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**THE ANTIOXIDANT AND ANTIATHEROGENIC EFFECTS
OF MAK-4 IN WHHL RABBITS**

DISSERTATION

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

By
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* * * * *

The Ohio State University
1995

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FIELDS OF STUDY

Major Field: Pathology

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LIST OF ABBREVIATIONS

ADP	Adenosinediphosphate
ANOVA	Analysis of variance
ATPase	Adenosine triphosphatase
BCS	Bathocuproine disulfonate
BE.....	Base excess
BP	Benzo(a)pyrene and blood pressure
cAMP	Cyclic adenosine monophosphate
CAR	Carotenoids
CCl ₃ [°]	Carbon trichloride radical
CCl ₄	Carbon tetrachloride
CF	Clastogenic factor
cGMP	cyclic Guanosine 5'-monophosphate
CHD	Coronary heart disease
Chol	Cholesterol
CNS	Central nervous system
CONT	Control
DEA	Diethanolamine
DETAPAC	Diethylenetriamine pentaacetic acid
DHA	Dehydroascorbic acid
Diene C.....	Diene conjugation

DMBA.....	7,12-dimethylbenz(a)anthracene
DNA	Deoxyribonucleic acid
DP	Density of plasma
DS.....	Density of salt
EDRF	Endothelial cell-derived relaxing factor
EC.....	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
FH.....	Familial hypercholesterolemia
GC-MS	Gas chromatography-mass spectroscopy
GSH.....	Glutathione reduced form
GSH-Px	Glutathione peroxidase
GSSG	Glutathione oxidized form
GSSG-R	Glutathione reductase
HDL	High density lipoprotein
HMG-CoA	β -Hydroxy- β -methylglutaryl-coenzyme A
HOCl	Hypochlorous acid
HPLC	High-performance liquid chromatography
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin
IL	Interleukin
IQ	Intelligence quotient
K	Potassium
LDH	Lactate dehydrogenase
LDL.....	Low density lipoprotein
LLC	Lewis lung carcinoma

LOH	Fatty acid alcohol
LOO°	Peroxy radical
LOOH.....	Free acid hydroperoxide
LP	lipid peroxide
MAK-4	Maharishi Amrit Kalash-4
MDA	Malondialdehyde
MPO	Myelin peroxidase
Na	Sodium
NADPH.....	Nicotinamide adenine dinucleotide phosphate reduced
NBT.....	Nitroblue tetrazolium
NO °	Nitric oxide radical
O °	Superoxide
OH °	Hydroxyl radical
ORAC.....	Oxygen-radical absorbance capacity
Ox-LDL.....	Oxidized low density lipoprotein
PGI ₂	Prostacyclin
PLase.....	Phospholipase
PLOOH	Hydroperoxide
PR	Pulse radiolysis
PUFA	Polyunsaturated fatty acid
QC	Quality control
RBC.....	Red blood cell
r-OHdG	r-Hydroxy-2'-deoxy-guanosine
ROO °	Peroxy radical
ROS.....	Reactive oxygen species

RTE	Rat tracheal epithelial
SD	Standard deviation
SE	Standard error
Se-GSH-Px	Selenium glutathione peroxidase
SL	Soybean lipoxygenase
SO	Superoxide anions
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric-acid-reactive substances
t-BuOOH	tert butylhydroperoxide
Trig	Triglyceride
UV	Ultra violet
VERIS	Vitamin E research and information science
VLDL	Very low density lipoprotein
VP	Volume of plasma
VS	Volume of salt
WHHL	Watanabe heritable hyperlipidemic

CHAPTER I INTRODUCTION

I-A. Introduction

Coronary heart disease is the leading cause of death in the United States. More than half a million Americans die from heart attacks annually. Three times that number will have heart attacks. The principal pathologic condition in most cases is atherosclerosis leading to coronary heart disease (CHD), peripheral vascular disease and strokes.

The initiation of atherosclerosis is possibly linked to free radical reactions, for example, lipid peroxidation [1-2], and oxidative modification of low density lipoproteins (LDLs) [3-4]. Other lipid peroxidation products react with LDL to change the LDL so that it is loosely bound to LDL receptors, thus prolonging its circulation and enhancing its uptake by macrophages [5]. The oxidative modification of LDL could contribute to the formation of foam cells and fatty streaks by generating a form of LDL recognized and taken up more rapidly by the acetyl-LDL receptor, the so called "scavenger receptor" macrophages [6]. The lipid peroxidation products of free radical-induced oxidation of LDL are toxic to endothelial cells, and this damage is a major factor in the formation of atherosclerotic plaques. Free radicals and reactive oxygen species (ROS) are being implicated in the pathogenesis of other diseases such as atherosclerosis [6,

7-13], carcinoma [14-18], rheumatoid arthritis [19, 20], diabetes mellitus [21, 22], hepatitis [23], cataract [24], chronic bronchitis [25], pulmonary fibrosis [25], vascular damage [26], cardiac arrhythmia [27], aging [28, 29], mental disorders [30-33], liver damage [34, 35], pancreas dysfunction [36, 37], heart injury [38, 39], kidney damage [40, 42], hemolytic anemia [43], immune disorders [44], and the abnormal release of enzymes [45, 46].

If these disorders are indeed caused by free radicals and ROS, they could be prevented by antioxidants, such as vitamins, minerals, enzymes, and Maharishi Amrit Kalash-4 (MAK-4) [47].

