and their abundance in fruits and vegetables has nourished the idea that they may partly explain the positive health effects of plant foods by their antioxidant actions, e.g. their ability to quench potentially damaging radical reactions. There is little doubt that polyphenols are indeed able to affect our capacity to deal with radicals; however, their ability to decrease damage to tissue components in humans needs to be further investigated**.**

1.4.2 EPIDEMIOLOGICAL EVIDENCE FOR PREVENTIVE ACTION

Epidemiological studies promote the notion that an increased consumption of plant products is an important factor in reducing the risk of degenerative diseases like heart diseases and cancer (36-38). Accordingly, there is evidence that high intake of various soy products, rich in isoflavonoids and lignans, protects against breast cancer and prostate cancer. This claim is supported by the fact that subjects with breast cancer or at

high risk of breast cancer excrete low amounts of lignans and isoflavonoids, but subjects living in areas with low risk of hormone-dependent cancers have higher levels (39).

The pleiotropic effects of dietary polyphenols has been emphasized especially in relation to degenerative diseases (40). Several epidemiological studies suggest that black tea consumption is associated with a reduced risk of degenerative diseases such as cardiovascular disease(35;41). It have been reported that the incidence of coronary mortality and lung cancer is higher among a population with low dietary intake of flavonoids (33;42).

In 1992 a fascinating hypothesis was postulated: the so called "French Paradox" (43). It claims that French subjects who have similar intakes of saturated fatty acids, similar risk factors and comparable plasma cholesterol levels exhibit a much lower incidence of death from CHD than U.S. or West European subjects with comparable intakes of fat. In subsequent reports the effects of red wine have been confirmed (44-46). The essential questions remains unanswered: whether alcohol *per se* (47) or the non-alcoholic fraction of wine represented mainly by polyphenols, is the primary factors responsible for the protective effect. Indeed, a recent study showing antioxidant properties of alcohol-free red wine, is an actuating notion in this respect (46).

In conclusion, there is little doubt that the preventive effects of plant products are intrinsically related to the presence of phytochemicals. Longitudinal dietary changes indicate that an increasing share of the population responds positively to current health management suggestions to include to more fruits and vegetables in their diets (48). Recently, positive associations of exercise and fruit consumption with cardiovascular

health have been reported in female adolescents and adults (49). Available data reinforce the advantages for human health of a diet containing plenty of fruits, vegetables, cereals, and reduced amount of fat. Based on available epidemiological studies, the U.S Government recommends a consumption of 400-600 g of vegetables and fruits daily, which comprises three to five portions of vegetables, and two to three portions of fruit (50).

1.4.3 PREVENTIVE ACTION OF BIOLOGICALLY ACTIVE FOOD COMPONENTS

During the last 30 years, research in the field of nutrition and chronic disease causation has led to significant progress in providing an understanding of specific risk factors and chemopreventive agents. The major health problems considered are cardiovascular and nutritionally related cancers. One aspect involved in the initiation and development of both cardiovascular diseases and cancer is abnormal oxidative processes, leading to the generation of hydroxyl radicals and peroxyl compounds. In part, the protective role of vegetables, fruits, and tea is thus, to provide antioxidant vitamins and specific phytochemicals that display a powerful inhibitory effect in oxidative reactions. Epidemiological studies (*vide supra*), as well as laboratory experimentation have yielded solid data and evidence in support of the fact that vegetables, fruits, tea and specific antioxidants therein account mechanistically for the inhibition (51-54).

1.4.4 THE ANTIOXIDANT HYPOTHESIS

The hypothesis that dietary antioxidants prevent chronic disease is well established and remains interesting to the scientific community. This may be because it seems immediately plausible. It is very likely that aging in humans is partly caused by free radical reactions just like aging and rancidity of foods. It is also known that radical reactions are involved in plaque formation in the intima of blood vessels (55) Furthermore, radicals may react with DNA to cause damage, some of which can lead to mutation, the putative initiating event in cancer. Thus, radical reactions are likely involved in aging, atherosclerosis, and cancer. As such, antioxidants should in theory be able to partially prevent these conditions. The idea that the preventive action of fruits and vegetables towards chronic disease, which has been observed in many epidemiological studies, was caused by the high levels of antioxidants in these food items, has given further plausibility to the antioxidant hypothesis. Most research has been directed towards some antioxidant vitamins and pro-vitamins in plant foods, including vitamin E and beta-carotene. However, large human intervention studies with these nutrients have not given immediate support to the antioxidant hypothesis and these nutrients seem either neutral or even harmful in controlled human studies (56). Thus, an increase in any antioxidant does not necessarily lead to decreased health risk. Some antioxidants may even be detrimental to health at doses above physiological levels. However, it may still be true that specific antioxidants are preventive, and since polyphenols act as potent antioxidants and are abundant in plant foods, they are likely candidates for fulfillment of the antioxidant hypothesis. The available methodology to evaluate such a hypothesis

consists of animal and human intervention studies using a combination of biomarkers for oxidative damage and antioxidative defense.

1.4.5 FREE RADICALS

Free radicals are highly-reactive molecules containing one or more unpaired electrons. Several potentially damaging species, such as reactive oxygen, nitrogen and chlorine species (ROS/RNS/RCS), as well as superoxide ($O_2.^-$) and hydroxyl (OH*) radicals arise as by-products of normal metabolism and from chemical accidents (57). Superoxide is a one-electron reduction product of molecular oxygen that is formed during normal respiration in mitochondria and by auto-oxidation reactions. The hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion-controlled rates. It therefore will not diffuse a significant distance within a cell before reacting and has an extremely short half-life but is capable of causing great damage within a small radius of its site of production.

Hydrogen peroxide (H_2O_2) is a non-radical reactive species formed during normal metabolism and its main significance lies in its being a source of hydroxyl radicals in the presence of reactive transition metal ions (e.g. $Fe2^+$). One example is the Fenton reaction:

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

Not all ROS/RNS/RCS production is accidental, since the body can use these substances for its own benefit. Nitric oxide (NO*) finds a multiplicity of uses, for

example as a regulator of vascular tone and as a messenger in the central nervous system (58). Reactive species produced by activated neutrophils, macrophages and several other cell types are active in killing bacteria. Indeed ROS/RNS/RCS may have a variety of functions including regulation of gene expression (59) and induction of apoptosis (60). The ability of radicals such as NO* and $O_2.^-$ to kill invading bacteria means that these substances are also capable of damaging normal tissues. All the major classes of biomolecules may be attacked by free radicals but lipids are probably the most susceptible. Polyunsaturated fatty acids (PUFAs), major components of cell membrane, are readily attacked by oxidizing radicals.

The oxidative destruction of PUFAs, known as lipid peroxidation, is particularly damaging because it proceeds as a self-perpetuating chain-reaction. The general process of lipid peroxidation can be envisaged as in the scheme below, where LH is the target PUFA and R* the initiating, oxidizing radical. Oxidation of the PUFA generates a fatty acid radical (L*) that rapidly adds oxygen to form a fatty acid peroxyl radical (LOO*). The peroxyl radicals are the carriers of the chain-reaction; they can oxidize further PUFA molecules and initiate new chains, producing lipid hydroperoxide (LOOH) that can break down to yet more radical species and to a wide range of compounds, notably aldehydes (61).

$$LH + R^* \longrightarrow L^* + RH$$

$$L^* + O_2 \longrightarrow LOO^*$$

$$LOO^* + L'H \longrightarrow LOOH + L'^*$$

$$LOOH \longrightarrow LO^*, LOO^*, \text{aldehydes}$$

In summary, lipid peroxidation is of particular significance as a damaging reaction consequent to free radical production in cells because: (i) it is a very likely occurrence, given the availability and susceptibility of PUFA in membranes; (ii) it is a very destructive chain-reaction that can directly damage the structure of membranes and indirectly damage other cell components by the production of reactive aldehydes. Therefore, lipid peroxidation alters functional properties of membranes and delivery of lipids to tissues (62). Thus, ROS/RNS/RCS may have extremely deleterious effects which under normal circumstances are counteracted by the antioxidant defenses in healthy individuals.

Polyphenols can scavenge free radicals involving LOO* and OH*, depending on the number and site of the hydroxyl group and double bonds in their structures. Furthermore, some polyphenols possess a capacity to chelate the transition metal ions responsible for the generation of reactive species and to inhibit lipoxygenase reaction (63). It is therefore likely that polyphenols act as preventive antioxidants, as well as chain-breaking antioxidants.

1.4.6 ANTIOXIDANT DEFENSES

Since reactive species are produced *in vivo,* organisms have evolved antioxidant defense systems either to prevent the generation of reactive species or to intercept any that are produced; see Figure 1.3 for antioxidant defence system in the cell. They exist in both the aqueous and membrane compartments of cells and can be enzymes or non-enzymes. Catalase and glutathione peroxidase are enzymes which can safely decompose peroxides, particularly H_2O_2 produced during the respiratory burst involved in microbial killing in phagocytic cells, while superoxide dismutase intercepts or "scavenges" free radicals. Most free-radical scavengers are not enzymes, and many are obtained through the diet. In cell membranes the most important is α-tocopherol, the major member of the vitamin E family. This molecule is known as a "chain-breaking antioxidant", because its function is to intercept lipid peroxyl radicals (LOO*) and to terminate lipid peroxidation chain reactions.

$$\text{LOO*} + \alpha\text{-tocopherol} - \text{OH} \rightarrow \text{LOOH} + \alpha\text{-tocopherol} - \text{O*}$$

Another group of lipid-soluble compounds that can act as antioxidants are the carotenoids, such as β-carotene, lycopene, and lutein, found in highly pigmented fruits and vegetables. The polyene structure of these compounds allows the molecules to quench, or inactivate, singlet oxygen and free radicals. A major water soluble free radical scavenger is ascorbic acid, which also plays a role in "sparing" vitamin E, by

regenerating α-tocopherol from the oxidized tocopheroxyl radical (64). More recently, attention has focused on the antioxidant properties of dietary plant polyphenols (65;66).

Many plant polyphenols inhibit lipid peroxidation and lipoxygenase enzymes *in vitro* (67;68) and thus, may be important dietary antioxidants (32;33;69). It has been speculated that polyphenols in red wine could explain the "French paradox". On the other hand, some polyphenolics are pro-oxidant *in vitro* if mixed with copper or iron ions (68;70). More data are needed on absorption and bioavailability of polyphenols, but there is a growing body of evidence that many dietary polyphenols can be absorbed (71); and the proof of polyphenols' bio-activity must come from application of reliable *in vivo* models where markers of baseline oxidative damage are examined from the standpoint of how they are affected by changes in diet or by antioxidant supplementation.

1.4.7 DIETARY α-TOCOPHEROL (VITAMIN E)

Studies of human subjects and animals, in either states of deficiency or at supra dietary levels, suggest strongly that α-tocopherol is involved in maintaining immune cell function. As mentioned above, vitamin E is the most effective chain-breaking lipid soluble antioxidant present in cell membranes; therefore, it is considered likely that it may play a major role in maintaining cell membrane integrity by limiting lipid peroxidation by reactive species. Vitamin E deficiency states are associated with depressed B-cell antibody production and T-cell proliferation in response to mitogenic stimulation, and with an increased rate of infection (72). As with other dietary

antioxidants, a marked improvement in immune indices can be seen in the elderly following supplementation with vitamin E (72). There is also evidence that increased intakes can modulate the function of immune cells in younger individuals. For example, supplementation with 300 mg vitamin E/day depressed the bactericidal activity of leucocytes from a group of healthy young men (73).

Recently, Deveraj et al. (74) examined the effect of high dose α-tocopherol supplementation (1200 mg/day) on *ex vivo* monocyte function. After 8 weeks of supplementation, the *in vitro* release of reactive species and lipid oxidation was decreased, both in the resting state and in lipopolysaccharide stimulated cells compared to both baseline and a 6-week washout period (when levels had returned to baseline). A similar effect was observed after culturing the cells in the presence of the protein kinase C (PKC) inhibitor, Calphostin C, suggesting that inhibition of PKC activity might be a mechanism by which α-tocopherol can inhibit reactive species release and lipid oxidation. It is also possible that vitamin E, as well as other antioxidant nutrients, can influence a variety of inflammatory processes by inhibiting the activity of the redox-controlled transcription factor nuclear factor-κβ (NF-κβ) (75). NF-κβ is required for maximal transcription of many cytokines, including interleukin-1β, and it is thought that the generation of reactive species is a vital link in mediating NF-κβ activation by a variety of stimuli (76;77).

Several studies have examined the effect of vitamin E in cigarette smokers. Cigarette smoke contains millions of free radicals per puff, and other compounds present can stimulate the formation of other highly reactive molecules (78). Circulating

phagocytes from smokers produce high levels of free radicals, which probably in part account for the depressed immune function observed in smokers (79). There is some evidence that vitamin E supplementation can reduce the over-production of reactive species by phagocytic cells from current smokers (80).

1.4.8 OXIDATIVE STRESS AND DIET

Because production of reactive species and antioxidant defenses is approximately balanced *in vivo,* it is easy to tip this balance in favor of the reactive species and create a situation of oxidative stress (81). Oxidative stress may occur in several ways: (i) by inadequate diet-derived antioxidants, and (ii) by excess production of O2*- and H_2O_2 , e.g. by exposure to drugs or toxins that are metabolized to produce free radicals, or by excessive activation of "natural" radical-producing systems (e.g. phagocytes in chronic inflammatory diseases).

Damaged tissues undergo more free radical reactions than healthy ones (82;83). In most human diseases, oxidative stress is a secondary phenomenon, not the primary cause of the disease (82). This does not mean that oxidative stress is not important; for example, secondary oxidative damage to lipids in blood vessel walls is a significant contributor to the development of atherosclerosis (84). DNA damage by reactive species (ROS and RNS) probably contributes to the age-related development of cancer (85). Oxidative stress contributes to tissue damage in rheumatoid arthritis (86) inflammatory bowel diseases and Parkinson's disease (87). There is growing evidence supporting the fact that the major human killers, cardiovascular diseases and cancer, can be prevented or

delayed to some extent by dietary changes, such as an increased consumption of fruits and vegetables, and reduction in fat intake (38;88). Since our endogenous antioxidant defenses are not 100% efficient, it is reasonable to propose that dietary antioxidants are important in diminishing the cumulative effects of oxidative damage over the human lifespan, and that they account for some of the beneficial effects of fruits and vegetables. For example, if continuous free radical damage to DNA and inefficient repair are involved in the development of spontaneous cancers, then a good intake of dietary antioxidants should be preventative (38;85).

1.5 LIST OF FIGURES AND TABLES

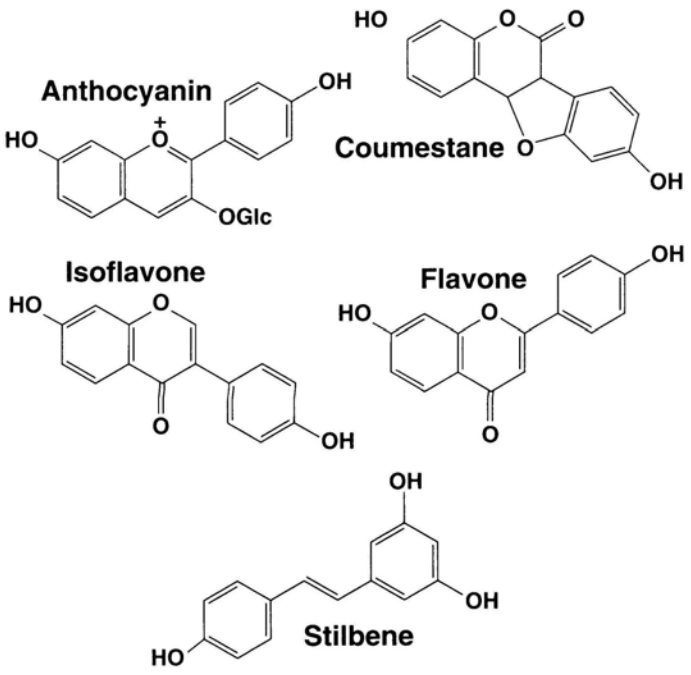


Figure 1.1 Chemical structures of the principal classes of polyphenols.
The examples shown are of polyphenols with hydroxyl groups in the 7- and 4”-positions.

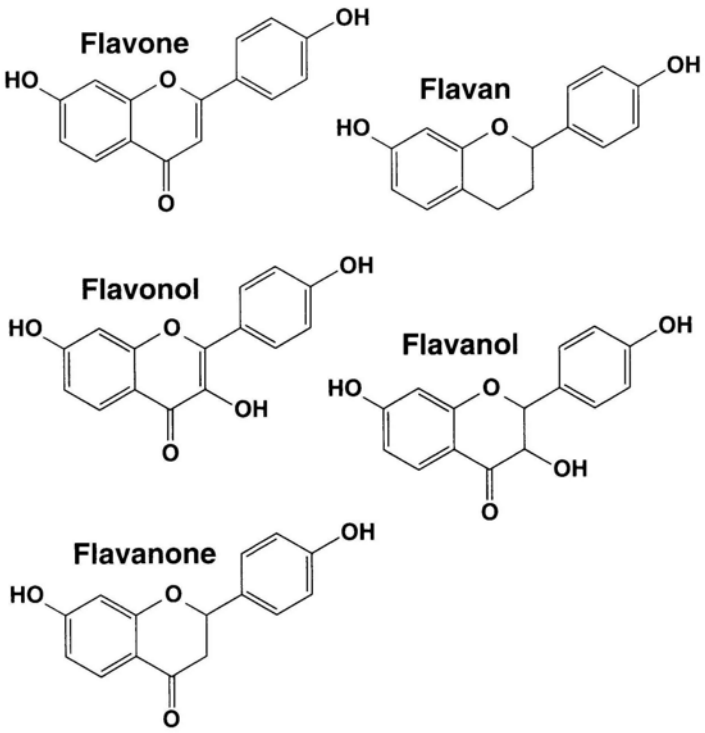


Figure 1.2 Chemical structures of various flavonoids.
The flavone heterocyclic ring can be reduced or oxidized in various ways, which leads to different structures.