According to Ayurvedic literature, a “rasayana” is an herbal mixture that is taken as a food supplement to enhance overall well-being, boost immunity to disease, promote vitality and increase longevity [48]. MAK-4 is characterized as an effective “rasayana” for the treatment of carcinoma, gastrointestinal disorders, anxiety, and atherosclerosis. Maharishi Amrit Kalash (MAK-4 and MAK-5) was effective in inhibiting platelet aggregation in whole blood caused by collagen and ADP [49] and was shown to have antioxidant activity [50-53]. Recent work has demonstrated that MAK-4 has more antioxidant potency in preventing human LDL oxidation than did certain vitamins and probucol [54]. MAK-4 inhibited microsomal lipid peroxidation in the rat liver [52-53].

The purpose of this study was to evaluate the antioxidant and in-vivo antiatherogenic property of MAK-4 on Watanabe Heritable Hyperlipidemic (WHHL) rabbits.

CHAPTER II

LITERATURE REVIEW

II-A. What Are Free Radicals?

A free radical is any species such as an atom or molecule that is capable of independent existence and that contains one or more unpaired electrons in its outer orbit [55]. A molecule becomes a free radical through either oxidation, i.e., the loss of an electron which yields a net positive charge, or reduction, i.e., the gain of an electron which yields a net negative charge [19].

Different kinds of free radicals vary widely in their chemical reactivity, but free radicals are more unstable and therefore more reactive than nonradicals in general. When two free radicals react, their unpaired electrons can join to form a pair and thus both lose their free radical property. But, because most physiological products do not have unpaired electrons, endogenous and exogenous free radicals formed in vivo will react with nonradical compounds generating new sets of free radicals that will react with other nonradicals. Therefore, a free-radical chain reaction will proceed until enzymes such as superoxide dismutase or antioxidants such as vitamin C or vitamin E stop the chain reaction. The most thoroughly studied chain reaction in living organisms is lipid peroxidation [56].

The study of free radicals began at the end of the nineteenth century; fat spoilage and the polymerization reactions in rubber and plastics involve

free radicals [55]. The fields of free radical research in biology and medicine have become active since McCord and Fridovich isolated the enzyme superoxide dismutase (SOD) from biological samples [57]. Free radicals and reactive oxygen species (ROS) that are considered very important in human pathology are superoxide ($O_2^{\circ-}$), hydroxyl (OH°), peroxy (ROO°), singlet oxygen, and hypochlorous acid (HOCl). Hydrogen peroxide (H_2O_2) is also important since it can generate hydroxyl radical (Fig. 1) [58]. Singlet oxygen is not a free radical, but is ROS because of its high reactivity and generation of other free radicals [19].

II-B. Production of Free Radicals in the Human Body

Free radicals in vivo have both endogenous and exogenous origins [59]. Free radicals are generated spontaneously in the body to protect against invading foreign objects or as a byproduct of metabolic activity. They are generated as the result of chemical reaction in the energy generating system of the mitochondria, or are caused by certain chemicals and pollutants. Mental and physical stress are believed to generate free radicals [19, 47, 60, 61]. Free radicals can be generated in cells spontaneously by flavin oxidation or by certain enzymes such as electron transport enzymes (e.g., cytochrome p 450), oxidases such as xanthine and NADPH, dehydrogenases, peroxidases, lipoxygenases, and cyclooxygenases [7].

Superoxide has a beneficial effect in human physiology. Lymphocytes and fibroblasts constantly produce small amounts of superoxide to regulate

growth. Neutrophils, monocytes, macrophages, and eosinophils generate NADPH-dependent superoxide [56] and hypochlorous acid [62]. Production of free radicals by phagocytic cells is useful in the immune system; however, it harms the surrounding tissues [19, 47]. The endothelial cells of blood vessels produce nitric oxide (NO°) to relax the smooth muscle in the vessel wall in response to acetylcholine and bradykinin [56] (Figure 1). Reduction of molecular oxygen is carried out by the cytochrome oxidase system in the mitochondria. About 1% to 2% of the oxygen consumed by the cells accidentally travels a different pathway to produce superoxide, hydrogen peroxide, and the hydroxyl radical [63]. Free radicals are generated in lysosomes and peroxisomes, nuclear endoplasmic reticulum, plasma membranes, and cytosol [64]. Oxygen produces free radicals through the electron transferring mechanism [58].

The human body metabolizes drugs, poisons, and pollutants to free radicals (Table 1) [47, 56]. Cigarette smoke and carbon tetrachloride (CCl_4) can generate free radicals in the body [56]. Cigarette smoking not only depletes both vitamin C and vitamin E, it also activates phagocytes in the lung to make superoxide. CCl_4 is metabolized by the cytochrome P-450 system in the liver to generate CCl_3° which reacts rapidly with oxygen to form a peroxy radical.

Exposure of water to ionizing radiation splits the water molecule into two radicals, hydrogen and hydroxyl radicals [56]. The dangerous OH° radical can attack almost all types of molecules in the cells. The body generates the superoxide radical by adding one electron onto an oxygen molecule [56]. Superoxide reacts with hydrogen ions to generate hydrogen

peroxide. H_2O_2 is also produced in the peroxisomes by oxidation of certain amino acids [60]. H_2O_2 is not as reactive as $\text{O}_2^{\circ-}$; however, H_2O_2 produces OH° and other reactive metal ion-oxygen radical complexes when it interacts with $\text{O}_2^{\circ-}$ in the presence of iron or copper ions [65].

II-C. Target Molecules of Free Radical Attacks

An approximate equilibrium exists between free radical generation and the capacity of the antioxidant systems in the human body. The body has an adaptive capability to generate more anti-oxidant enzymes and chemicals if the concentration of free radicals increases. When cellular defenses are overloaded, oxidative damage to surrounding tissue results. Free radicals and their by-products damage biological molecules such as lipids, proteins, nucleic acids, and carbohydrates [15, 19]. Table 2 shows a list of known products of oxygen free radicals that attack various biomolecules [15, 55].

II-C-a. Lipids

Lipids that contain double bonds and that are part of membranes are most susceptible to oxidation [66]. Free radicals attack the double bonds in polyunsaturated fats in the cell membrane and oxidize them to peroxides; this is known as lipid peroxidation [67]. Membrane lipid peroxidation will change the structure of the membrane, altering the rigidity of the membrane and changing the activity of membrane proteins such as Na/K-ATPase. The consequences of the structural and physiological changes may result in an abnormal ion pumping rate of the membrane [15]. Two well-known

degradation products of lipid peroxidation, malondialdehyde and 4-hydroxynonenal, react with other biomolecules to cause cell edema, increased vascular permeability, and inflammation [55]. Some of the adverse biological effects of 4-hydroxynonenal are cell lysis, inactivation of enzymes, inhibition of DNA and protein synthesis, chemotactic activity, and activation of phospholipase D to signal endothelial cell transduction [68]. Several aldehydes, including malondialdehyde, react with nucleic acids to cause possible mutagenesis and carcinogenesis [69, 70]. Other lipid peroxidation products react with low density lipoproteins (LDL), enhancing their uptake into macrophages, which could lead to foam cell formation and atherogenesis [5].

There are many assay techniques for the measurement of lipid peroxidation. Some widely used techniques are the determination of absorbance of conjugated dienes at 234 nm [71, 72], assay for thiobarbituric acid reactive substances (TBARS) [73], fluorometric determination of peroxide [74], assay of lipid peroxide by chemiluminescence [75], iodometric measurement [76], high-performance liquid chromatography (HPLC) [77], and gas chromatography-mass spectrometry (GC-MS) [77, 78]. The simplest method for the determination of lipid peroxidation is the measurement of TBARS. The "gold standard" method is GC-MS because of its superior chemical specificity [79].

II-C-b. Proteins

Oxygen free radicals attack proteins, producing fragmentation products that accumulate in aged cells [15], products that are cross-linked

with malondialdehyde (MDA), and site-specific lesions that change the protein structure [56]. Lipid radicals play an important role in the damage of proteins [80]. Byproducts of peroxidation, MDA and hydroxynonenal, attack proteins, especially thiol (-SH) and amino (-NH₂) groups, injure human cells, and change the protein structure [56]. During lipid peroxidation, membrane proteins are altered and the loss of polypeptides and accumulation of high-molecular weight materials occurs [81]. MDA can modify proteins by adduct formation and damage tissue via an immunologic mechanism in a rabbit model [82]. Proteins modified by aldehydes, malondialdehyde and 4-hydroxynonenal, were detected in atherosclerotic lesions by an immunological technique [70].

Such structural and functional changes in specific proteins have significant physiological results, such as enzyme inactivation and/or inhibition. Oxidation of an amino acid residue in the α 1-protease inhibitor eliminates the inhibitory effect; the nonactive protease leads to emphysema [83]. Oxidation of the amino acid residues of glutamine synthetase causes inactivation that may lead to stroke, owing to an accumulation of glutamate [84]. Free radicals produce hemoglobin damage and stimulate protein degradation, lipid peroxidation, and hemolysis [85].

II-C-c. Nucleic Acids

Oxidation of nucleic acids by free radicals creates two basic lesions: strand breaks and altered bases [15]. Superoxide generated from the xanthine/xanthine oxidase system causes DNA strand breaks [86] and hydroxyl free radicals produced by the ascorbate/iron system damage DNA

[87]. Strand breaks are caused by attack of hydroxyl free radicals on the sugar portion of the nucleic acid. Enzymes normally repair the DNA strand breaks, but the enzymes damaged by free radicals cannot restore the altered DNA to its original form. A consequence of incorporating the wrong base into DNA is the pathogenesis of many diseases [15]. Superoxide radicals may produce the clastogenic factor (CF) that stimulates further superoxide production by cells and may lead to cancer [88].

Three altered bases, 8-hydroxyguanine, 5-hydroxymethyluracil, and thymine glycol, are produced by hydroxyl free radical attacks on DNA. The formations of these modified bases are believed to be major reasons for causing mutagenesis and carcinogenesis [15]. 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation in DNA by hydroxyl radical attack [89] and by attack of singlet oxygen free radicals [90] has been found.

II-C-d. Carbohydrates

Free radicals produced by the food coloring, carminic acid, damage the carbohydrate deoxyribose in the presence of trace amounts of iron salts [91]. Hydroxyl radicals produced by tetracycline antibiotics cause the degradation of carbohydrates in the presence of a ferric salt [92]. Radiolytically generated peroxy and hydroxy radicals destroy galactose in immunoglobulin G (IgG) [93]. Free radicals depolarize polysaccharides, such as hyaluronic acid, causing loss of structural and functional properties of these molecules [55].

II-D. Free Radical-Induced Tissue Damage and Diseases

Free radicals and ROS play an important role in the pathogenesis of numerous diseases. Eighty-five percent of degenerative diseases and more than 50 human diseases may be caused by derivatives of oxygen [56, 94]. Table 3 shows a list of diseases that may be caused by free radicals.