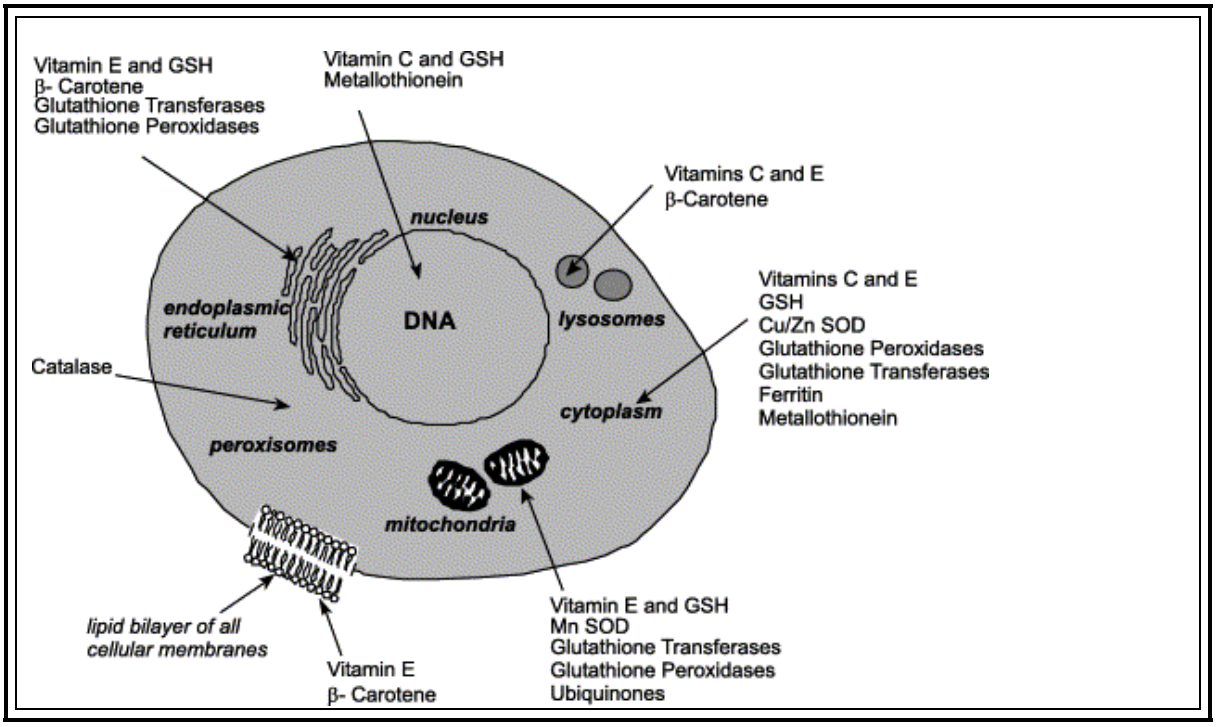


Figure 1.3 Antioxidant defence systems in the cell.

CHAPTER 2: EFFECT OF PROTEIN HYDROLYSATES ON HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS

2.1 INTRODUCTION

High blood pressure, commonly known as hypertension, is one of the most common cardiovascular diseases. In terms of dietary intervention, any food component with the capacity to reduce blood pressure is a potential candidate in the prevention/treatment of cardiovascular disease (CVD).

The seventh Joint National Committee report by the National Heart, Lung, and Blood Institute recommended changes to the pre-existing guidelines used to classify adult blood pressure (89). Due to the fact that the risk of heart disease and stroke increases at blood pressures above systolic blood pressure/diastolic blood pressure values of 115/75 mm Hg, health experts have now decreased the previously accepted blood pressure range in order to encourage a more proactive and earlier treatment of high blood pressure. The new guidelines divide blood pressure into 4 categories as follows: (i) normal, with a systolic blood pressure (SBP) of <120 mmHg, and diastolic blood pressure (DBP) of <80 mmHg; (ii) prehypertension, with a SBP 120-139 mmHg, and DBP 80-90 mmHg; (iii) stage 1 hypertension, with a SBP of 140-159 mmHg, and DBP 90-99mmHg; and stage 2 hypertension, with a SBP $\geq$ 160 mmHg, and DBP $\geq$ 100 mmHg. As a result of these changes many individuals whose blood pressure was previously considered normal or borderline will now fall into the "prehypertension" category.

Hypertension is a controllable risk factor in the development of a number of CVDs including stroke, coronary infarct, heart failure, and end-stage diabetes (1;90).

According to a recent report, hypertension, affect approximately 25% of the U.S. population (91). The annual drug costs associated with the treatment of hypertension and related diseases is estimated to be $15 billion annually in the U. S. (92). The risk of developing CVD is directly related to the reduction of the blood pressure level. It is estimated that for each 5 mmHg reduction in blood pressure the risk of CVD is drop by about 16% (93;94). Two main strategies are currently recommended for the regulation of blood pressure: changes in lifestyle and drug treatments. Studies such as the Dietary Approaches to Stop Hypertension (DASH) trial have shown that a diet rich in fruits, vegetables, and low fat dairy products is an effective means of lowering blood pressure (95). Alternatively, a range of targeted drug treatments may be employed including angiotensin-I-converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers, vasodilators, and diuretics.

The physiological control of blood pressure is carried by a number of different interacting biochemical pathways. Classically, blood pressure control has been associated with the renin-angiotensin system (RAS). The RAS is one of the major regulators of blood pressure, electrolyte balance, renal, nueronal, and endocrine functions associated with cardiovascular control in the body (96). As seen in Figure 1, RAS begins with the inactive precursor angiotensinogen (ATN). ATN is the only known precursor of angiotensin I as well as the only known substrate for renin.

Renin is an asparctic acid proteinase. The main source of renin is the juxtaglomerular cells of the kidney. It is generated from the inactive precursor prorenin, by the action of kallikrein. Several factors influence the release of renin, including renal

perfusion presure, salt depletion, and stimulation of β_2-receptors by aldosterone (97). Renin is responsible for liberation of angiotensin I from ATN. Inhibition of renin activity may be achieved as a result of angiotensin II production and by pharmacological agents.

Angiotensin I is the decapeptide released from the N-terminal portion of ATN by the action of renin. Angiotensin-I-converting enzyme (ACE) regulates the balance between the vasodilatory and natriuretic properties of bradykinin and the vasoconstrictive and salt-retentive properties of angiotensin II. ACE inhibitors alter this balance by decreasing the formation of angiotensin II and the degradation of bradykinin. The formation of angiotensin II from angiotensin I results in a number of responses within the body. The major effects of angiotensin II are the control of blood pressure, fluid volume and neurotransmitter interactions, as well as the control of gonadotropic-releasing hormones and pituitary hormones activities (98;99). Angiotensin II is a substrate for the angiotensinase group of enzymes resulting in the generation of other biologically active peptides; see Figure 2.1 for ACE inhibitors mechanism.

Various side effects are associated with the use of ACE inhibitory drugs in the control of blood pressure. These effectss include hypotension, increased potassium levels, reduced renal function, and cough (100-102). Natural inhibitors of ACE have been identified within the primary sequences of a range of proteins derived from food (yeast, casein, tuna and sardines), which could ultimately influence blood pressure without side effects.

A limited number of human studies have associated milk protein-derived peptides with hypotensive effects. The double blind placebo controlled study of Hata et al., was

the first to demonstrate that a fermented sour milk drink could significantly reduce SBP and DBP following oral ingestion of 95 ml Calpis per day by mildly hypertensive human volunteers (7). In this study, 30 elderly male and female patients were divided into two groups and administered either the Calpis soured milk or acidified milk as a placebo. In the test group, significant SBP reductions of -9.4 and -14.1 mm Hg were recorded at 4 and 8 weeks after initiation of the trial, respectively. DBP was reduced by 6.9 mm Hg at the end of the trial. No significant changes in blood pressure were observed in the placebo group. In addition, ingestion of the test material or the placebo had no effect on heart rate, body weight or blood serum variables, i.e. HDL. In another study, the ingestion of fermented milk, containing similar quantities of the bioactive peptides as those found in Calpis, resulted in a larger hypotensive effect at 8 weeks following ingestion of the fermented milk (103).

Another material which has been studied in humans for its potential hypotensive properties is the Katsuobushi oligopeptide. This material is derived from Katsuobushi, dried tuna (a fish from the tuna / mackerel family), by thermolysin catalyzed hydrolysis. The manufacturer of this hydrolysate, Nippon Synthetic Chemical Industry, Co., Ltd., has conducted and published three separate clinical trials on mildly hypertensive adult volunteers using this material (104-106). The first two trials using the raw material (labeled KO Type L) induced a clinically significant decrease in both systolic (~ 12 - 13 mm Hg decrease) and diastolic (~ 6 - 7 mm Hg decrease) blood pressure after consuming a daily dose of 3 grams of the hydrolysate for 8 weeks. The studies were conducted in a blinded crossover design. In the first study, the Katsuobushi oligopeptide was tested raw

(104), while in the second study (105), the same ingredient was part of a soup. A follow-up study was done using an enriched product derived from the original material treated by ultrafiltration (labeled KO type S) and enriched with the active material (Katsuobushi oligopeptide) (106). This study was also a crossover design, and showed similar effects as the previous two studies using a daily dose of 1.5 grams. These effects were also shown in the generally used spontaneously hypertensive rat model (SHR) (106), and compared to the previously used Katsuobushi oligopeptide material (KO Type L). Intragastric gavage of either type "L" (1000 mg/kg) or type "S" (500 mg/kg) material in an SHR model produced an ~ 15 mm Hg drop in systolic blood pressure within 2-4 hours, which persisted for at least 6 hours post gavage and this effect was dose dependent. An active peptide sequence, LKPMN (Leu-Lys-Pro-Asn-Met, was claimed by Fujita's group (2001).

Evidence is also begining to emerge suggesting that consumption of whey protein hydrolysates may result in significant reductions in blood pressure. In a recent study, a whey protein hydrolysates (20 g/d) and a whey protein isolate control (20 g/d) were orally ingested by 30 male and female, borderline hypertensives over a 6 weeks period (107). The study indicated that significant reductions in SBP (-11.0 mm Hg) and DBP (-7.0 mm Hg) occurred 1 week after ingestion of the hydrolysate and that these blood pressure reductions persisted for the remaining 5 weeks of the study.

A most recent study carried out by Abbot Pharmaceutical: Ross Products Division, used a hydrolysate generated from corn, which produced a pronounced effect in the SHR model using an intragastric gavage of 0.15 g/kg. The effect was maximal at

about 2 - 4 hours post gavage, and showed a tendency to regress toward baseline values by 8 hours post-treatment; however, the effect was still significant at that point. In addition, a normotensive, genetically related rat (WKY) was completely unaffected by this treatment and the control material (casein, 0.15g/kg) had no effect.

An important observation from the *in vivo* studies is that a consumption of specific hydrolysates or fermented dairy products has no effect on blood pressure in either normotensive rats or humans (104;106;108). Futhermore, dose-dependent effects are generally observed and there is a lag time between consumption of a given test sample and the appearance of hypotensive effects; hypotensive effects are observed between 1-5 hours in 24 hours studies and from 1-4 weeks in long-term studies. Finally, no adverse effects have been reported following oral consumption of the different materials.

In summary, these data suggest that there is a strong potential to identify food-derived components beyond nutrients such as mineral (Na^{+}, K^{+}, Ca^{++}, Mg^{++}) that could modulate blood pressure without side effects, and specifically dairy –based peptides. In our study, the main aim was to determine the influence of four protein hydrolysates on blood pressure in the SHR model.

2.2 MATERIALS AND METHODS

Experimental Design, Animals, and Treatment

All animal studies were performed with the approval of the University of Arizona animal review committee.

Animals, Care and Initial Testing - Hypertensive rats (SHR, n = 48) exhibiting systolic blood pressures of approximately 180-200 mm Hg by the time they are 10 weeks of age were used in this study. Animals were purchased from a Taconic, Germantown, New York. Individual rats were labeled by tail marking. After a period of quarantine and acclimation to the animal care facility (12 hour light/dark cycle, ad libitum access to rodent chow and water), animals (3 per cage) were subjected to blood pressure measurements taken 2 times (ie. 8 am and 4 pm), twice a week (ie. Monday and Thursday) for a run-in period of 2 weeks. During this period body weights and food intake were also recorded.

Blood Pressure Measurements - Blood pressure measurements were made using a non-invasive tail cuff method (II TC, Model 179-2, Harvard Apparatus, Holliston, MA). The 179-2 model uses a pneumatic cuff and measures systolic and diastolic blood pressure from the tail.

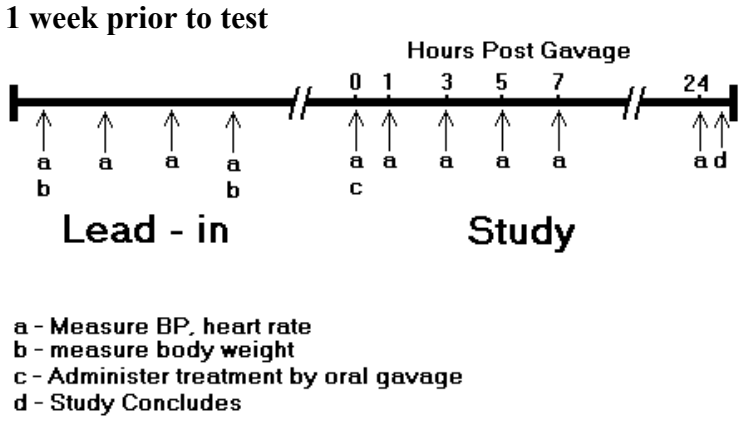
Treatment Groups - Blood pressure and body weight were measured before initiating the experiment (within 1 week of testing) and the rats were randomized (based on systolic blood pressure) into the following test groups:

Group	Treatment
1	0.9% saline (carrier)
2	CPH (0.15 g/kg body weight, in 0.9% saline) - Positive Control #1
3	Caseinate (0.15 g/kg body weight, in 0.9% saline) - Negative Control
4	NZMP WPH (0.15g/kg body weight, in 0.9% saline)
5	Biozate-1 (0.15g/kg body weight, in 0.9% saline
6	SPH (0.15g/kg body weight, in 0.9% saline)
7	FPH (0.15g/kg body weight, in 0.9% saline)
8	Captopril (10 mg/Kg body weight, in 0.9% saline) - Positive Control #2

CPH= corn protein hydrolysate; SPH= soy protein hydrolysate; FPH= fish protein hydrolysate; NZMP WPH= known only by sponsor. Number of rats per group= 1.

The test materials were administered by gastric gavage and blood pressure (systolic, diastolic, mean arterial pressure) and heart rate were measured at 0 (immediately prior to gavage), 1, 3, 5, 7 and 24 hours post-dose.

Work Plan



Statistical analysis **-** Statistical analyses were carried out using SAS (SAS Institute, Cary, NC, USA). Variables were compared with One Way Anova; considered significant at $p < 0.05$.

HYPOTHESIS

Protein-hydrolysates will lower blood pressure in this rat model.

2.3 RESULTS

We tested whether a single dose of oral hydrolysates derived from different protein sources (soy, fish, Biozate 1 (Calpis), and NZMP WPH) could significantly lower blood pressure in a well established animal model for hypertension. At 0, 1, 3, 5, 7, and 24 hours after oral administration of the potential anti-hypertensive protein hydrolysates NZMP WPH, Biozate-1, soy protein, and fish protein the SBP of the animals was

measured. The major results for the four outcome measures are shown in Figures 2.2 through 2.5. All the cardiovascular parameters mean profile over time, shows a tendency to lower values, but once compared, there was no significant difference between any of the protein hydrolysates tested and the control group. Time is the only result to be significant but test of the interaction between treatment/time was not significant. The mean values and SEM are shown for the four outcomes measures in Table 2.1. One Way ANOVA analysis to estimate the difference between groups is shown in Table 2.2.

2.4 DISCUSSION

Over the past 10 years a vast amount of time and resources have been devoted to studying the potential hypertensive effects of milk protein derived peptides (109-111). The major purpose for screening these peptides is for their ability to inhibit angiotensin-I-converting enzyme activity *in vitro* since this enzyme plays a central role in controlling blood pressure. *In vivo* studies with spontaneously hypertensive rats and hypertensive human volunteers have shown a significant blood pressure reducing effect by consuming specific milk protein hydrolysates and fermented dairy products (7;104-106).

The present study evaluated the effect of four protein hydrolysates on blood pressure in the SHR rat model. Under this study design, treatment of SHR with the different protein hydrolysates did not significantly decrease blood pressure. A number of factors could explain these results. First, the ability to inhibit ACE *in vitro* is indicative of the potential of a given protein hydrolysates to act as a hypotensive agent *in vivo.* However, in order to mediate a hypotensive effect *in vivo*, the hydrolysates must reach the target organ. Hydrolysates with potent *in vitro* ACE inhibitory activity did not show

a hypotensive effect *in vivo*, presumably due to degradation to inactive fragments during oral ingestion. These peptides need to survive degradation by gastrointestinal proteinases and peptidases in order to be absorbed; therefore, they need to pass from the intestine into the serum where they may be susceptible to brush border and intracellular enzymatic activities. The peptides also need to be resistant to degradation by serum peptidases. In this study, the hydrolysates were administered orally and thus, the oral ingestion of the protein hydrolysates containing ACE inhibitory peptides could have affected the stability of the peptides therein.

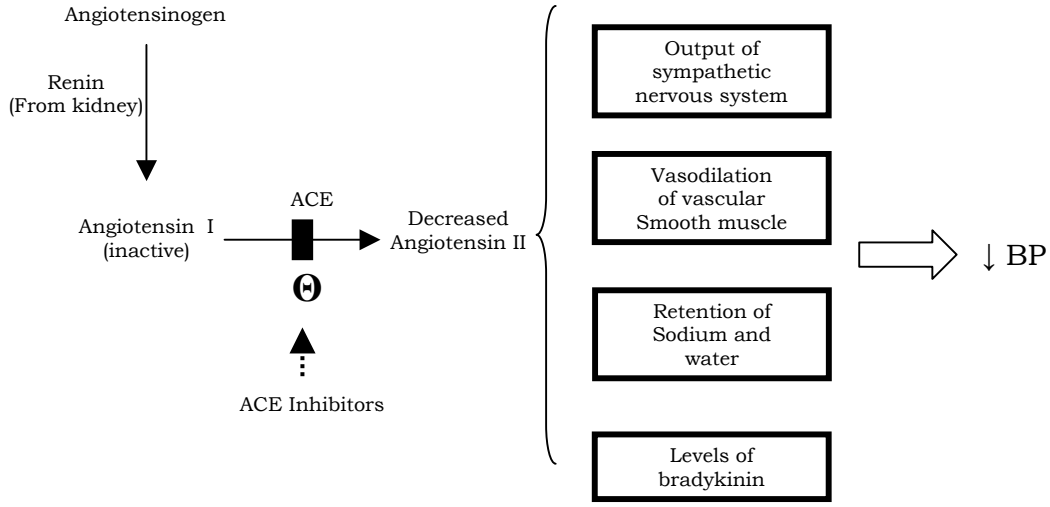
Besides, the peptide could have been transported intact to its target organ. However, the concentration transported could have been too low to exert an ACE inhibitory effect *in vivo*. While valuable information can be obtained from *in vitro* model systems with respect to the proteolytic/peptideolytic stability and susceptibility to intracellular passage, it is only through *in vivo* studies that the hypotensive effects of a given peptide or peptide preparation can be reliably assessed so the results obtained in this study may be indicative of no hypotensive action from the tested protein hydrolysates.

Finally, numerous rat studies have been performed to determine the hypotensive effects of milk protein derived ACE inhibitors. The differences in the responses of SBP and other cardiovascular parameters responses in this study and in the literature may not only relate to compositional differences in the test material investigated but also to the study design where the sample number, dosage, duration, route of administration, and choice of control differed.

More detailed studies are required for a better understanding of the blood pressure reducing mechanism(s) of food derived peptides as the hypotensive effect may not be entirely due to inhibition of ACE activity. For example, it was recently shown that α-lactorphin (whey protein-derived opioid peptide), reduced blood pressure in spontaneously hypertensive rats and in normotensive Wistar Kyoto rats in a dose-dependent manner following subcutaneous administration. However, the blood pressure reducing effect was absent in the presence of naloxone, an opioid receptor antagonist, indicating that the hypotensive effect was mediated through the vasodilatory action of binding to opiate receptors (108). The blood pressure reducing effect of complex systems such as fermented milk drinks and milk protein hydrolysates may only be in part due to ACE inhibition. Furthermore, the hypotensive effects of fermented milk drinks may also be in part due to the high levels of biologically available calcium present in these products (103). The hypotensive effects of high calcium, low fat dairy product diets have been well documented (95). There is need for detailed conclusive evidence demonstrating the hypotensive effects of consuming specific milk protein based products.

2.5 LIST OF FIGURES AND TABLES

Figure 2.1 ACE inhibitors - Mechanism



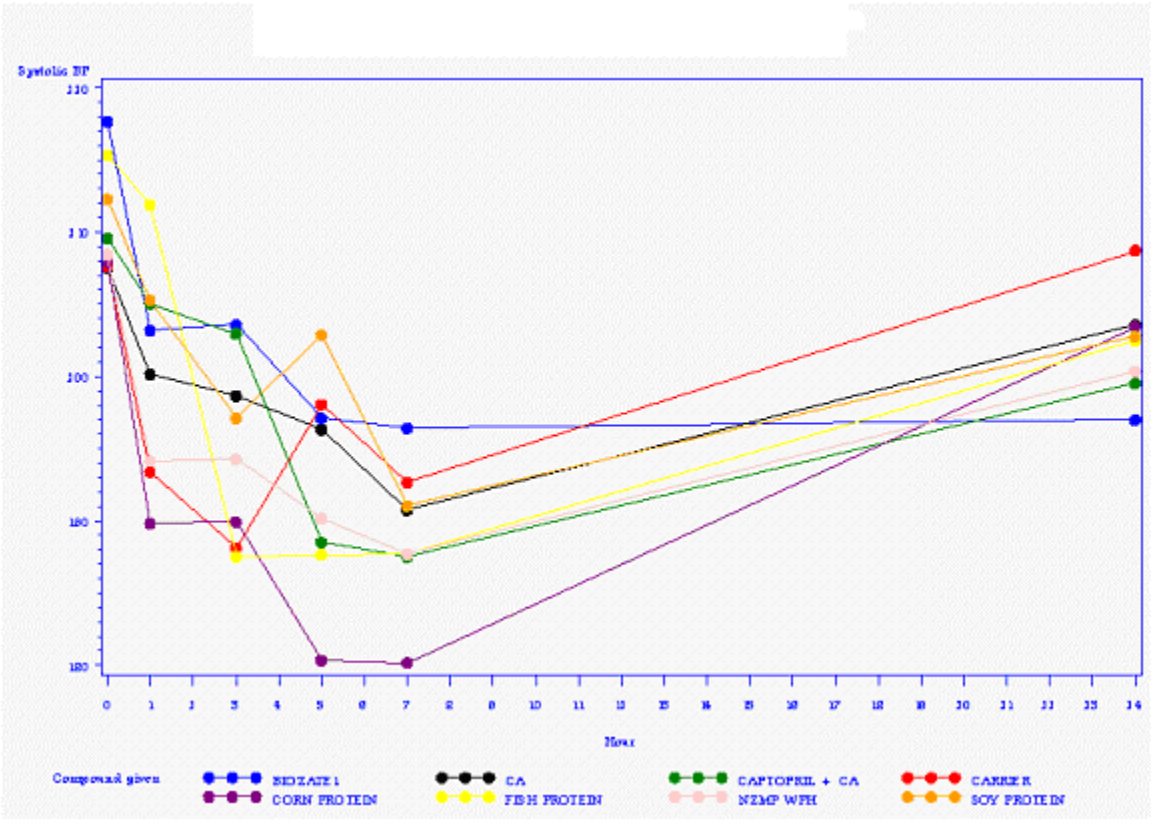


Figure 2.2 Mean profile of systolic blood pressure over time.

n= 1/group; for values and ±SEM refer to **Table 2.1**.

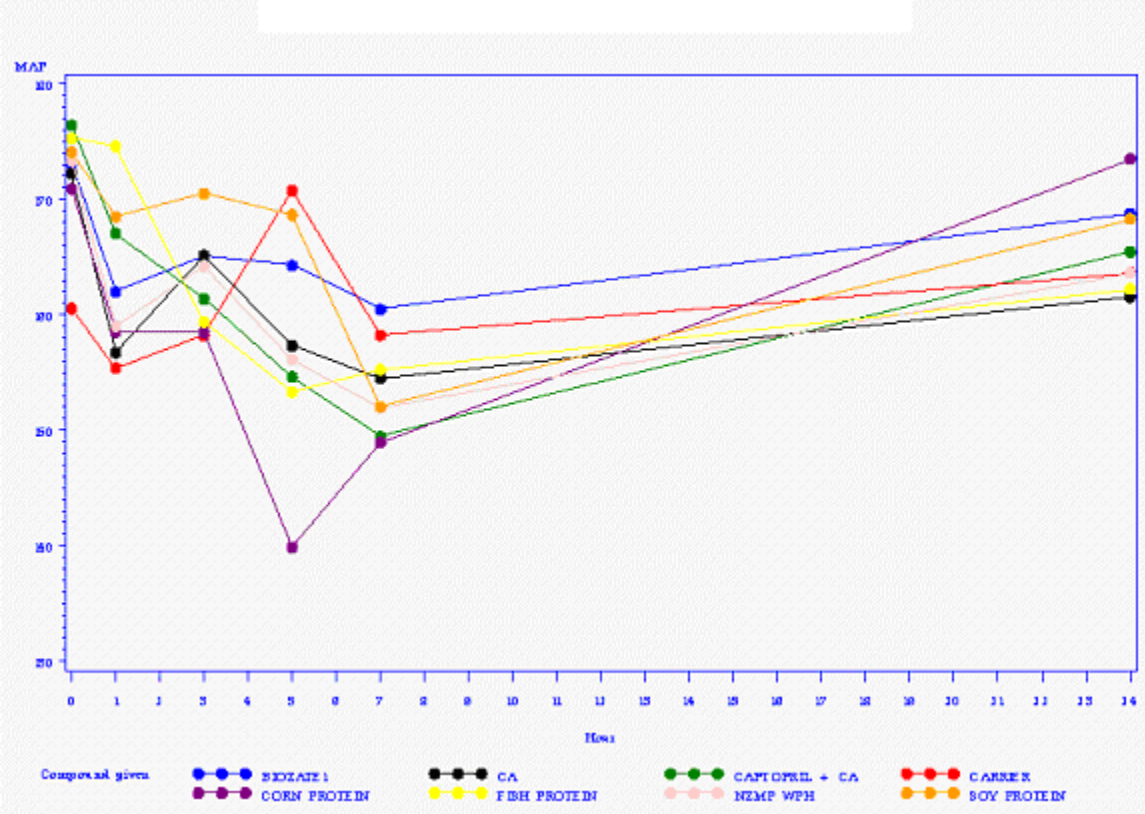


Figure 2.3 Mean profile of mean arterial pressure over time.

for values and ±SEM refer to **Table 2.1**.

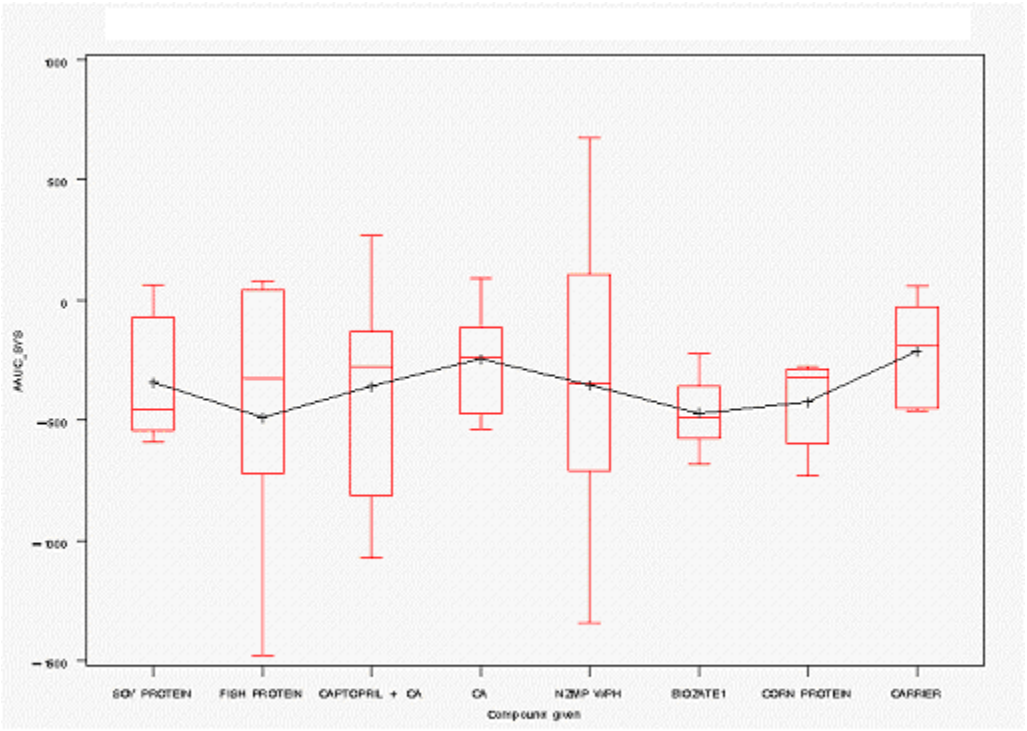


Figure 2.4: Changes in SBP of animals following administration of test materials.

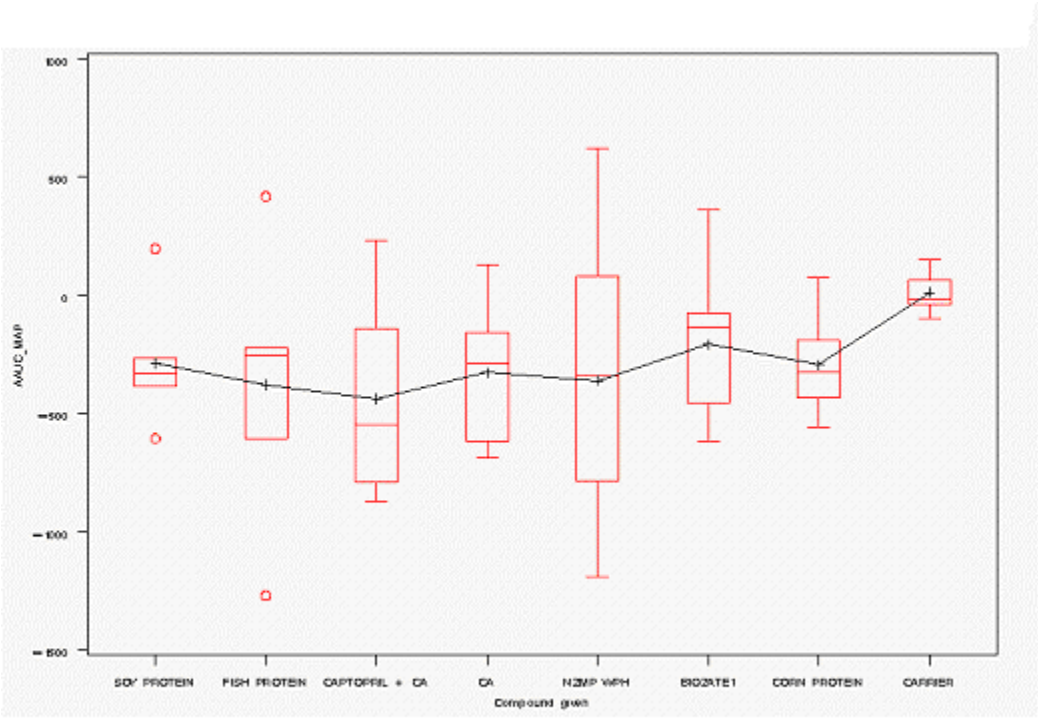


Figure 2.5: Changes in mean arterial pressure of animals following administration of test materials.

Table 2.1 Cardiovascular parameters of animals treated with different protein hydrolysates; values are expressed as mean (± SEM); n= 6 – 7.