II-D-a. Membrane Damage

Cell membranes are composed of phospholipids, the backbone of membrane structure; proteins; and cholesterol. Many fatty acids in phospholipids are polyunsaturated fatty acids (PUFAs) which are essential in maintaining membrane functions, e.g. fluidity [56, 95]. One of the targets of free radical attack in cells is the lipid in cell membranes. Once a radical attacks lipids, it initiates a chain reaction [96]. One way to stop this chain reaction is to use chain-breaking antioxidants (scavenger molecules) [57, 97].

The functions of the cell membrane are compromised following an attack by free radicals [47]. Isolated rat-liver mitochondria are swollen in the presence of oxygen free radicals [98]. When oxidant production exceeds the capacity of the RBC's antioxidant system, membrane damage may occur and cause red cell destruction [99]. Another important function of the membrane protein which is compromised by free radical attack is the sodium/potassium pump, a way of transporting vital raw materials such as glucose and amino acids across cell membranes [47].

II-D-b. Endothelial Damage and Vascular Effects

Endothelial cell injury by free radicals and ROS is very important because of the implication for atherosclerosis [1]. Endothelial cell injury is caused by free radicals such as superoxide, hydroxyl radical, and hydrogen peroxide during the cellular pro-oxidant state. Superoxide generated in the endothelial cells during oxidation of hypoxanthine by xanthine oxidase plays an important role in the injury of endothelial cells [100]. Hypochlorous acid and hydroxyl radicals generated during granulocytic phagocytosis via the action of membrane-bound NADPH oxidase can cause lipid peroxidation of membranes, and produce irreversible tissue injury [101]. ROS produced by platelets, macrophages, and smooth muscle cells are probably responsible for damage to the endothelium and the development of atherosclerosis. Another cause of atherosclerotic vascular disease is endothelial dysfunction owing to altered permeability of plasma lipoproteins, increased cytokine and growth factor production, high coagulability of blood, and hyper-adhesiveness of white blood cells to the endothelium [96].

II-D-c. Lipid Peroxidation

Lipid peroxidation is a free radical-induced degradative process of both free polyunsaturated fatty acids (PUFAs) and those structures that incorporate lipids that are attacked by free radicals. Attacked lipids are oxidized and become lipid peroxides that are toxic and damage all tissues [102-105].

The initiation of lipid peroxidation occurs at the double bonds which are more susceptible to free radical attack. Since a hydrogen atom contains

only one electron, the removal of a hydrogen electron from carbon produces a carbon-centered radical that can rearrange its chemical structure. This creates a lipid peroxy radical under the action of O_2 . Peroxy radicals can (1) react with amino acid residues on membrane proteins, (2) oxidize LDL which is very important in atherogenesis, (3) induce the free radical chain reaction of lipid peroxidation, (4) react with one another when they collide within the membrane, and (5) react with antioxidants to stop the chain reaction [56].

II-E. Atherosclerosis

Atherogenesis involves environmental and genetic factors, endothelial and smooth muscle cells, monocytes, platelets, LDL, connective tissue, elements of the arterial intima, and fluid mechanical factors. Among them, oxidized-LDL (ox-LDL) clearly plays an important role in atherogenesis [6-12].

The presumed involvement of ox-LDL in atherogenesis is based on: 1) the presence of ox-LDL and lipid peroxides in areas of the atherosclerotic plaque [106], 2) the inhibition of macrophage uptake of native LDL by the antioxidant probucol [107], 3) the anti-atherogenicity of antioxidant therapy [108], 4) the increased lipid peroxidation of LDL isolated from atherosclerotic patients [10], 5) the antibodies developed against ox-LDL, and MDA-conjugated LDL on the aortic lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits [109], 6) the finding from Western blot analysis showing the reactivity of LDL extracted from human and rabbit lesions to antibodies to ox-LDL [106], and 7) the presence of autoantibodies

that cross-react with MDA-conjugated LDL in sera of humans and WHHL rabbits [106, 110].

For a better understanding of ox-LDL and its involvement in atherosclerosis, the pathophysiology of LDL, LDL receptors, mechanisms of oxidation of LDL, properties of ox-LDL, a theory of atherosclerosis and its prevention by antioxidants will be discussed in detail.

II-E-a. Structure and Metabolism of LDL

The major cholesterol-carrying lipoprotein in plasma is LDL. It consists of mostly cholesterol ester inside a double lipid layer, and also contains unesterified cholesterol, phospholipids, and apo B-100. The average diameter of LDL particles is 22 nm and they have a mean molecular weight of 2.5 million [11, 112]. LDL is smaller, but has a higher density (1.006 to 1.063 g/mL), than VLDL and chylomicrons [113].

LDL can be cleared from the blood by the liver (75%) or by extrahepatic tissues (25%). About 75% of LDL can be removed by receptors in the liver cells and extrahepatic tissues; 25% is removed by nonreceptor pathways. An average of 30% to 40% of the total plasma LDL is metabolized per day [111]. LDL receptors are transported and migrate to coated pits on the cell surface. LDL receptors get together on the coated pits and combine with the circulating LDL, which then are taken into the lysosomes. LDL receptors are dissociated from LDL and recycled to the surface of the cell for reuse [114]. The unesterified cholesterol that is hydrolyzed from LDL cholesterol esters can serve as an essential ingredient of cell membranes, can be stored after re-esterification to cholesterol ester,

and/or can be metabolized into bile acids. The cholesterol quantity entering the cell controls both the activity of HMG-CoA reductase and the rate of synthesis of LDL receptors [111].

II-E-b. LDL Receptors

The concentration of serum LDL depends on the quantity of VLDL that is produced in the liver cells as a precursor of LDL, and the percentage of VLDL remnants cleared by the liver. Both the rate of formation and clearance of LDL are affected by the number of LDL receptors. The LDL receptor consists of approximately 820 amino acids and a series of carbohydrate moieties that increase the molecular weight to 120,000 daltons. The receptor has several portions with a different number of amino acids, each with a unique function such as a portion that binds LDL, an epidermal growth factor-like structure with unknown function, and a bridge to a carbohydrate residue that spans the cell's membrane and projects into the cytoplasm [114, 115].

The concentration of cholesterol in the cell has a negative feed-back control on the synthesis of LDL receptors [116]. Each cell regulates its own production of LDL receptors based on the demands of cholesterol [117]. This regulation can be achieved through conversion of a small portion of cholesterol into an active form, oxy-sterol, that easily penetrates into the nucleus. This oxy-sterol suppresses the activity of the promoter area on the gene encoding for the LDL receptor. When the cholesterol concentration decreases, the promoter is activated to produce more LDL receptors [118]. An important regulator of plasma LDL is the number of functional LDL

receptors [119, 120]. About 1 in 500 humans are familial heterozygotes for hypercholesterolemia (FH); these patients have one-half the normal number of receptors and a two-fold normal plasma LDL concentration. FH homozygotes (1/million) have a six-fold or higher LDL concentration [118]. One of the best animal models for FH is the WHHL rabbit [121]. An increase in human LDL owing to a defect in LDL receptors has been well-documented [122, 123].

II-E-c. Oxidative Modification of LDL

Oxidized LDL plays a role in the development of atherosclerosis [11, 124]. LDL, the major cholesterol carrier in the blood, can be modified by enzymatic and non-enzymatic processes [9]. Oxidation of LDL occurs by cells in the arterial wall, endothelial cells [125-127], smooth muscle cells [127-1219], macrophages [130] or monocytes [131, 132], T-lymphocytes [133], and polymorphonuclear leukocytes [134, 135]. Modification of LDL by cells involves both enzymatic and non-enzymatic processes [125]. These oxidized forms are recognized by the acetyl LDL receptor, also known as the scavenger receptor. Another way of oxidative modification of LDL in vitro is achieved by incubating LDL with a high concentration (μ moles) of copper and iron [128, 136]. The mechanism of LDL oxidation by endothelial cells is unknown; however, these cells may generate free radicals to cause lipid peroxidation and dissociate the apolipoprotein B molecule [9, 137]. A second possibility is the generation of lipo-oxygenase that acts directly on the LDL [138, 139]. Another possible way is through oxidized free fatty acids that initiate peroxidation of LDL [138].

II-E-d. Characteristics of Ox-LDL

Functional changes of oxidized LDL are manifested in its uptake by macrophages, chemotaxis and adhesiveness, cytotoxicity, enhanced coagulation, increased platelet aggregation, vasoconstriction, inflammation, and altered immune function by destroying galactose in IgG [93].

The most important of these properties is the markedly increased rate of uptake and degradation through the acetyl LDL or scavenger receptor, leading to foam cell formation [140, 141]. Uptake of ox-LDL by scavenger receptors on macrophages and the resulting accumulation of lipid in the cells is a result of the peroxidative changes in apo B induced by oxidation of LDL [5]. The cellular uptake and accumulation of modified LDL is not caused by changes in the apo B structure; rather, it is caused by the net charges of the apo B protein, especially lysine residues [142].

Ox-LDL enhances chemotaxis of human monocytes [143] and human T-lymphocytes [144, 145]. The responsible component for the chemotaxis is believed to be lyso-phosphatidylcholine that is generated by phospholipase A2 during oxidation [143]. This same ox-LDL strongly inhibits the motility of mouse resident peritoneal macrophages [146]. It is believed that once LDL is oxidized at the intima, it recruits monocytes and favors their retention after they become phenotypically modulated [6]. LDL modified by monocytes and endothelial cells [147] and modified by superoxide [148] stimulates adhesive properties of the endothelium and leukocytes, respectively, a process that is inhibited by antioxidants. A sub-lethal dose of ox-LDL may alter expression of intercellular adhesion molecule 1 (ICAM-1) to enhance monocyte binding capability [149].

Cytotoxicity has already been discussed in the sections on Endothelial Damage and Vascular Effects (II-D-b), and Lipid Peroxidation (II-D-c). Research has demonstrated the cytotoxicity of ox-LDL and its inhibition by antioxidants [129, 150], and the atherogenesis caused by endothelium injury which is due to ox-LDL [151-153].

Ox-LDL markedly decreases the activation of protein C, an important ingredient in vascular anticoagulation in both human venous and arterial endothelial cells [154, 155]. Ox-LDL increases the human arterial and venous endothelial cell tissue factor activity [155], an effect that was prevented by the antioxidant probucol [154]. Ox-LDL and its extracted lipid stimulate plasminogen activator inhibitor-1 and inhibit tissue-type plasminogen activator release from endothelial cells [156]. Ox-LDL increases the prothrombin and partial prothrombin times in vitro. The mechanism is ox-LDL combining with calcium ions [157]. Ox-LDL probably promotes favorable conditions for thrombus formation.

The high binding affinities of both LDL and HDL to isolated intact platelets has been described, and the receptor proteins that are responsible for the binding of lipoproteins to platelet surfaces have been identified [158]. LDL induces platelet aggregation of human blood at a concentration above 2 g protein/L and is followed by secretion of dense granules by the platelets [159]. Prostacyclin (PGI₂) is a potent inhibitor of platelet aggregation, but its action was reversed by LDL by decreasing the synthesis of cAMP [160]. Uptake of ox-LDL by macrophages can be stimulated by a protein-like factor secreted by the alpha granules of activated platelets [161, 162]. This mechanism facilitates the formation of foam cells.