SBP	mean (0)	SEM	mean (1)	SEM	mean (3)	SEM	mean(5)	SEM	mean (7)	SEM	mean (24hr)	SEM
Compound												
Soy	212.28	8.35	205.28	10.95	197.11	6.46	202.89	6.94	191.06	9.74	202.78	8.07
Fish	215.33	3.63	211.9	8.08	187.52	11.61	187.67	13.46	187.69	10.18	202.48	4.76
Captopril	209.57	4	205.05	4.76	202.95	4.24	188.52	5.58	187.52	11.16	199.52	8.29
CA	207.52	4.11	200.14	3.98	198.67	3.16	196.33	4.16	190.76	7.18	203.57	2.71
NZMP	208.43	7.78	194.14	7.05	194.29	4.35	190.19	4.77	187.71	5.61	200.38	6.85
Biozate1	217.67	4.44	203.19	4.72	203.57	5.98	197.1	6.07	196.43	5.24	197	3.31
Corn	208	4.22	189.83	5.06	189.94	4.22	180.33	9.91	180.17	6.5	203.44	6.63
Saline	207.67	6.49	193.4	6.31	188.13	4.96	198.07	4.51	192.67	6.26	208.73	2.82
DBP												
Compound												
Soy	154.78	8.96	149.86	12.17	158.39	3.97	153.78	10.49	132.61	11.38	151.06	7.45
Fish	155	3.22	155.95	9.7	144.43	11.8	136.1	10.36	139.26	11.26	141.79	7.56
Captopril	159.95	4.71	147.95	6.9	142.71	7.45	137.36	6.23	130.24	17.77	148.38	7.18
CA	154.81	6.9	135.4	9.61	148.48	6.97	138	9.75	136.36	9.63	140.52	6.94
NZMP	156.76	8.85	141.17	11.8	149.05	6.77	139.19	7.32	134.1	8.76	145.62	3.06
Biozate1	154.71	5.23	142.29	6.6	145.76	11.44	147.9	3.41	142.57	6.86	154.62	5.2
Corn	152.44	4.79	142.78	6.1	143.33	4.44	119.22	9.94	133.33	5.22	158.67	8.64
Saline	136.7	6.09	136.2	10.38	143.27	10.35	156.87	7.46	141	2.47	141.37	7.07
MAP												
Compound												
Soy	174.11	8.51	168.53	9.91	170.5	5.17	168.67	7.64	152.06	9.91	168.31	6.63
Fish	175.29	2.76	174.62	8.38	159.4	11.52	153.33	11.16	155.26	10.51	162.24	6.3
Captopril	176.43	2.82	167.05	5.36	161.38	5.25	154.64	5.26	149.48	15.28	165.48	7.44
CA	172.24	5.38	156.74	7.41	165.14	5.62	157.33	6.89	154.52	8.52	161.57	5.35
NZMP	173.36	8.09	159.05	10.27	164.24	5.45	156.14	6.1	151.98	7	163.62	3.85
Biozate1	173.17	4.71	162	4.76	165.05	9.16	164.29	3.49	160.48	5.32	168.79	3.68
Corn	170.94	4.17	158.5	5.62	158.53	2.53	139.89	9.54	148.94	5.69	173.5	7.9
Saline	160.57	5.05	155.4	8.29	158.2	8.49	170.77	6.32	158.27	1.56	163.63	5.01
HR												
Compound												
Soy	395.06	12.23	368.06	11.01	343.5	44.64	375.56	19.68	368.28	9.83	363.83	15.82
Fish	388.67	16.14	388.29	12.19	367.9	5.63	369.05	7.32	362.9	10.78	391.95	14.72
Captopril	383.52	12.12	379.1	12.8	380.19	13.83	363	13.15	358.14	16.52	389.62	18.25
CA	407.05	12.34	364.05	15.08	361	13.01	347.38	9.17	344.1	9.94	372.29	11.22
NZMP	387.14	19.83	377.14	10.63	367.24	6.84	363.1	14.07	355.9	9.65	382.38	10.53
Biozate1	373.24	13.1	392.43	15.55	372.62	11.17	370.52	14.9	347.05	8.02	383.33	11
Corn	396.94	17.95	378.56	5.65	377.22	11.51	363.33	13.91	349.39	10.03	377.78	19.06
Saline	393.33	8.38	397.4	21.07	370.87	13.68	373.6	8.45	350.8	5.65	388.47	9.53

Table 2.2 Comparison of the groups with One Way ANOVA; time is the only result to be significant but test of the effect of treatment*time interaction was not significant.

Variable	Effect	Result
Systolic blood pressure	Treatment	NS
	Time	<0.0001
	Treatment*time	NS
Diastolic blood pressure	Treatment	NS
	Time	<0.001
	Treatment*time	NS
Mean arterial pressure	Treatment	NS
	Time	<0.0001
	Treatment*time	NS
Heart rate	Treatment	NS
	Time	<0.001
	Treatment*time	NS

CHAPTER 3: EVALUATION OF POTENTIAL IMMUNOMODULATORY COMPOUNDS USING A MURINE MODEL FOR IMMUNE SUPRESSION

3.1 INTRODUCTION

Immunomodulation is a process that can alter the immune response of an organism by interfering with its normal functions. If the action of the compound results in an enhancement of immune reactions it is named an immunostimulative compound, which primarily stimulates a non-specific system, i.e. granulocytes, macrophages, complement, and T-lymphocytes. Immunosuppression refers to reduced resistance against infections and stress, which arises from environmental or chemotherapeutic factors (112).

Immunomodulation helps boost or prevent decline of the immune responses, as seen in aging. Despite the recent development of new immunomodulatory drugs, there is need for less toxic and more widely applicable compounds (113). Hence, a search for better compounds exerting the above mentioned activities has become a field of major interest (114;115). Once identified, one can evaluate compounds for their ability to immunomodulate, as well as for their effectiveness in reducing immune response dysfunction.

The interrelationship between diet and the immune system has become the focus of increasing attention as an increasing number of bioactive food components are being identified as having immune-modulating function. Amino acids such as glutamine, cysteine, and dimethylglycine, as well as the protein lactoferrin, may have immunomodulating activity. Glutamine appears to be required to support proliferation of

mitogen-stimulated lymphocytes, as well as the production of interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) Rohde T Scan J Immunol 1996; 44:648; same author Med Sci Sports Exerc 1998; 30:856]. It is also required for the maintenance of lymphokine-activated killer cells [ ]. Glutamine can enhance phagocytosis by neutrophils and monocytes [ ]. N-acetyl cysteine is a delivery form for cysteine, which serves as a major precursor for synthesis of glutathione, thus, cysteine immunomodulatory potential could relate to reduction of oxidative stress. Dimethyglycine (DMG) potential modulatory action comes from research were it enhanced both humoral and cell-mediated immune responses [Reap EA 1990; j lab clin med 115:481].

A few mechanisms are proposed for lactoferrin's possible immunomodulatory activity. Lactoferrin (LF) appears to bind uniquely in the region of major histocompatibility (MHC) proteins and the CD4 and CD8 determinant on T4 (helper) and T8 (suppressor) lymphocytes; it bears sequence homologies with MHC Class II determinant [Legrand D Biometals; 2004; 17:225). LF also appears to play a role in the regulation of cytokines and lymphokines, such as tumor necrosis (TNF)-alpha and IL-6.

Despite the development of antibiotics and vaccines, infectious diseases are a continuing threat to humans, especially the elderly where the age-associated decline of immune function contributes to the increased susceptibility to infection (116). Changes in immune functions with aging include dysregulation of cytokines, including a change in T helper (Th) 1/Th2 cytokine balance (117).

Watson et al. (118-120) studied the effects of age on immune function in an aged mice model (C57BL/6, 12-20 months of age). Their results shows a significant decrease ($P<0.05$) on splenocyte proliferation due to aging, which could be overcome by nutritional intervention with vitamin E and dehydroepiandrosterone. Similar responses had also been observed in human studies (11;121).

Since data support a role of nutrition in enhancing the immune response in the aged mice model, in the current study, the same model was used to assess diets containing ingredients that could potentially overcome immune dysfunction. In this study, we evaluated the activity of six compounds, provided by Ross Product Division, Abbot Pharmaceutical, that could potentially overcome immune dysfunction through immunomodulation.

OBJECTIVE

The aim of the study is to evaluate the immunomodulatory effect of varying doses of nutritional compounds on specific immunological parameters using an aged murine model with impaired immunological system. Specifically, the current study aims are to 1) reproduce previous immunological findings in an aged mice model, and 2) evaluate the effect of different doses of several nutritional compounds on specific immunological parameters (cytokine production and splenocytes proliferation) in this model. This research was designed to determine the optimum dose of select immunomodulators required to restore immune functions, as the optimal dose will be used to design future studies in this area.

3.2 MATERIALS AND METHODS

Experimental Design, Animals, and Treatment

All animal studies were performed with the University of Arizona animal review committee approval.

Animals

Twelve C57BL/6 female mice, three-month old (± 1 month), and 156 C57BL/6 retired-breeder female mice, 14-month-old (± 1 month) were purchased from Charles River Laboratories (Wilmington, DE). These animals were housed in the University of Arizona animal facility, accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were housed in groups of four per cage in a pathogen free facility with constant temperature of 70ºF, 35-40% relative humidity, and a twelve-hour light/dark cycle.

Measurements and Assays

Potential Immunomodulatory Compounds

Initially, the sponsor company chose a series of polyphenol/flavonoids compounds for testing. However, in the end only one polyphenol compound/mixture was provided for testing along with five other compounds chosen by Ross Production Division as potential immunomodulatory candidates from the literature. Thus, following are the compounds evaluated.

EID - Ensure Immune Defense
DMG - Dimethyglycine
LAC - Lactoferrin

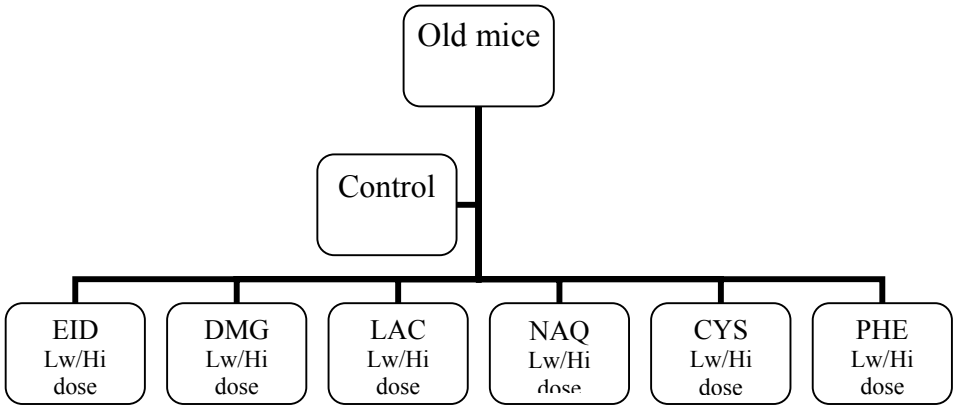
NAQ	-	N-acetyl glutamine
CYS	-	Cysteine Peptide
PHE	-	Polyphenol combo
CON	-	Control Diet

Diet

Twelve mice from each of the 3- and 14-month-old animals were fed a purified balanced synthetic diet, the American Institute of Nutrition diet (AIN-93 diet), and served as control groups. One hundred forty-four mice 14-month-old were divided into six groups of 24; each group was further subdivided into two subgroups of twelve mice each. The subgroups were treated with a LOW and HIGH dose for each compound, respectively. Doses were defined by Ross Production Division. The compounds were mixed on a base of AIN 93 synthetic diet. Water and the synthetic diet were provided *ad libitum* for 6 weeks. Once the feeding period was completed, mice were euthanized using carbon dioxide asphyxiation.

The work plan is illustrated as follows:

n= 156 24/group; 12/dose



***IL-2 and IL-4 determination* (17):**

Cytokines were measured by enzyme-linked immunosorbent assays (ELISA). Spleens were gently teased with forceps in culture medium (CM, RPMI 1640 containing 10% fetal bovine serum, 1% penicillin and streptomycin), resulting in a suspension of spleen cells. Red blood cells were removed by addition of a lysis buffer (0.16 M ammonia chloride Tris buffer, pH 7.2) at 37^{o}C for 3 minutes. After lysis, splenocytes were washed twice with culture medium. The concentration of cells was determined and adjusted to 1 X 10^7 cell/ml. Splenocytes (0.1 ml/well; 1x10^7 cell/ml) were cultured in triplicate on 96-well flat-bottomed culture plates (Falcon 3072, Lincoln Park, NJ) with CM. Splenocytes were then stimulated with Con A (0.1 ml/well; 10 μg/ml; Sigma) for 24 hours and incubated at 37^{o}C, 5% CO_2 to induce IL-2, and IL-4 production. After incubation, the culture media were collected and analyzed. The presence and type of cytokines were determined by ELISA using a commercial ELISA MiniKit (Endogen, Cambridge, MA) following the instructions of the manufacturer.

DATA ANALYSIS

One Way Analysis of variance (ANOVA), was used to evaluate the statistical significance of changes in all indices as function of dose and intervention. Tests of feeding group main effects were two-sided and declared significant at the 0.05 level. These results are reported in bold in the tabulation of tests. P-values between 0.05 and

0.10, while not statistically significant, are reported in parentheses in the results section as they would possibly show a trend in the data. Summary and analysis of data were run using SAS Version 8.2 on the PC (SAS Institute, Cary, NC, USA).

HYPOTHESIS

The following hypothesis was tested in this study:

1. The immunological responses (splenocyte proliferation, Th1 and Th2 cytokines) of the aging animals fed the control diet will show a difference from that of the young animals fed the control diet.

3.3 RESULTS

Cytokine production of splenocytes

IL-2

1. The *in vitro* production of Th1 cytokine, IL-2, by ConA –stimulated splenocytes from old mice was significantly lower (p=0.0032) when compared to splenocytes from young mice. See discussion for meaning.

2. T-helper 1 cells produced cytokines, including IL-2 to regulate cellular immunity, and suppressed cytokine production by T-helper 2 cells. Of the six compounds evaluated in old mice, only NAQ-High dose diet caused a significant (p=0.0086) change in IL-2 production, where LAC-High dose was marginally significant (p=0.0643). For all other

compounds the level determined was less than or equal to the control. As a result, no minimum effective dose could be determined.

IL-4

1. Release of Th2 cytokine, IL-4, by mitogen-stimulated splenocytes was significantly ($p=0.0370$) increased in old mice when compared to young mice. See discussion for meaning.

2. T-helper 2 cells produced cytokines including IL-4, which suppress cytokine secretion by T-helper 1 cells. Intervention with the six compounds/two doses in old mice caused no significant change of IL-4 production by cells. As with IL-2, this result means that the level determined was less than or equal to the control; therefore, there is no minimum effective dose.

3.4 DISCUSSION

From ancient times, medicinal treatment has derived from natural sources, either plants or microorganisms. Gradually, the amount of knowledge about chemical compounds acting as drugs increased, as well as the number of compounds from natural origin and their role in modern medicine. Examples of these compounds include immunomodulators as Cyclosporin A and Sanglifehrin A used in organ transplantation (113;122). In recent years, intense research interest has been focused on identifying biologically active food or food-components within our diet that could have an impact on immune function.

For the current study, six nutritional compounds, two doses per compound, were tested. These nutritional compounds were chosen for their potential immunomodulatory properties based on compounds already existing in the market (Ensure Immune Defense), as well as from novel compounds at the Ross Production Division's own *in vitro* testing (dimethyglycine, lactoferrin, N-acetyl glutamine, cysteine peptide), and literature background (polyphenol combo).

It is well known that natural products, such as flavonoids, can affect mitogenesis and cytokine production via stimulation (IL-2) or suppression (IL-4) of T cells. (123). In this study, the production of T-cells was determined by the amount of ^{3}H-thymidine incorporated into the nucleus from newly replicating DNA, which takes up thymidine for growth. The chosen compounds, including a mixture of polyphenols, were tested; however, problems with the assay invalidated the results obtained.

Cytokines are regulatory polypeptides secreted during the generation of an immune response by lymphocytes, macrophages, endothelial cells, and a variety of other cell types (124). Certain cytokines can accelerate macrophage production, enhance their function, or potentially decrease their function (125). Thus, cytokines are essential mediators of cellular responses. T-helper 1 cells stimulate secretion of IL-2 and T-helper 2 cells stimulate secretion of IL-4. IL-2 is a potent stimulatory factor for NK cells, which induces proliferation, cytokine production, and increased cytotoxic activity (126). Polyphenols, in general, have been found to decrease the level of basal and induced secretion of cytokines in a variety of cells. Nair et al. (127) found that quercetin down-regulated IL-4 gene expression, as well as IL-4 production by peripheral blood mononuclear cells. In another investigation, production of IL-8 by human bronchial gland cells was decreased by genistein in a dose dependent manner (128). The data in our study suggest that levels of IL-2 were significantly increased by N-acetyl glutamine (NAQ) and marginally significant by lactoferrin (LAC). However, IL-2 levels of all the other compounds were below the control levels, indicating that the dose used in this study were not an effective Th1 stimulator in ageing mice.

Dysregulation of IL-4 production may be a major contributing factor in aging (129). IL-4 production also increases as immune systems become unregulated, as an apparent normal consequence of the aging process. In this study, the compounds used did not lower IL-4 production in old mice when compared to the control. This result might be due to (i) the compounds themselves not being effective in modulating this immune parameter or (ii) the doses used and duration of the intervention was not

sufficient to promote metabolic changes that would have had an impact on the immune response of the animals or (iii) time point in lifespan was not appropriate. In the case of the polyphenol diet and perhaps other compounds tested here also, this result might be due to the fact that polyphenols are in conjugated forms in the circulatory system and would undergo metabolism, such as methylation, in the liver. The metabolism would largely reduce the effectiveness of the polyphenolic compounds. Recently, Arts et al. (130) reported that albumin can mask the antioxidant capacity of flavonoids.

In the present study, consumption of the test compounds gave no indication of effectiveness in preventing immune dysfunction related to aging, contrary to initial expectations. However, the immunological response (Th1 and Th2 cytokines) of the aging mice did show a significant difference from that of the young mice fed the control diet, results consistent with previously inmmunological findings produced in an aged mice model (118;131).

A possible cause for the negative results obtained after dietary intake of the six compounds might be that the constituents, which may have led to an increase in cytokine production under *in vitro* conditions, either cannot be absorbed in the small intestine or are inactivated by digestion. In animal studies with extracts, it was helpful to provide the compound of interest by gavage (132) or intraperitoneally (133) to avoid potential inactivation of the active ingredients by digestion and/or metabolism.

Another factor to consider in this study is the gender of the mice used. The negative effects in immunity of the compounds tested might be related to the immune response in female mice. There does not appear to be evidence in the literature

suggesting gender specific differences in immune function response in mice. On the other hand, there is evidence for gender differences in the non-specific and specific immune response in humans (134). A further factor to consider relates to the use of combinations of the compounds. Mixtures of different plant extracts have been shown to exert different, stronger, weaker, or even opposing, effects on immune function than individual plant extracts (135;136). Finally, more than two doses should be tested since some of the compounds showed indications of increasing cytokine production with the doses used but the response was not large enough to demonstrate significant differences.