Ox-LDL selectively inhibits vascular smooth muscle relaxation that leads to a decrease in vasodilation [163]. In the intact porcine aorta, ox-LDL increased the production of a peptide that could be a source of vasoconstriction, smooth muscle cell proliferation, and progression of atherosclerosis. It also increased the release of endothelin found in the atherosclerotic patient [164]. Ox-LDL not only inactivates the endothelial cell-derived relaxing factor (EDRF), a mediator of blood vessel relaxation, but also directly impairs the release of nitric oxide-induced cGMP accumulation in detector cells [165]. An experiment performed on vascular tone in isolated, perfused rabbit femoral arteries shows that ox-LDL increases agonist-induced vasodilation by a direct effect on the vascular smooth muscle; therefore, ox-LDL increases the risk of vasoconstriction in hypercholesterolemia [166].

Immunologic processes and inflammation may be involved in the development of atheroma [167, 168]. This theory is supported by the fact that: 1) atherosclerotic lesions contain immunoglobulins and complement components [169], T-cells [170], and immunostained antibody to ox-LDL [171]; 2) antibodies against ox-LDL are present in human and rabbit sera; and 3) human and rabbit atherosclerotic lesions contain IgG that recognizes epitopes of ox-LDL [172]. Moderately oxidized LDL induces IL-1 β in human monocyte-derived macrophages by 9-hydroxyoctadecadienoic acid [173]. These characteristics further support a critical role for ox-LDL in atherosclerosis.

Other chemical properties of ox-LDL are: 1) increased density, greater mobility on agarose gel electrophoresis, and loss of cholesterol esters; 2) decreased content of polyunsaturated fatty acids; 3) increased amount of aldehydes [174]; 4) increased aggregation of particles that lead to enhanced macrophage degradation [175] and macrophage recognition [176]; 5) release of lactate dehydrogenase in to the medium and a decrease in viable cells; and 6) the incorporation of [^3H] thymidine into DNA [177].

II-E-e. Atherosclerosis Caused by Ox-LDL

Oxidatively modified low-density lipoprotein (ox-LDL) is a known major factor leading to atherosclerosis. Its principal atherogenic properties have been discussed in detail in this section, especially the characteristics of ox-LDL (II-E-d). Several new lines of evidence show that ox-LDL occurs in vivo and that antioxidant therapy slows progression of atheroma formation, supporting the atherogenic role of ox-LDL [178].

Early atherogenic mechanisms seem to trace the following events in the experimental hypercholesterolemic animal [12]:

- 1). Hypercholesterolemia with increased LDL
- 2). Increased adhesion and infiltration of monocytes/macrophages into the intima
- 3). Ox-LDL uptake by macrophages and foam cell formation
- 4). Fatty streaks are formed though endothelium is still intact
- 5). Appearance of smooth muscle cells in the intima
- 6). Endothelial cell retraction by exit of foam cells from the intima
- 7). Adhesion of platelets on the exposed sub-endothelial matrices

8). Formation of proliferative lesions

9). Fibrous plaque formation

A key event in the above sequence is the process of forming foam cells. Some of the circulating LDL, especially when the LDL concentration is high, is entrapped in the subendothelial space by the binding of apo B-100 to glycosaminoglycans [179]. The LDL is oxidized when the subendothelial space favors oxidation because of a low concentration of antioxidants. This ox-LDL enhances adhesion of monocytes to the endothelial cells [147] and activates the adherent monocytes to penetrate into the intima by chemotaxis [143, 145]. Chemotaxis-driven monocytes in the intima undergo phenotypic transformation into macrophages whose motility is then inhibited by ox-LDL. Therefore, macrophages cannot return to the circulation. The more LDL that is oxidized by the entrapped macrophages, the greater the atherogenic actions of ox-LDL, including the cascade of atheroma formation. Macrophages become foam cells after taking up abundant ox-LDL by scavenger or acetyl-LDL receptors that are found only on monocytes/macrophages, Kupffer cells, and some endothelial cells. Finally, fatty streaks develop [7].

While more foam cells are generated, their volume is increased and the endothelial cells overlying them are stretched thin and eventually rupture exposing the underlying macrophages to the circulating blood components. Platelets aggregate at the exposed structural elements of the artery wall and release growth factors causing both recruitment of smooth muscle cells into the intimal layer and stimulation of their proliferation [11].

II-E-f. Prevention of Ox-LDL as an Anti-Atherosclerotic Mechanism

If ox-LDL is implicated in the atherosclerotic process, then treatment with antioxidants or any scavengers of free radicals could be beneficial. The antioxidant probucol decreases the rate of progression of atherosclerosis in WHHL rabbits [180-182]. A detailed discussion of this topic is in the Antioxidants section (II-G).

II-F. Measurement of Free Radicals

Free radicals have short half-lives (Table 4), therefore direct measurement of free radicals is difficult [63]. Direct measurement methods have another drawback; they require special instruments. There are many indirect methods that require only ordinary laboratory equipment.

II-F-a. Direct Methods

- 1). Pulse Radiolysis (PR) is used for the controlled generation of radicals by ionizing radiation, and UV-visible spectroscopy is used to detect the intermediates.
- 2). Electron Spin Resonance (ESR) spectroscopy is used to detect long-lived or short-lived species that have been spin-trapped.

II-F-b. Indirect Methods

- 1). The markers of free radical formation, hydroperoxides, epoxides, aldehydes, volatile breath by-products (ethane, pentane), oxidized proteins and DNA (purines and pyrimidines), and damaged chromosomes (micronuclei) can be analyzed by GC, and HPLC

methods.