This study was product-driven. This means, at a practical level, that resources and effort were directed toward a particular facet of the immune response which may have been deemed commercially important. The immune system comprises a complex interplay between cells and molecules, and between effector immune system cells and remote signals from "non-immune system" sources. Thus, it should never be considered that research which homes straight in on to an end-point effector mechanism will discern immediately whether a particular food or food component is or is not important in immune modulation.

3.5 LIST OF FIGURES AND TABLES

Table 3.1 Comparison of the two doses of the six nutritional compounds on cytokines IL-4 and IL-2 production in old mice; n = 5 to 12 mice in each group

IL-4 (pg/ml)

Young vs Old fed Control Diet Determine MED by Compound	**Old > Young p=0.0370**
EID	No MED
DMG	No MED
LAC	No MED
NAQ	No MED
CYS	No MED
PHE	No MED
Compare MED	No Comparison Made

IL-2 (pg/ml)

Young vs Old fed Control Diet Determine MED by Compound	**Young > Old p=0.0032** The mean IL-2 for the resulting MED for each compound below are all less than the CON mean.
EID	No MED
DMG	No MED
LAC	MED□High (p=0.0643)
NAQ	**MED□High p=0.0086**
CYS	No MED
PHE	No MED
Compare MED	NS

The results in the table show that none of the doses used were effective in cytokine production, except for NAQ-High dose ($p<0.0086$), meaning that the doses used were less efficient than the control in stimulating cytokine production. Note that all p values

are not significant except for the comparison between old and young mice fed the Control diet ($p < 0.05$); and compound NAQ, High dose; LAC, High dose was marginally significant.

Splenocyte IL-2 Production

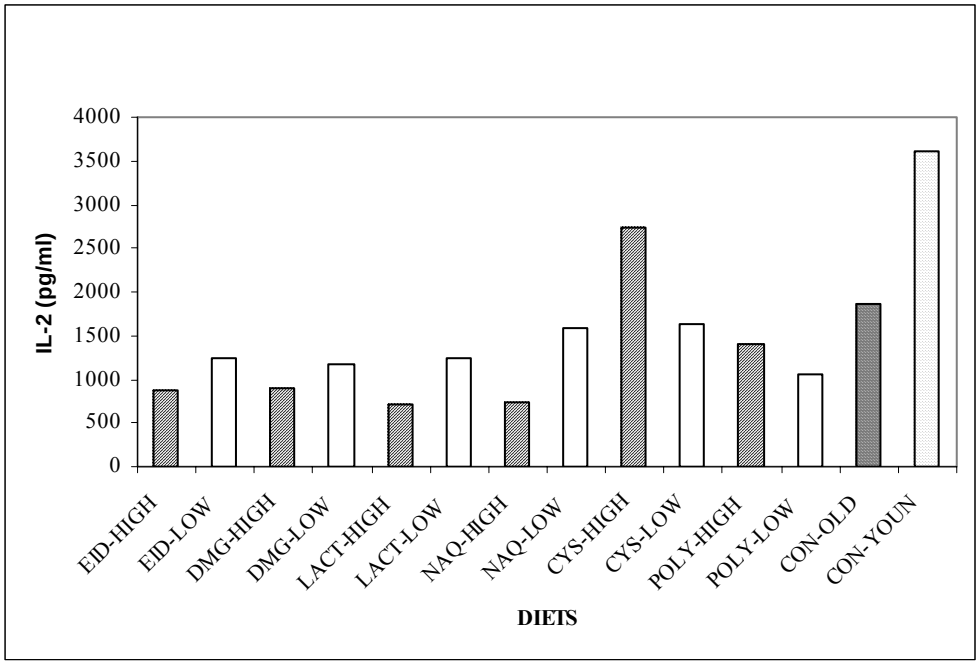


Figure 3.1 Mean *in vitro* splenocytes IL-2 production of the two doses (low and high) of the six nutritional compounds in old mice.

The assay was conducted by ELISA. Values represent the mean ± SEM of 6 to 12 mice in each group. EID = Ensure Immune Defense; DMG = Dimethylglycine; LACT = Lactoferrin; NAQ = N-acetyl glutamine; CYS = Cysteine peptide; POLY = Polyphenol combo; CON = control diet

[a] $P < 0.05$ compared control diet young mice to control diet old mice [b] $P < 0.05$ compared NAQ minimum effective dose (MED) to high dose

Table 3.2 Mean values and SEM for differences in IL-2 production for the six compounds tested, two doses

Diet	IL-2 (pg/ml)	
	Mean	**±SEM**
EID-HIGH	878.9	219.8
EID-LOW	1251.8	358.8
DMG-HIGH	903.6	268.4
DMG-LOW	1174	217
LACT-HIGH	717.2	291.1
LACT-LOW	1231.3	285.4
NAQ-HIGH	741.1	285.3
NAQ-LOW	1577.3	396.1
CYS-HIGH	2739.3	935
CYS-LOW	1621.5	413.7
POLY-HIGH	1401.5	396.3
POLY-LOW	1056.5	252.7
CON-OLD	1851.7	295.5
CON-YOUN	3609.2	409

Some samples were done in duplicate, others in triplicate. Values represent the mean ± SEM of 6 to 12 mice in each group. IL-2 = interleukin 2; EID = Ensure Immune Defense; DMG = Dimethylglycine; LACT = Lactoferrin; NAQ = N-acetyl glutamine; CYS = Cysteine peptide; POLY = Polyphenol combo; CON = control diet

Splenocyte IL-4 Production

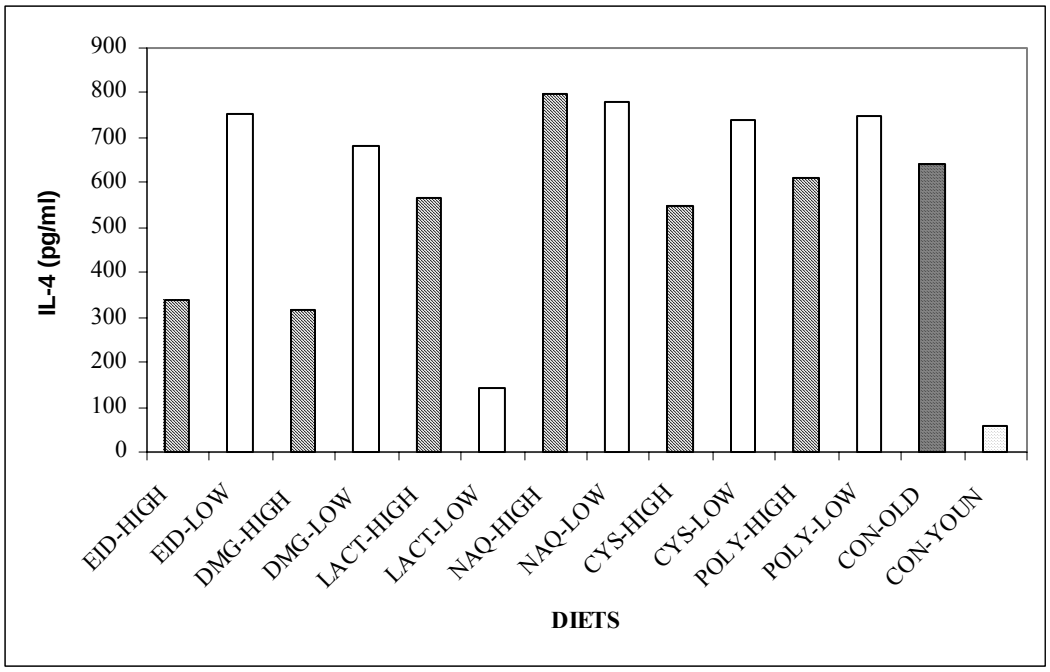


Figure 3.2 Mean values of the two doses (low and high) of the six nutritional compounds on *in vitro* splenocyte IL-4 production in old mice.

The assay was conducted by ELISA. Values represent the mean ± SEM of 5 to 11 mice in each group; EID = Ensure Immune Defense; DMG = Dimethylglycine; LACT = Lactoferrin; NAQ = N-acetyl glutamine; CYS = Cysteine peptide; POLY = Polyphenol combo; CON = control diet; none of the comparisons was statistically significant.

Table 3.3 Mean values and SEM for differences in IL-4 production for the six compounds tested, two doses

Diet	IL-4 (pg/ml)	
	Mean	**±SEM**
EID-HIGH	338.9	102.4
EID-LOW	751.8	260.7
DMG-HIGH	318.4	110.3
DMG-LOW	683.2	251.4
LACT-HIGH	566.4	289.8
LACT-LOW	140.7	41.2
NAQ-HIGH	799.4	280.9
NAQ-LOW	777.5	318.2
CYS-HIGH	548.6	179.9)
CYS-LOW	740.1	342
POLY-HIGH	609	429.5
POLY-LOW	749.6	312
CON-OLD	641.5	340.9
CON-YOUN	57.5	16

Some samples were done in duplicate, others in triplicate. Values represent the ± SEM of 5 to 11 mice in each group. IL-4 = interleukin 4; EID = Ensure Immune Defense; DMG = Dimethylglycine; LACT = Lactoferrin; NAQ[a] = N-acetyl glutamine; CYS = Cysteine peptide; POLY = Polyphenol combo; CON = control diet

Acknowledgement

This study was funded in part by an NIH fellowship T32 AT001287-01 from the National Center for Complementary and Alternative Medicine (NCCAM).

CHAPTER 4: *IN VIVO* EFFECTS OF BIOACTIVE COMPONENTS PRESENT IN HOP AND BORAGE EXTRACTS ON OXIDATIVE STRESS INDUCED BY RETROVIRAL INFECTION

4.1 INTRODUCTION

Currently, there is a strong interest worldwide in searching for new and safe antioxidants from natural sources for human consumption to improve physiological and immunological responses to oxidative stress caused by environmental factors. A large number of natural antioxidants have been isolated from different types of plant derivatives and products such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (137-141). Among natural antioxidants, polyphenolic antioxidants are widely distributed in the plant kingdom. For example, polyphenolic compounds occur in oilseeds, as the hydroxylated derivatives of benzoic and cinnamic acids, flavonoids compounds, and lignins (142). Polyphenolic extracts of plant derivatives have been shown to neutralize free radicals in various model systems, by quenching oxygen-derived free radicals, as well as the substrate-derived free radicals (143;144). The quenching process occurs by electrophilic attack of the polyphenol molecule to a hydrogen atom or an electron of the free radical, as illustrated in the following reactions:

$$ROO^* + PPH \rightarrow ROOH + PP^*$$

$$RO^* + PPH \rightarrow ROH + PP^*$$

The phenoxy radical intermediates are relatively stable and, as a consequence, a new chain reaction is not easily initiated. The phenoxy radical intermediates act also as terminators of the propagation route by reacting with other free radicals (145):

$$ROO^* + PP^* \rightarrow ROOPP$$

$$RO^* + PP^* \rightarrow ROPP$$

Several authors have reported that extracts of various oilseed meals exhibited antioxidant properties and in some cases exerted better antioxidant effects than synthetic antioxidants at the same concentrations (139).

Plants synthesize phenolics compounds to counteract the oxidative effects of reactive species, such as reactive oxygen species (ROS), and substrate derived-free radicals produced during the process of photosynthesis. ROS generated by metabolic subproducts or environmental cues play an important role in tissue damage in humans (83). Reaction of reactive oxygen species (ROS) with biomolecules such as membrane lipids, proteins, and DNA can induce irreversible modifications in their molecular structure (146). Membrane lipid peroxidation has particularly been associated with many tissue injuries and disease conditions (147;148). Plant polyphenolics can delay the onset of lipid oxidation and decomposition of hydroperoxides in food products, as well as in living tissues (149). Polyphenolic acids such as ferulic, sinapic, and caffeic acids have been involved in various stages of chain initiation and propagation reactions during the oxidations of pure triacylglycerols and methyl esters of sunflower oil (150). In addition, studies in animal models and *in vitro* systems have shown that polyphenolic antioxidants can inhibit free radical-induced damage to macromolecules (151). One of the most extensively investigated polyphenolic antioxidants is green tea catechins

One natural product that has caused great interest among medical and nutritional research groups is borage (*Borago officinalis* L) oil due to its high content of γ-linolenic

acid (GLA). While a great number of different spices and aromatic herbs have been tested for their antioxidant activity, the knowledge about antioxidant properties of crude extract of borage is scarce.

Studies done by Bandoniene (2002), and Wettasinghe (1999, 2002), showed borage as a potential natural antioxidant and indicated its applicability in the food industry as a substitute for synthetic antioxidants in bulk oils and meat products to retard lipid oxidation. Wettasinghe et al. (152) found that borage meal and its extract have antioxidant properties, which are concentration-dependent. In a later study by the same group, it was reported that borage extracts possess strong ROS scavenging properties, which are either comparable or superior to those of authentic catechin and sinapic acid at the same concentration (153). Another study detected the activity of several radical scavenging compounds in crude extract of borage leaves by a new on-line HPLC-DPPH method (154).

In the brewing industry, the female inflorescences of the hop plant (*Humulus lupulus* L.) called hops are widely used in the brewing industry to add bitterness and aroma to beer. Hop cones are also used in folk medicine as a tranquilizer or bitter stomachic (138). Hop constituents are reported to have a variety of interesting properties including antioxidant (155), antibacterial (156), and potential anticancer (157;158) activities. Hops also contain the potent phytoestrogen 8-prenylnaringenin (159). Hop contains hundreds of secondary metabolites comprising many different groups of organic compounds, among which the hop polyphenols are one of the main classes (160). Hops polyphenols are complex mixtures consisting predominantly of flavonoids. As

antioxidants, the flavonoids inhibit lipid peroxidation induced by several prooxidants as demonstrated with liver homogenate, microsomes, mitochondria and liposomes (151). As mentioned earlier, lipid peroxidation initiated by free radicals has been implicated in the pathogenesis of a number of disease conditions such as cancer, atherosclerosis, neurological disorders, and toxic cell injury**.** Since the antioxidant activities of flavonoids are related to their ability to react with ROS, flavonoids exerting antioxidant activities may be useful to help maintain human health by protecting cells from oxidative damage.

There are several lines of evidence suggesting hop as a potential material for antioxidant development. Studies on structure-activity relationships (SAR) have shown that a catechol group in the B ring and a 3- and -5 hydroxyl group are important for the antioxidant activity of flavonoids (161). In a recent study, flavonoids such as chalcones and flavanones with prenyl or geranyl groups were isolated from hops (162). As phenolic compounds, these unique hop flavonoids may have antioxidant activities since chalcones with hydroxyl and methoxy groups have exhibited antioxidant activities (163). In addition, flavones with prenyl groups such as artonins and cycloheterophyllin isolated from *Artocarpus heterophyllis* Lam are powerful antioxidants while other prenylflavones were shown to be ineffective (164). Furthermore, chalcones, including xanthohumol, have been shown to inhibit the production of nitric oxide (165)

In summary, these findings suggest that current information on the chemical and biological properties of hops and borage are insufficient and further investigations are needed. Borage and hop extracts may be used as alternative compounds to prevent or

treat human diseases associated with oxidative tissue damage. However, such applications have to be adequately justified using animal and clinical studies to support the research.

OBJECTIVE

The objective of this study was to evaluate the antioxidant properties of two doses of borage and hop extracts on oxidative stress using a murine model for leukemia virus (LP-BM5) infection.

Hypothesis: The dietary consumption of the extracts, hops and borage extracts, by young leukemia-infected mice will significantly reduce hepatic oxidative damage relative to infected mice that consume a control diet.

4.2 MATERIALS AND METHODS

Experimental Design, Animals, and Treatment

Plant Extracts

The antioxidant capacity of two plant extracts, hops and borage, were evaluated. These compounds were obtained from Dr. Food, Dr. Watson's New Zealand collaborator.

Concentration

A diet containing plant extracts at concentrations of 10 mg/kg diet or 50 mg/kg diet concentrations was chosen. The concentrations chosen were based on results obtained in previous experiments in the laboratory of Dr. Ronald Watson using other plant extracts from boysenberries, evening primrose seeds, or black currants. Their data suggest plant

extracts used to treat mice at these concentrations suppressed hepatic lipid peroxidation and prevented hepatic α-tocopherol loss (manuscript in process).

Animals

Seventy-two C57BL/6 female mice, four-month-old, were housed in groups of four mice per cage in a pathogen free facility with constant temperature of 70 F, 35-55% relative humidity**,** and a twelve-hour light/dark cycle. After one week of acclimatization, sixty mice were infected via intraperitoneal with 0.1 ml inoculum containing the LP-BM5 virus in minimum essential medium with an esotropic titer (XC) of 4.5 x 10^{-3} $\log_{10}$ plaque-forming units /L. Two weeks following LP-BM5 infection, the mice were fed with the supplemented diets for 12 consecutive weeks and the animals body weight and diet consumption were recorded weekly. Animals were sacrificed at the end of the feeding period of 12 weeks.

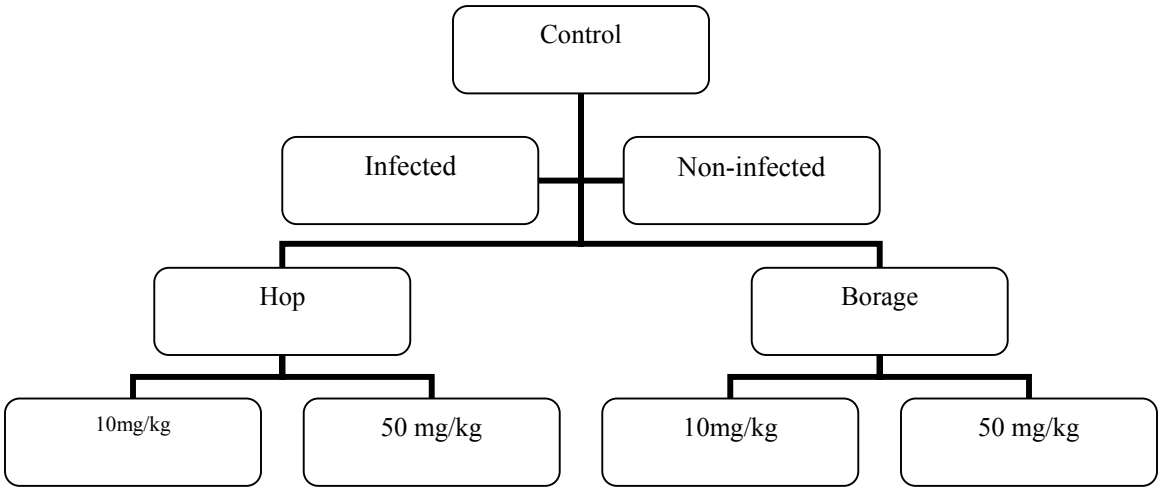
Diet Treatments

Mice were fed a purified balanced synthetic diet, American Institute of Nutrition Diet (AIN-93 diet, Dyets Inc. Bethlehem, PA, USA) supplemented with either 10 mg hops/kg of diet, 50 mg hops/kg diet, 10 mg borage/kg diet, 50 mg borage/kg diet, or a control diet (AIN-93 base diet). Water and the diets were provided *ad libitum* for 12 weeks.

Work Plan

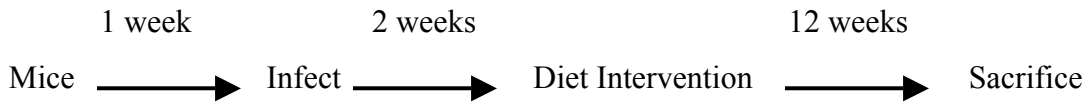
Seventy-two C57BL/6 female mice, 4 months old infected with retrovirus (LP-BM5) Duration: 12weeks of dietary intervention.

The work plan is illustrated as follows:



n= 12/group

Overall design consisted of : seventy-two C57BL/6 female mice, 4 month's old infected with retrovirus (LP-BM5);



Tests

Mitogen stimulation and proliferation of splenocytes:

Splenic T-cell proliferation was determined by ^{3}H-thymidine incorporation. Splenocytes ($1x10^{7}$ cell/L) in 0.1 ml of RPMI 1640 supplemented with 10% FBS (fetal bovine serum) and 1% antibiotics (penicillin-streptomycin) were cultured in 96-well flat-bottom tissue culture plates with concanavilin A (Con A, 10 μg/ml) and lipopolysaccharide (LPS, 10 μg/ml) added in triplicate. Plates were incubated at 37^{0}C with 5% CO_2 for about 44 hours for Con A induced T-cell proliferation, and then pulsed with ^{3}H-thymidine (0.5 μCi/well, New England Nuclear, Boston, MA). After 4 hours, plates were harvested by a cell-sample harvester (PHD Cell Harvester, Cambridge Technology Inc., Cambridge, MA). Radioactivity was determined by a liquid scintillation counter (LS 6500 Multi-purpose Scintillation Counter Beckman). Data obtained from this experiment are presented as counts per minute (cpm).

Measurement of hepatic tissue lipid peroxides (LPO):

Liver tissues were removed gently and stored at -70^{0} C. Quantitative determination of lipid peroxides in the liver was done using the LPO-CC K-ASSAY (Kamiya Biomedical Company, Seattle, WA). Phospholipids were extracted from approximately 0.2 g of liver in a chloroform/methanol mixture (2:1), v/v). After centrifugation at 2,800-3000 RPM, the chloroform layer was mixed with 0.6 ml saline to separate protein. The chloroform layer was evaporated in a steady flow of nitrogen gas, and lipid residues were dissolved with 100 μl of isopropanol. Experimental samples, standards, and controls were added

in triplicate in different wells of the same 96-well microplate. Lipid peroxides were quantified colorimetrically by measuring methylene blue at 675 nm wavelength in a spectrophotometer (Titertek Multiskan, EALAB, Finland).

Lipid peroxide values were calculated using the following formula:

LPO value in nmol/ml = (sample absorbance – blank absorbance) x 50.0 (absorbance of 50 nmol/ml standard – blank absorbance). LPO value was converted to nmol/g wet tissue for illustration purposes.

Determination of tissue α-tocopherol levels:

α-tocopherol levels in liver were measured by HPLC using a modification of a previously described method (166). Briefly, about 0.2 g of wet tissue was homogenized in 1.0 ml of distilled water. Butylated hydroxytoluene was added to prevent oxidation of α-tocopherol from homogenates. Pentane, ethanol, and sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. Extracts were evaporated under a steady flow of nitrogen gas at 20^0C and redissolved in 1.0 ml of methanol injection onto a C18 column (Lichro CA®T 125mm x 4mm LiChrosphe® 100 PR-18 [5μm]. The mobile phase was composed of methanol and distilled water in a 93:7 volume ratio and a flow rate of 0.8 ml/min. α-tocopherol, eluting around 40 min, and was monitored by an HP 1046A programmable fluorescence detector (HP Company, Wilmington, DE) at 290 nm

excitation and 340 nm emission wavelengths. A set of α-tocopherol standard concentrations was analyzed to make a standard curve and to verify calibration.

STATISTICAL ANALYSIS

Statistical analyses were conducted by using STATA 7, (College Station, TX) statistical package. All data were reported as mean ± SEM. Variables were compared using a one-way analysis of variance (ANOVA) and subsequent comparisons of two groups were analyzed by Scheffé's. A level of $p < 0.05$ was defined as statistically significant.

4.3 RESULTS

In the current study, the work plan was to run the dietary intervention for 12 weeks followed by animal sacrifice. However, by week 12 the animals were showing either minimum signs of having caught the viral infection or no signs at all. The decision was made to re-infect them with the LP-BM5 virus and re-run the study for another 12 weeks. After 10 weeks into the re-infection, some animals showed stronger signs of the infection (swollen lymphatic nodules), but not the majority of them.

Weight

There was no change in food consumption due to the intervention. Likewise, the body weight of the mice was not significantly affected (data not shown). The spleen weight was elevated in the infected mice, which gave the indication that the viral

infection may had progressed to murine AIDS. However, there was no difference in weight between the spleen of infected and uninfected mice indication that viral infection did not progress as expected.

Hepatic liver peroxides production

The liver is the major organ that has been studied for tissue lipid peroxidation. Data analysis indicates that hepatic lipid peroxides were not significantly decreased by any of the extracts (Table 4.1). However, data do show a lowering trend, except for hop (50mg/kg) dose.

Hepatic α-tocopherol levels

Hepatic α-tocopherol levels decreased as a result of the interventions (Table 4.2). Hepatic α-tocopherol levels were significantly reduced by borage 10 mg relative to uninfected control ($p = 0.02$).

4.4 DISCUSSION

The proposed hypothesis suggested that dietary consumption of hop and borage extracts by infected mice would show reduced oxidative damage. Our results however, indicate that hop and borage extracts have no significant statistical effect in reducing oxidative stress in a murine model of leukemia. Despite the statistical fact, data do show a tendency towards less oxidative damage which may have physiological significance (Figure 4.1 and Figure 4.2). One possible explanation of this outcome is the reduced virulence observed with the LP-BM5 retrovirus used in the study.

During the progression of the LP-BM5 retroviral infection to murine AIDS, mice immune system step forward to increased lipid peroxidation concomitantly with reduced concentrations of antioxidants vitamins in the tissue (167). In the current study, the development and progression of lymphadenopathy associated with MAIDS was delayed when the animals were infected with the virus and fed with plant extract for 12 weeks. A possible explanation for the observed delayed progression to MAIDS would be that dietary components have the potential to interfere with viral replication and/or infectivity. Naturally occurring polyphenols with antiviral activity, i.e. flavonoids have been identified since the 1940s (151). Among other polyphenols, quercetin, morin, rutin, catechin, and naringin have been reported to possess antiviral activity against 11 types of viruses (151). Quercetin, which is one of the constituents of hop, was reported to exhibit both anti-infective and anti-replicative abilities (168). An interesting interaction between ascorbate and quercetin was observed by Vrijsen et al. (149). In this study, quercetin exhibited antiviral activity only when oxidative degradation was inhibited by ascorbate.

A finding of possible physiological relevance given the frequent co-occurrence of these diet-derived compounds. It is possible to assume that a similar interaction could have taken place between vitamin E and the plant extracts used in our study. Possibly, the oxidative degradation in the liver was prevented by vitamin E while the plant extracts inhibited the virus growth. Clearly, this assumption would require further investigation.

A second explanation for the observed outcome has to do with polyphenols redox potential. Although there appears to be evidence that natural products, i.e. polyphenols, affect oxidant stress in *in vitro* experimental conditions (63;143;144), the broader picture of results from *in vivo* experimental studies using extracts has provided only marginal support for the beneficial effects of natural products. The liver is a major organ subjected to free radicals attack. In the present study, it appears that the 10 mg/kg hop treatment resulted in a decreased hepatic lipid peroxidation. This was also observed with borage treatment at 50 mg/kg, but to a lesser extent (Table 4.1). Furthermore, there seem to be indications of toxicity when using the hop 50 mg dose exemplified by the observed increased levels of lipid peroxidation in this group relative to the control-retrovirus infected values.

It is well known that the lipid-soluble α-tocopherol (vitamin E) is an integral part of cell membranes and acts as a cytosolic antioxidant by scavenging free radicals that promote peroxidative chain reactions (169). Thus, the observed decrease in hepatic α-tocopherol levels (Table 4.2) could indicate that α-tocopherol was involved in antioxidant defense against oxidative stress as opposed to being preserved by the potential antioxidant effect of the plant extracts used. There is evidence that a decrease in

antioxidant defense in hepatocytes can promote oxidative stress (170). However, observations mentioned above need to be considered carefully because of the difficulties in producing an active viral infection in the animals during the first 12 weeks and the fact that re-infection had to be applied, thus increasing the experimental time to 24 weeks.

The effectiveness of a dietary antioxidant will depend on different factors such as the specific reactive oxygen or nitrogen specie being scavenged, as well as the mechanism and location where they are being generated and the accessibility of the antioxidant to possible sites of damage. Due to the nature of its solubility, for instance, a water soluble antioxidant may be less able to penetrate a lipid particle or cell membrane. Polyphenols are compounds whose solubility covers a wide spectrum. Some polyphenols are water soluble while others are lipid soluble, and others show a solubility behavior between the two states. Consequently, the lack of knowledge regarding the exact molecular structure and concentration of the constituent(s) in the extracts used in our study makes difficult to determine the possible cause(s) of treatment failure. Therefore, a study should be designed to evaluate different exposure mechanisms that would account for more than one solubility state and to examine their antioxidant properties.

It is also important to remember that, under some conditions, redox active polyphenolic compounds can act as pro-oxidants (151). This is particularly the case in metal ion catalyzed systems, as demonstrated with flavonoids (171). If metal ions do play a role in lipoprotein oxidation *in vivo*, then potential pro-oxidants effects of polyphenolic compounds should not be ignored.

Most of the dietary studies examining *in vivo* antioxidant effects of polyphenolic compounds have used indirect methods to measure lipid and lipoprotein damage or non-specific measures of plasma antioxidant capacity. These indirect quantitative approaches, such as those involving spectrophotometry, are becoming less acceptable because they are non-specific and subject to interferences. Future research in this area requires specific markers of oxidative damage that can be exactly characterized and distinguished from interferences (172). In this regard, nitrated/hydroxylated protein residues may be effective markers of protein damage by RNS/ROS and can be analyzed by GC-MS (gas chromatography-mass spectrometry) using negative chemical ionization, which offers selective and highly sensitive detection. Modification of guanine residues in DNA to 8-hydroxyguanine or 8-nitroguanine can be used to assess exposure of DNA to oxidation/nitration by HPLC measurement of these compounds in urine. For lipid oxidation, measurement of 8-isoprostane has gained considerable acceptability (173). Measurement of 8-isoprostane in plasma or urine currently uses GC-MS as the method of choice. An immunoassay is available, although it has not provided results that are comparable to GC-MS (174).

Another explanation for the observed results relates to the question of bioavailability and the fate of metabolites of the polyphenolic components in the two plant extracts used. Available evidence indicates that polyphenols may have limited bioavailability and may be extensively metabolized (31). More in-depth studies on bioavailability should facilitate a correlation of mechanisms determined *in vitro* with *in*

vivo situations, increase the understanding of dose-response relationships, and facilitate extrapolation of results from animal studies to humans.

Taken together, the findings from this study suggest that dietary consumption of 2 doses of hops and borage (10 and 50mg/kg) for 24 weeks does not reduce oxidative damage among animals. Nevertheless, data do show a trend towards reduction of oxidative damage. It is unclear whether the negative results are due to a true lack of efficacy or to the difficulties encountered during the study.

4.5 LIST OF FIGURES AND TABLES

Table 4.1 Effects of hop and borage exposure on hepatic lipid peroxidation (LPO) in murine AIDS

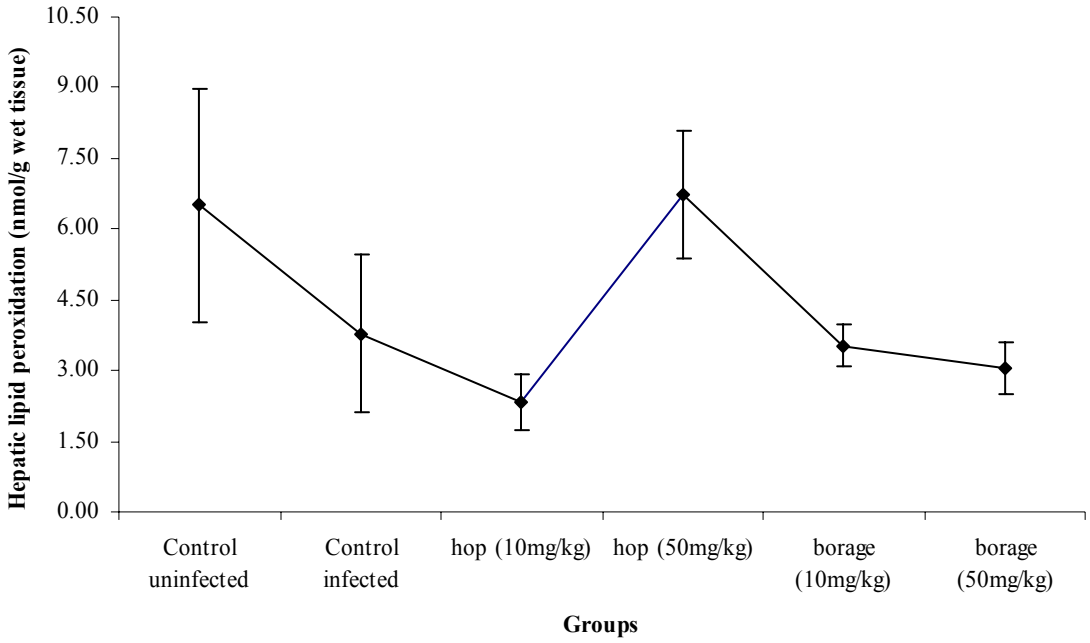
Intervention	Retrovirus	LPO (±SEM)
Control not infected	-	6.50 (2.49)
Control infected	+	3.78 (1.68)
Hop (10mg/kg)	+	2.33 (0.58) [a, b]
Hop (50mg/kg)	+	6.72 (1.36)
Borage (10mg/kg)	+	3.52 (0.44)
Borage (50mg/kg)	+	3.03 (0.55)

Lipid peroxide was determined with 0.2g of mouse liver tissue. Phospholipids in the liver tissue were extracted by the chloroform/methanol 2:1, v/v method. LPO was measured at 675 nm. Data are presented as mean ±SEM of triplicate wells, 6 to 9 mice in each group.