- 2). Biological reactions as indications of free radical damage can be used; typical examples are thiobarbituric acid-reactive substances (TBARS) and conjugated diene formation.
- 3). Total antioxidant capacity of serum [183] can be assayed by the oxygen-radical absorbance capacity (ORAC) test.
- 4). In vivo functional assays for cell viability, surface markers and membrane function (fluidity) can be done.

II-G. Antioxidants

An antioxidant is "a substance added to a product to prevent or delay its deterioration by the oxygen in air" [184]. A definition of a biological antioxidant is "any compounds, of natural or artificial in origin, that protect biological systems against any effects of oxidative action or process of oxidable substances by their actions of (1) scavenging of free radicals and ROS, (2) interception (chain breaking) of reactive radical species, (3) suppression of amplification of free radical damages by further intercepting secondarily derived radicals, and (4) inhibition of the recruitment/adhesion of blood cells and chemotaxis" [185].

Many antioxidants have been discovered and utilized for experiments and treatments. Different kinds of antioxidants are subgrouped into: 1) biological antioxidants such as enzymes, vitamins and small molecular weight biomolecules (Table 5), 2) plasma proteins acting as antioxidants (metalloenzymes) (Table 6), and 3) other chemicals [7] (Tables 7).

II-G-a. Mechanisms and Functions of Biological Antioxidants

The major extracellular antioxidants are located mostly in the plasma, and their actions include the removal of metal ions that generate ROS (Table 8) [185]. The possible anti-carcinogenic effects of vitamins A, E, C, and β -carotene are summarized in Table 9 [186]. Intracellular antioxidants are more lipophilic, and are compartmentalized in the membrane, cytoplasm and organelles (Fig. 2) [7]. The structures of some common lipid-soluble and water-soluble antioxidants are shown in the Fig. 3 and Fig. 4 [185].

Mechanisms of antioxidants commonly occurring in the human physiology are as follows:

1. Superoxide dismutase (SOD) catalyzes the conversion of superoxide to oxygen and hydrogen peroxide as follows:

SOD has been used to protect rat liver from radical-mediated peroxidation and to preserve hepatic energy stores upon reperfusion [187], to protect rabbit retina from ischemic injury [188], and to treat inflammatory diseases and skin ulcers [189]. Other investigations of the antioxidant effects of SOD have been carried out on psychiatric diseases [190] and Down's syndrome [191]. One of the limitations of SOD therapy is its short half-life, being less than 10 minutes [192].

2. Catalase catalyzes the conversion of H_2O_2 (including that produced SOD) as follows:

In the absence of catalase, the extremely reactive hydroxyl radical (OH°) is generated in the presence of iron and copper as follows:

Beneficial effects of using a combined treatment with catalase and SOD have been shown [188, 193]. Catalase alone prevents rat oral mucosal damage caused by induction of hydrogen peroxide [194], and protects rat jejunum from oxidative damage [195].

3. Selenium glutathione peroxidase (Se-GSH-PX), with the help of phospholipase A2 (Plase A2), converts harmful phospholipid hydroperoxides (PLOOH) to free fatty-acid hydroperoxides (LOOH) and lysophospholipids, then to harmless fatty acid alcohol (LOH) as follows:

Glutathione peroxidase removes harmful hydroperoxides from tissues [196], especially from the human brain [197].

4. Phospholipid hydroperoxide glutathione peroxidase (PLOOH-GSH-Px) directly converts harmful phospholipid hydroperoxides to phospholipid-fatty acid alcohol (P-LOH):

This enzyme can also remove membrane-associated cholesterol hydroperoxides. Unlike glutathione peroxidase that requires phospholipase A2 before it acts, phospholipid hydroperoxide glutathione peroxidase protects membranes directly from damage caused by lipid peroxidation by reducing membrane phospholipid hydroperoxides [198].

5. Tocopherol (Vitamin E) can react with two peroxy radicals, as shown below:

The α -tocoperoxyl radical (α -tocopherol $^\circ$) can react with another peroxy radical to form a stable compound. Vitamin E is a major lipid-soluble, chain-breaking, membrane-bound antioxidant. Vitamin E is oxidized by quenching free radicals and is reduced by reaction with ascorbic acid in the aqueous phase [199]. Vitamin E inhibits the oxidation of protein

kinase C that is involved in cell proliferation [200], platelet phospholipase A2 [201], and LDL oxidation [202]. The anti-atherogenic role of vitamin E is reviewed by Ferns [203]. The Vitamin E Research and Information Service (VERIS) published a booklet that contains hundreds of abstracts published during 1991 and 1992 worldwide [204].

6. Carotenoids (CAR) are widely distributed. There are 600 identified in nature, and these are efficient antioxidants for singlet oxygen owing to their highly conjugated double bonds [205]. β -carotene may react with two peroxy radicals as does vitamin E [206]. The antioxidative ability can continue to produce carbon-centered radicals on a single carotene molecule, followed by radical-radical reaction as follows:

Studies show that β -carotene reduces the risk of coronary artery disease [207], cancer in a human epidemiological study [208], and cancer in an animal study [209]. The intake of β -carotene-rich vegetables was shown to correlate with a lower risk of cancer (especially lung cancer) [210], however this was not conformed by another research group [211]. Bendich

[212] reviewed articles demonstrating that β -carotene enhances immune functions, including B- and T-lymphocyte proliferation, induction of tumor killer cells, and secretion of immunological communication factors.