[a] $p = 0.12$ compared to not infected control mice

[b] $p = 0.07$ compared to infected control mice

Figure 4.1 Effects of hop and borage exposure on hepatic lipid peroxidation in murine AIDS



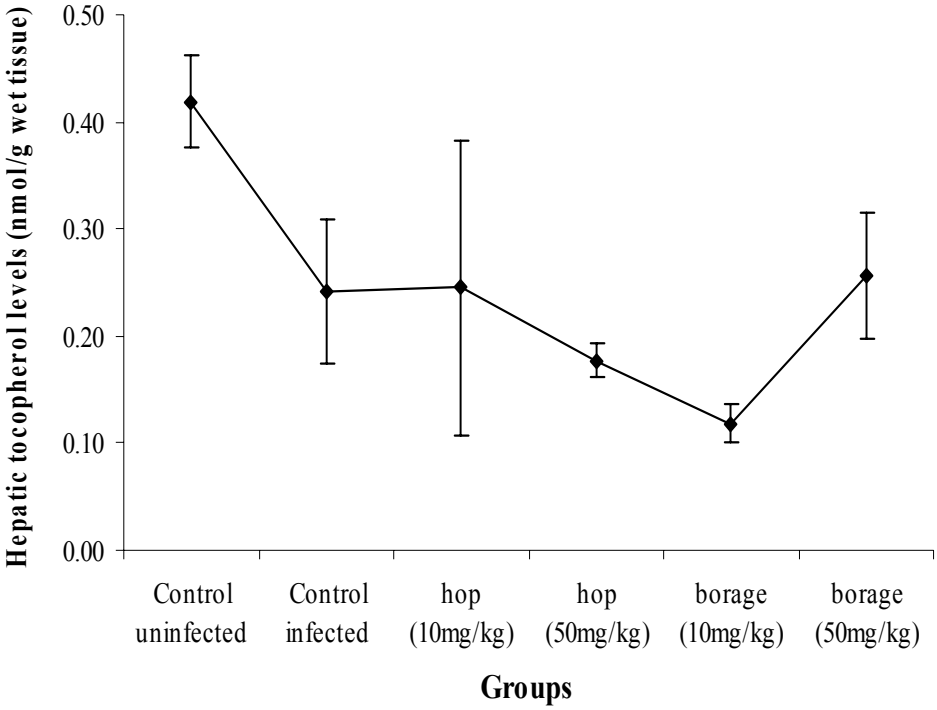
Lipid peroxide was determined with 0.2 g of mouse liver tissue. Phospholipids in the liver tissue were extracted by the $CHCl_3$ / methanol 2:1, v/v method. LPO was measured at 675 nm. Data are presented as mean ±SEM of triplicate wells, 6 to 9 mice in each group. [a] $p = 0.12$ compared to not infected control mice; [b] $p = 0.07$ compared to infected control mice;

Table 4.2 Effects of hop and borage exposure on hepatic α-tocopherol levels in murine AIDS

Intervention	Retrovirus	α-tocopherol (±SEM)
Control not infected	-	0.42 (0.04)
Control infected	+	0.24 (0.07)
Hop (10mg/kg)	+	0.25 (0.14)
Hop (50mg/kg)	+	0.18 (0.02)
Borage (10mg/kg)	+	0.12 (0.02) [a]
Borage (50mg/kg)	+	0.26 (0.06)

Hepatic α-tocopherol level was used as an indicator for oxidative stress. The assay was performed by HPLC. Values are mean ±SEM of 6 to 9 mice in each group. [a] $p = 0.02$

Figure 4.2 Effects of hop and borage exposure on hepatic α-tocopherol levels in murine AIDS



Hepatic α-tocopherol level was used as an indicator for oxidative stress. The assay was performed by HPLC. Values are mean ±SEM of 6 to 9 mice in each group; $^{a}p = 0.02$

Acknowledgement

This study was funded in part by an NIH fellowship T32 AT001287-01 from the National Center for Complementary and Alternative Medicine (NCCAM).

CHAPTER 5: CONCLUSION AND FUTURE STUDIES

Conclusions

Our study to evaluate the effect of four protein hydrolysates on blood pressure in SHR model showed that all cardiovascular parameters mean profile over time exhibited a tendency to lower values, but once compared, there was no significant difference between any of the protein hydrolysates tested and the control group.

The study which evaluated the potential immunomodulatory effect of six nutritional supplements, gave no indication of effectiveness in preventing immune dysfunction caused by aging; however, the immunological response (Th1 and Th2 cytokines) of the aging mice did show a significant difference from that of the young mice fed the control diet which reproduce previous immunological findings in an aged mice model. Ross Laboratories, sponsor for this study, and based on determination of the minimum effective dose (MED), selected 2 compounds (NAQ and LAC, High dose) to proceed to a Phase 2 study were animals will be challenge with Coxsackie virus.

We found that dietary consumption of 2 doses of hops and borage as a dietary supplement, (10 and 50mg/kg) for 24 weeks did not prevent hepatic liver peroxidation or preserve α-tocopherol among animals. However, data showed a tendency towards reduced oxidative damage.

Taken together, it appears that none of the food or food-derived bioactive compounds tested had a significant effect *in vivo*. However, data do indicate a trend towards positive physiological significance. It is unclear whether the negative results are

due to a true lack of effect, to weakness on the design, and/or to the difficulties encountered during the studies.

Perspective on future directions

The link between diet and health is well established, but renewed interest in which dietary components are biologically active and how they exert their effects is being fuelled by the development of nutritional genomics. Nutritional genomics is the application of high throughput functional genomic technologies in nutrition research, where a massive array of molecular events can be observed simultaneously. In our studies we focused on only 2-5 protein messages and could have missed other significant changes.

The food we eat contains thousands of biologically active substances, many of which may have the potential to provide substantial health benefits. Indeed, several food derived compounds, such as polyphenols, are among the most promising chemopreventive agents being evaluated (175). The full extent of biologically active components in our diet is unknown, and our understanding of their mechanisms of action is even more limited. Much of the available data have been derived from *in vitro* studies with purified compounds in forms and concentration to which the tissues in our bodies may never be exposed. While this work provides a starting point, more physiologically relevant model systems, including characterization of the extent and rate of absorption, tissue dispersal, and site specific targeting of metabolically relevant compounds, and comprehensive studies of time and dose effects, is required to interpret the true potential of these constituents.

Innovative food products are bioactive compounds with health promoting or disease preventing properties. One example is the antihypertensive effect of dietary peptides derived from milk protein, mediated by angiotensin converting enzyme inhibition. Although epidemiological data and preclinical studies are promising, clinical studies of the effect of these milk peptides on human blood pressure have not yet been done. It is crucial for prospective clinical trials to incorporate nutrigenomic technologies, especially when comparing these nutritionally derived peptides with synthetically produced angiotensin converting enzyme inhibitors, because responses to the latter possibly depend on gene polymorphism (176). Another example is the antioxidant effect of dietary compounds such as polyphenols in oxidative stress. Future studies about antioxidant supplementation should provide a global assessment of dietary intervention (e.g. plant extracts) at the molecular level and its biological effects; for example, determine the impact of retroviral infection on mouse liver gene expression and mitochondrial function. It will also be important to determine whether these changes could be prevented or attenuated by the antioxidant diet. Functional genomics techniques are ideal for elucidating the effects of novel bioactive food-derived compounds on global gene expression and cell function without making assumptions about what to look for in terms of risk.

CHAPTER 6: REFERENCES

References

(1) Unger T. The role of the renin-angiotensin system in the development of cardiovascular disease. Am J Cardiol 2002; 89(2A):3A-9A.

(2) Ondetti MA, Cushman DW. Enzymes of the renin-angiotensin system and their inhibitors. Annu Rev Biochem 1982; 51:283-308.

(3) Ariyoshi Y. Angiotensin-converting enzyme inhibitors derived from food proteins. Trends in Food Science & Technology 1993; 4(5):139-144.

(4) FitzGerald RJ, Meisel H. Lactokinins: whey protein-derived ACE inhibitory peptides. Nahrung 1999; 43(3):165-167.

(5) Yamamoto N, Maeno M, Takano T. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4. J Dairy Sci 1999; 82(7):1388-1393.

(6) Karaki H, Doi K, Sugano S, Uchiwa H, Sugai R, Murakami U et al. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. Comp Biochem Physiol C 1990; 96(2):367-371.

(7) Hata Y, Yamamoto M, Ohni M, Nakajima K, Nakamura Y, Takano T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. Am J Clin Nutr 1996; 64(5):767-771.

(8) Kelley DS, Bendich A. Essential nutrients and immunologic functions. Am J Clin Nutr 1996; 63(6):994S-996S.

(9) Chandra RK. Nutrition and the immune system. Proc Nutr Soc 1993; 52(1):77-84.

(10) Kubena KS, McMurray DN. Nutrition and the immune system: a review of nutrient-nutrient interactions. J Am Diet Assoc 1996; 96(11):1156-1164.

(11) Bogden JD, Bendich A, Kemp FW, Bruening KS, Shurnick JH, Denny T et al. Daily micronutrient supplements enhance delayed-hypersensitivity skin test responses in older people. Am J Clin Nutr 1994; 60(3):437-447.

(12) Meydani SN, Meydani M, Blumberg JB, Leka LS, Siber G, Loszewski R et al. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. JAMA 1997; 277(17):1380-1386.

(13) Jeng KC, Yang CS, Siu WY, Tsai YS, Liao WJ, Kuo JS. Supplementation with vitamins C and E enhances cytokine production by peripheral blood mononuclear cells in healthy adults. Am J Clin Nutr 1996; 64(6):960-965.

(14) Herberman RB, Holden HT. Natural cell-mediated immunity. Adv Cancer Res 1978; 27:305-377.

(15) Santos MS, Meydani SN, Leka L, Wu D, Fotouhi N, Meydani M et al. Natural killer cell activity in elderly men is enhanced by beta-carotene supplementation. Am J Clin Nutr 1996; 64(5):772-777.

(16) Murata T, Tamai H, Morinobu T, Manago M, Takenaka H, Hayashi K et al. Effect of long-term administration of beta-carotene on lymphocyte subsets in humans. Am J Clin Nutr 1994; 60(4):597-602.

(17) Hughes DA. Effects of dietary antioxidants on the immune function of middle-aged adults. Proc Nutr Soc 1999; 58(1):79-84.

(18) Yamada K, Tachibana H. Recent topics in anti-oxidative factors. Biofactors 2000; 13(1-4):167-172.

(19) Isolauri E. Probiotics in human disease. Am J Clin Nutr 2001; 73(6):1142S-1146S.

(20) Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. Pediatr Res 1992; 32(2):141-144.

(21) Solis-Pereyra B, Aattouri N, Lemonnier D. Role of food in the stimulation of cytokine production. Am J Clin Nutr 1997; 66(2):521S-525S.

(22) Roberfroid MB. Prebiotics and probiotics: are they functional foods? Am J Clin Nutr 2000; 71(6 Suppl):1682S-1687S.

(23) Ha CL, Lee JH, Zhou HR, Ustunol Z, Pestka JJ. Effects of yogurt ingestion on mucosal and systemic cytokine gene expression in the mouse. J Food Prot 1999; 62(2):181-188.

(24) Marin ML, Tejada-Simon MV, Lee JH, Murtha J, Ustunol Z, Pestka JJ. Stimulation of cytokine production in clonal macrophage and T-cell models by Streptococcus thermophilus: comparison with Bifidobacterium sp. and Lactobacillus bulgaricus. J Food Prot 1998; 61(7):859-864.

(25) Miettinen M, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, Kurimoto M et al. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and

gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 1998; 66(12):6058-6062.

(26) Tejada-Simon MV, Lee JH, Ustunol Z, Pestka JJ. Ingestion of yogurt containing Lactobacillus acidophilus and Bifidobacterium to potentiate immunoglobulin A responses to cholera toxin in mice. J Dairy Sci 1999; 82(4):649-660.

(27) Tejada-Simon MV, Ustunol Z, Pestka JJ. Effects of lactic acid bacteria ingestion of basal cytokine mRNA and immunoglobulin levels in the mouse. J Food Prot 1999; 62(3):287-291.

(28) Muscettola M, Massai L, Tanganelli C, Grasso G. Effects of lactobacilli on interferon production in young and aged mice. Ann N Y Acad Sci 1994; 717:226-232.

(29) Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 1998; 56(11):317-333.

(30) Le Marchand L. Cancer preventive effects of flavonoids--a review. Biomed Pharmacother 2002; 56(6):296-301.

(31) Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000; 130(8S Suppl):2073S-2085S.

(32) Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993; 342(8878):1007-1011.

(33) Knekt P, Jarvinen R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Finland: a cohort study. BMJ 1996; 312(7029):478-481.

(34) Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary flavonoids and cancer risk in the Zutphen Elderly Study. Nutr Cancer 1994; 22(2):175-184.

(35) Hertog MG, Sweetnam PM, Fehily AM, Elwood PC, Kromhout D. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. Am J Clin Nutr 1997; 65(5):1489-1494.

(36) Chatenoud L, Tavani A, La Vecchia C, Jacobs DR, Jr., Negri E, Levi F et al. Whole grain food intake and cancer risk. Int J Cancer 1998; 77(1):24-28.

(37) Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med 1995; 155(4):381-386.

(38) Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc 1996; 96(10):1027-1039.

(39) Adlercreutz H, Mazur W. Phyto-oestrogens and Western diseases. Ann Med 1997; 29(2):95-120.

(40) Candlish JK, Das NP. Antioxidants in food and chronic degenerative diseases. Biomed Environ Sci 1996; 9(2-3):117-123.

(41) Keli SO, Hertog MG, Feskens EJ, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. Arch Intern Med 1996; 156(6):637-642.

(42) Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E et al. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 1997; 146(3):223-230.

(43) Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 1992; 339(8808):1523-1526.

(44) Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 1993; 341(8843):454-457.

(45) Maxwell S, Cruickshank A, Thorpe G. Red wine and antioxidant activity in serum. Lancet 1994; 344(8916):193-194.

(46) Serafini M, Maiani G, Ferro-Luzzi A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. J Nutr 1998; 128(6):1003-1007.

(47) Kiechl S, Willeit J, Rungger G, Egger G, Oberhollenzer F, Bonora E. Alcohol consumption and atherosclerosis: what is the relation? Prospective results from the Bruneck Study. Stroke 1998; 29(5):900-907.

(48) Prevost AT, Whichelow MJ, Cox BD. Longitudinal dietary changes between 1984-5 and 1991-2 in British adults: association with socio-demographic, lifestyle and health factors. Br J Nutr 1997; 78(6):873-888.

(49) Lloyd T, Chinchilli VM, Rollings N, Kieselhorst K, Tregea DF, Henderson NA et al. Fruit consumption, fitness, and cardiovascular health in female adolescents: the Penn State Young Women's Health Study. Am J Clin Nutr 1998; 67(4):624-630.

(50) De Winter K, Rodriguez G. Consumers' views on nutrition and public health. Proc Nutr Soc 1997; 56(3):879-888.

(51) Weisburger JH. Lifestyle, health and disease prevention: the underlying mechanisms. Eur J Cancer Prev 2002; 11 Suppl 2:S1-S7.

(52) Akita S, Sacks FM, Svetkey LP, Conlin PR, Kimura G. Effects of the Dietary Approaches to Stop Hypertension (DASH) diet on the pressure-natriuresis relationship. Hypertension 2003; 42(1):8-13.

(53) Pereira MA, Liu S. Types of carbohydrates and risk of cardiovascular disease. J Womens Health (Larchmt) 2003; 12(2):115-122.

(54) Nyberg F, Hou SM, Pershagen G, Lambert B. Dietary fruit and vegetables protect against somatic mutation in vivo, but low or high intake of carotenoids does not. Carcinogenesis 2003; 24(4):689-696.

(55) Westhuyzen J. The oxidation hypothesis of atherosclerosis: an update. Ann Clin Lab Sci 1997; 27(1):1-10.

(56) Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996; 334(18):1150-1155.

(57) Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull 1993; 49(3):481-493.

(58) Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radic Res 1999; 31(6):577-596.

(59) Jabs T. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. Biochem Pharmacol 1999; 57(3):231-245.

(60) Stewart BW. Mechanisms of apoptosis: integration of genetic, biochemical, and cellular indicators. J Natl Cancer Inst 1994; 86(17):1286-1296.

(61) Halliwell B. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward?
HALLIWELL2000. Cardiovasc Res 2000; 47(3):410-418.

(62) Halliwell B. Mechanisms involved in the generation of free radicals. Pathol Biol (Paris) 1996; 44(1):6-13.

(63) da Silva EL, Piskula MK, Yamamoto N, Moon JH, Terao J. Quercetin metabolites inhibit copper ion-induced lipid peroxidation in rat plasma. FEBS Lett 1998; 430(3):405-408.

(64) Li X, Huang J, May JM. Ascorbic acid spares [alpha]-tocopherol and decreases lipid peroxidation in neuronal cells. Biochemical and Biophysical Research Communications 2003; 305(3):656-661.

(65) Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 1995; 22(4):375-383.

(66) Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Arch Biochem Biophys 1995; 322(2):339-346.

(67) Gorelik S, Kanner J. Oxymyoglobin oxidation and membrane lipid peroxidation initiated by iron redox cycle: prevention of oxidation by enzymic and nonenzymic antioxidants. J Agric Food Chem 2001; 49(12):5945-5950.

(68) Laughton MJ, Evans PJ, Moroney MA, Hoult JR, Halliwell B. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. Biochem Pharmacol 1991; 42(9):1673-1681.

(69) Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996; 20(7):933-956.

(70) Hodnick WF, Kung FS, Roettger WJ, Bohmont CW, Pardini RS. Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study. Biochem Pharmacol 1986; 35(14):2345-2357.

(71) Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C et al. How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr 2004; 80(1):15-21.

(72) Beharka A, Redican S, Leka L, Meydani SN. [22] Vitamin E status and immune function. Methods in Enzymology. Academic Press, 1997: 247-263.

(73) Prasad JS. Effect of vitamin E supplementation on leukocyte function. Am J Clin Nutr 1980; 33(3):606-608.

(74) Devaraj S, Li D, Jialal I. The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. J Clin Invest 1996; 98(3):756-763.

(75) Daynes RA, Enioutina EY, Jones DC. Role of Redox Imbalance in the Molecular Mechanisms Responsible for Immunosenescence. Antioxidants & Redox Signaling 2003; 5(5):537-548.

(76) Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J 1996; 10(7):709-720.

(77) Jackson MJ, McArdle A, McArdle F. Antioxidant micronutrients and gene expression. Proc Nutr Soc 1998; 57(2):301-305.

(78) Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci 1993; 686:12-27.

(79) Hughes DA, Haslam PL, Townsend PJ, Turner-Warwick M. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. Clin Exp Immunol 1985; 61(2):459-466.

(80) Richards GA, Theron AJ, Van Rensburg CE, Van Rensburg AJ, Van der Merwe CA, Kuyl JM et al. Investigation of the effects of oral administration of vitamin E and beta-carotene on the chemiluminescence responses and the frequency of sister chromatid exchanges in circulating leukocytes from cigarette smokers. Am Rev Respir Dis 1990; 142(3):648-654.

(81) Sies H. Role of reactive oxygen species in biological processes. Klin Wochenschr 1991; 69(21-23):965-968.

(82) Gutteridge JM. Free radicals in disease processes: a compilation of cause and consequence. Free Radic Res Commun 1993; 19(3):141-158.

(83) Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 1992; 119(6):598-620.

(84) Kaul N, Devaraj S, Jialal I. Alpha-tocopherol and atherosclerosis. Exp Biol Med (Maywood) 2001; 226(1):5-12.

(85) Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993; 90(17):7915-7922.

(86) Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radic Res 1999; 31(4):261-272.

(87) Jenner P. Oxidative damage in neurodegenerative disease. Lancet 1994; 344(8925):796-798.

(88) Willett WC. Diet and health: what should we eat? Science 1994; 264(5158):532-537.

(89) National Institutes of Health BM. National Heart, Lung, and Blood Institute; The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure. Publication no. 03-5233. 2003.
Ref Type: Report

(90) Neutel JM, Smith DH, Weber MA. Is high blood pressure a late manifestation of the hypertension syndrome? Am J Hypertens 1999; 12(12 Pt 3):215S-223S.

(91) National Centre for Health Statistics. Health/Hypertension prevalence among adults, according to age, race, sex, and Hispanic origin. Table 68:210. 2004.
Ref Type: Report

(92) FitzGerald RJ, Murray BA, Walsh DJ. Hypotensive Peptides from Milk Proteins. J Nutr 2004; 134(4):980S-9988.

(93) MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J et al. Blood pressure, stroke, and coronary heart disease. Part 1, Prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. Lancet 1990; 335(8692):765-774.

(94) Collins R, Peto R, MacMahon S, Hebert P, Fiebach NH, Eberlein KA et al. Blood pressure, stroke, and coronary heart disease. Part 2, Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context. Lancet 1990; 335(8693):827-838.

(95) Conlin PR, Chow D, Miller ER, III, Svetkey LP, Lin PH, Harsha DW et al. The effect of dietary patterns on blood pressure control in hypertensive patients: results from the Dietary Approaches to Stop Hypertension (DASH) trial. Am J Hypertens 2000; 13(9):949-955.

(96) Persson PB, Skalweit A, Thiele BJ. Controlling the release and production of renin. Acta Physiologica Scandinavica 2004; 181(4):375-381.

(97) Dezsi L. Fibrinolytic actions of ACE inhibitors: a significant plus beyond antihypertensive therapeutic effects. Cardiovascular Research 2000; 47(4):642-644.

(98) Phillips MI. Functions of angiotensin in the central nervous system. Annu Rev Physiol 1987; 49:413-435.

(99) Mazzolai L, Nussberger J, Aubert JF, Brunner DB, Gabbiani G, Brunner HR et al. Blood pressure-independent cardiac hypertrophy induced by locally activated renin-angiotensin system. Hypertension 1998; 31(6):1324-1330.

(100) Ames RP. Negative effects of diuretic drugs on metabolic risk factors for coronary heart disease: possible alternative drug therapies. Am J Cardiol 1983; 51(4):632-638.

(101) Sesoko S, Kaneko Y. Cough associated with the use of captopril. Arch Intern Med 1985; 145(8):1524.

(102) Nakamura H. Effects of antihypertensive drugs on plasma lipids. Am J Cardiol 1987; 60(9):24E-28E.

(103) Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. Am J Clin Nutr 2003; 77(2):326-330.

(104) Fujita H, et al. Human Volunteers Study on Antihypertensive Effect of "Katsuobushi Oligopeptide" (I). Jpn Pharmacol Ther 1997; 25(8):147-151.

(105) Fujita Heal. Human Volunteers Study on Antihypertensive Effect of "Katsuobushi Oligopeptide" (II). Jpn Pharmacol Ther 1997; 25(8):152-157.

(106) Fujita H, Yamagami T, Ohshima K. Effects of an ace-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. Nutrition Research 2001; 21(8):1149-1158.

(107) Pins J, Keenan J. The antihypertensive effects of a hydrolysed whey protein isolate supplement (Biozate 1): A pilot study. FASEB J 2003; 17:A1110.

(108) Nurminen ML, Sipola M, Kaarto H, Pihlanto-Leppala A, Piilola K, Korpela R et al. [alpha]-Lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. Life Sciences 2000; 66(16):1535-1543.

(109) Maeno M, Yamamoto N, Takano T. Identification of an Antihypertensive Peptide from Casein Hydrolysate Produced by a Proteinase from Lactobacillus helveticus CP790. J Dairy Sci 1996; 79(8):1316-1321.

(110) Saito T, Nakamura T, Kitazawa H, Kawai Y, Itoh T. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. J Dairy Sci 2000; 83(7):1434-1440.

(111) Abubakar A, Saito T, Kitazawa H, Kawai Y, Itoh T. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. J Dairy Sci 1998; 81(12):3131-3138.

(112) Makare N, Bodhankar S, Rangari V. Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. J Ethnopharmacol 2001; 78(2-3):133-137.

(113) Zhang LH., Liu JO. Sanglifehrin A, a novel cyclophilin-binding immunosuppressant, inhibits IL-2-dependent T cell proliferation at the G1 phase of the cell cycle. Journal of Immunology 2001; 166(9):5611-5618.

(114) Tiwari U, Rastogi B, Singh P, Saraf DK, Vyas SP. Immunomodulatory effects of aqueous extract of Tridax procumbens in experimental animals. J Ethnopharmacol 2004; 92(1):113-119.

(115) Liang B, Jiang S, Zhang Z, Inserra P, Lee J, Solkoff D et al. Anti-inflammatory effects of theophylline: modulation of immune functions during murine leukemia virus infection. Immunopharmacol Immunotoxicol 2001; 23(3):307-319.

(116) Glezen WP. Emerging infections: pandemic influenza. Epidemiol Rev 1996; 18(1):64-76.

(117) Miller RA. The aging immune system: primer and prospectus. Science 1996; 273(5271):70-74.

(118) Liang B, Zhang Z, Inserra P, Jiang S, Lee J, Garza A et al. Injection of T-cell receptor peptide reduces immunosenescence in aged C57BL/6 mice. Immunology 1998; 93(4):462-468.

(119) Zhang Z, Araghi-Niknam M, Liang B, Inserra P, Ardestani SK, Jiang S et al. Prevention of immune dysfunction and vitamin E loss by dehydroepiandrosterone and melatonin supplementation during murine retrovirus infection. Immunology 1999; 96(2):291-297.

(120) Wang Y, Huang DS, Wood S, Watson RR. Modulation of immune function and cytokine production by various levels of vitamin E supplementation during murine AIDS. Immunopharmacology 1995; 29(3):225-233.

(121) Chandra RK. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. Lancet 1992; 340(8828):1124-1127.

(122) Clark AM. Natural products as a resource for new drugs. Pharm Res 1996; 13(8):1133-1144.

(123) Middleton E Jr. Effect of plant flavonoids on immune and inflammatory cell function. Adv Exp Med Biol 1998; 439:175-182.

(124) Martinez F, Abril ER, Earnest DL, Watson RR. Ethanol and cytokine secretion. Alcohol 1992; 9(6):455-458.

(125) Roilides E, Pizzo PA. Modulation of host defenses by cytokines: evolving adjuncts in prevention and treatment of serious infections in immunocompromised hosts. Clin Infect Dis 1992; 15(3):508-524.

(126) Naume B, Espevik T. Immunoregulatory effects of cytokines on natural killer cells. Scand J Immunol 1994; 40(2):128-134.

(127) Nair MP, Kandaswami C, Mahajan S, Chadha KC, Chawda R, Nair H et al. The flavonoid, quercetin, differentially regulates Th-1 (IFNgamma) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. Biochim Biophys Acta 2002; 1593(1):29-36.

(128) Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E et al. Genistein inhibits constitutive and inducible NFkappaB activation and decreases IL-8 production by human cystic fibrosis bronchial gland cells. Am J Pathol 1999; 155(2):473-481.

(129) Wang J, Huang DS, Wood S, Giger PT, Watson RR. Kinetics of cytokine production by thymocytes during murine AIDS caused by LP-BM5 retrovirus infection. Immunol Lett 1995; 47(3):187-192.

(130) Arts MJ, Haenen GR, Voss HP, Bast A. Masking of antioxidant capacity by the interaction of flavonoids with protein. Food Chem Toxicol 2001; 39(8):787-791.

(131) Araghi-Niknam M, Liang B, Zhang Z, Ardestani SK, Watson RR. Modulation of immune dysfunction during murine leukaemia retrovirus infection of old mice by dehydroepiandrosterone sulphate (DHEAS). Immunology 1997; 90(3):344-349.

(132) Guo TL, McCay JA, Zhang LX, Brown RD, You L, Karrow NA et al. Genistein modulates immune responses and increases host resistance to B16F10 tumor in adult female B6C3F1 mice. J Nutr 2001; 131(12):3251-3258.

(133) Johnson VJ, He Q, Osuchowski MF, Sharma RP. Physiological responses of a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: III. Silymarin inhibits T-lymphocyte function at low doses but stimulates inflammatory processes at high doses. Planta Med 2003; 69(1):44-49.

(134) Bouman A, Schipper M, Heineman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. Am J Reprod Immunol 2004; 52(1):19-26.

(135) Matsuura K, Kawakita T, Nakai S, Saito Y, Suzuki A, Nomoto K. Role of B-lymphocytes in the immunopharmacological effects of a traditional Chinese medicine, xiao-chai-hu-tang (shosaiko-to). Int J Immunopharmacol 1993; 15(2):237-243.

(136) Zhang ZJ. Therapeutic effects of herbal extracts and constituents in animal models of psychiatric disorders. Life Sciences 2004; 75(14):1659-1699.

(137) Kogure K, Goto S, Nishimura M, Yasumoto M, Abe K, Ohiwa C et al. Mechanism of potent antiperoxidative effect of capsaicin. Biochim Biophys Acta 2002; 1573(1):84-92.

(138) Stevens JF, Page JE. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry 2004; 65(10):1317-1330.

(139) Shahidi F. Antioxidant factors in plant foods and selected oilseeds. Biofactors 2000; 13(1-4):179-185.

(140) Lampe JW. Spicing up a vegetarian diet: chemopreventive effects of phytochemicals. Am J Clin Nutr 2003; 78(3):579S-583.

(141) Kiefer I, Prock P, Lawrence C, Wise J, Bieger W, Bayer P et al. Supplementation with Mixed Fruit and Vegetable Juice Concentrates Increased Serum Antioxidants and Folate in Healthy Adults. J Am Coll Nutr 2004; 23(3):205-211.

(142) Oomah BD, Kenaschuk EO, and Mazza G. Phenolic Acids in Flaxseed. J Agric Food Chem 1995; 43:2016-2019.

(143) da Silva JM, Darmon N, Fernandez I, Mitjavila S. Oxygen Free Radical Scavenger Capacity in Aqueous Models of Different Procyanidins from Grape Seeds. J Agric Food Chem 1991; 39:1549-1552.

(144) Zhang D, Yasuda T, Yu Y, Zheng P, Kawabata T, Ma Y et al. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. Free Radical Biology and Medicine 1996; 20(1):145-150.

(145) Shahidi F, Wanasundara PK. Phenolic antioxidants. Crit Rev Food Sci Nutr 1992; 32(1):67-103.

(146) Iuliano L, Colavita AR, Leo R, Pratico D, Violi F. Oxygen Free Radicals and Platelet Activation. Free Radical Biology and Medicine 1997; 22(6):999-1006.

(147) Chiarpotto E, Scavazza A, Leonarduzzi G, Camandola S, Biasi F, Teggia PM et al. Oxidative Damage and Transforming Growth Factor 1 Expression in Pretumoral and Tumoral Lesions of Human Intestine. Free Radical Biology and Medicine 1997; 22(5):889-894.

(148) Weber C, Podda M, Rallis M, Thiele JJ, Traber MG, Packer L. Efficacy of Topically Applied Tocopherols and Tocotrienols in Protection of Murine Skin From Oxidative Damage Induced by UV-Irradiation. Free Radical Biology and Medicine 1997; 22(5):761-769.

(149) Vrijsen R, Everaert L, Boeye A. Antiviral activity of flavones and potentiation by ascorbate. J Gen Virol 1988; 69(7):1749-1751.

(150) Yanishlieva VI, Marinova EM. Effects of antioxidants on the stability of triacylglycerols and methyl esters of fatty acids of sunflower oil. Food Chemistry 1995; 54(4):377-382.

(151) Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52(4):673-751.

(152) Wettasinghe M, Shahidi F. Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (Borago officinalis L.) seeds. Food Chemistry 1999; 67(4):399-414.

(153) Wettasinghe M, Shahidi F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. Food Chemistry 2000; 70(1):17-26.

(154) Bandoniene D, Murkovic M. The detection of radical scavenging compounds in crude extract of borage (Borago officinalis L.) by using an on-line HPLC-DPPH method. Journal of Biochemical and Biophysical Methods 2002; 53(1-3):45-49.

(155) Tagashira M, Watanabe M, Uemitsu N. Antioxidative activity of hop bitter acids and their analogues. Biosci Biotechnol Biochem 1995; 59(4):740-742.

(156) Buckwold VE, Wilson RJ, Nalca A, Beer BB, Voss TG, Turpin JA et al. Antiviral activity of hop constituents against a series of DNA and RNA viruses. Antiviral Res 2004; 61(1):57-62.

(157) Honma Y, Tobe H, Makishima M, Yokoyama A, Okabe-Kado J. Induction of differentiation of myelogenous leukemia cells by humulone, a bitter in the hop. Leuk Res 1998; 22(7):605-610.

(158) Gerhauser C, Alt A, Heiss E, Gamal-Eldeen A, Klimo K, Knauft J et al. Cancer chemopreventive activity of Xanthohumol, a natural product derived from hop. Mol Cancer Ther 2002; 1(11):959-969.

(159) Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, De Keukeleire D. Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer. J Clin Endocrinol Metab 1999; 84(6):2249-2252.

(160) De Keukeleire D, De Cooman L, Rong H, Heyerick A, Kalita J, Milligan SR. Functional properties of hop polyphenols. Basic Life Sci 1999; 66:739-760.

(161) Bors W, Michel C, Stettmaier K. Antioxidant effects of flavonoids. Biofactors 1997; 6(4):399-402.

(162) Stevens JF, Ivancic M, Hsu VL, Deinzer ML. Prenylflavonoids from Humulus lupulus. Phytochemistry 1997; 44(8):1575-1585.

(163) Anto RJ, Sukumaran K, Kuttan G, Rao MNA, Subbaraju V, Kuttan R. Anticancer and antioxidant activity of synthetic chalcones and related compounds. Cancer Letters 1995; 97(1-4):33-37.

(164) Ko FN, Cheng ZJ, Lin CN, Teng CM. Scavenger and Antioxidant Properties of Prenylflavones Isolated From Artocarpus Heterophyllus. Free Radical Biology and Medicine 1998; 25(2):160-168.

(165) Zhao F, Nozawa H, Daikonnya A, Kondo K, Kitanaka S. Inhibitors of nitric oxide production from hops (Humulus lupulus L.). Biol Pharm Bull 2003; 26(1):61-65.

(166) Zhang J, Liu Y, Shi J, Larson DF, Watson RR. Side-Stream Cigarette Smoke Induces Dose-Response in Systemic Inflammatory Cytokine Production and Oxidative Stress. Experimental Biology and Medicine 2002; 227(9):823-829.

(167) Liang B, Zhang Z, Araghiniknam M, Eskelson C, Watson RR. Prevention of retrovirus-induced aberrant cytokine secretion, excessive lipid peroxidation, and tissue vitamin E deficiency by T cell receptor peptide treatments in C57BL/6 mice. Proc Soc Exp Biol Med 1997; 214(1):87-94.

(168) Wang HK, Xia Y, Yang ZY, Natschke SL, Lee KH. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. Adv Exp Med Biol 1998; 439:191-225.

(169) Wang X, Quinn PJ. Vitamin E and its function in membranes. Progress in Lipid Research 1999; 38(4):309-336.

(170) Loguercio C, Federico A. Oxidative stress in viral and alcoholic hepatitis. Free Radical Biology and Medicine 2003; 34(1):1-10.

(171) Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radic Biol Med 1997; 22(5):749-760.

(172) de Zwart LL, Meerman JHN, Commandeur JNM, Vermeulen NPE. Biomarkers of free radical damage: Applications in experimental animals and in humans. Free Radical Biology and Medicine 1999; 26(1-2):202-226.

(173) Morrow JD, Roberts LJ. The isoprostanes: Unique bioactive products of lipid peroxidation. Progress in Lipid Research 1997; 36(1):1-21.

(174) Proudfoot J, Barden A, Mori TA, Burke V, Croft KD, Beilin LJ et al. Measurement of Urinary F2-Isoprostanes as Markers of in Vivo Lipid Peroxidation--A Comparison of Enzyme Immunoassay with Gas Chromatography/Mass Spectrometry. Analytical Biochemistry 1999; 272(2):209-215.

(175) Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW et al. Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. J Nutr 2000; 130(2S Suppl):467S-471S.

(176) Baudin B. Angiotensin I-converting enzyme gene polymorphism and drug response. Clin Chem Lab Med 2000; 38(9):853-856.