7. Vitamin C is a strong antioxidant that works synergistically with vitamin E [213]. Vitamin C, found in the cytosol and extra-cellular fluids because of its water solubility, is oxidized to dehydroascorbate by oxidants, then reduced back to ascorbate by a GSH-dependent reaction [7]. Ascorbic acid scavenges radicals before they reach the membranes and LDL. Even though vitamin C cannot scavenge lipophilic radicals in the membrane, it helps in the regeneration of lipophilic vitamin E, working synergistically as seen below :

The tocopheroxyl radical may be reduced by ascorbate to regenerate tocopherol or may scavenge another peroxy radical to generate an adduct (EOOL) [214]. The ascorbyl radical produces dehydroascorbic acid (DHA) that is reduced enzymatically to ascorbate [214] or stabilized by urate due to its chelation of iron [215]. The biological properties of vitamin C are listed in Table 9 [186]. Additional characteristics of vitamin C include the protective ability of vitamin C on LDL oxidation [216], the protective effect on cell membranes in focal cerebral ischaemia [217], and the reductant activity for nitroxide radicals in erythrocyte membranes [218].

8. Vitamin A is a beneficial antioxidant as listed in Table 5 and 9; however, excessive amounts may cause liver damage [219]. Vitamin A deficiency is one of the world's major malnutrition problems and is associated with infections and increased child mortality [220]. Vitamin A has been shown to have protective effects in various transplantable tumor systems [221] and it protects against cancer [222]. Table 10 contains a list of immunological responses to vitamin A, and the subjects or models the researchers used for their studies [223].

9. Probucol is a strong antioxidant that is highly hydrophobic; its structure consists of two butylated hydroxytoluenes bridged by a sulfur-carbon-sulfur bond. The butylated hydroxytoluene groups are strong antioxidants. Only 2-8% of probucol is absorbed and it is transported by LDL, VLDL, and HDL in the plasma [224].

Probucol is a superoxide radical scavenger in vitro [225]. It inhibits oxidative modification of LDL and uptake of ox-LDL by macrophages [87, 226]. It also reduces plasma lipid peroxide in humans [227]. Animal studies in WHHL rabbits reported that probucol reduces atheroma formation [180-182, 228, 229].

Clinical experiments with probucol demonstrate that it also has a hypocholesterolemic effect, including lowering of HDL [230]. This is an undesirable effect since elevated concentrations of HDL are indicative of a lower risk factor for atherogenesis [231]. The hypothesis for the HDL-lowering effect of probucol is that probucol not only has an antioxidant property, but also the ability to modify plasma HDL metabolism leading to a

reduction in both HDL cholesterol and HDL2 concentrations by improving the removal of tissue cholesterol [232].

Probucol has a few mild side effects, mostly in the gastrointestinal system, for example diarrhea, loose stools and flatulence. Animal and human studies on probucol show an induction of ventricular fibrillation in dogs sensitized with epinephrine [224], a prolonged electrocardiographic QT interval in monkeys [233], and a small but significant prolongation of QT in a five-year prevention trial in humans [234].

10. Flavonoids in plants are polyphenolic compounds and are known to have antioxidant [235, 236] and cytotoxic effects [237]. Plant flavonoids inhibit cell proliferation significantly; however, more research is needed because their potent cytotoxic actions may not involve free radicals or lipid peroxidation reactions [238].

The National Cancer Institute has begun the investigation of plant compounds, e.g. phytochemicals, that affect cancer treatment or prevention [239]. Niwa et al. [94] demonstrated that many natural products contain ROS-scavenging compounds. Plants and herbs and their extracts have been used for the treatment or prevention of various disorders, even though their mechanisms of action are not well understood. They have been used as anti-inflammatory agents [240], anti-convulsants [241], immunomodulating agents [242], agents to prevent the biotransformation of arachidonic acid [243], agents for malaria and fever treatment [224], antimicrobial agents [245], agents that inhibit a human malignant melanoma cell line [246],

agents that inhibit human colon cancer growth in vitro [247], and agents for cytotoxicity to cultured cells [248].

11. Miscellaneous antioxidants or substances that scavenge free radicals are: catechin, glucose, bilirubin, riboflavin, 5-amino-salicylic acid, uric acid, sodium hydroxybutyrate, cysteine, creatinine, ubiquinol, ubiquinone, glutathione, 2-hydroxy estrone, 2-hydroxy estradiol, N-acetyl cysteine, allopurinol, lazaroids, ferulic acid, and ceruloplasmin (Table 5-7).

II-G-b. Synergistic Effects of Antioxidants

Antioxidants work best in combinations. Efficient prevention of membrane lipid peroxidation by vitamin E may be due to its regeneration by vitamin C [214]. The addition of ascorbate to vitamin E-containing liposomes provides marked synergistic protective effects [213], but such effects depend on the location of the antioxidants in the membrane [249]. The same synergistic antioxidant effect was found between vitamin E and cysteine in the prevention of oxidation, but the degree of regeneration of vitamin E was less than with vitamin C [250]. A combination of β -carotene and α -tocopherol exerted a more potent inhibiting effect on lipid peroxidation in membranes than α -tocopherol alone [251, 252]. Inhibition of LDL oxidation was enhanced by using a combination of probucol and ascorbate. The synergistic effects of antioxidants may play an important role in vivo [253]. A minimal amount of vitamin E was required, when combined with selenium, to produce a significant cancer-retarding effect in

a study on 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors [254]. Combined antioxidants such as vitamin E, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, beta-carotene, canthaxanthin, and (+)-catechin, provided better protection of liver slices from oxidative damage than that of the individual antioxidants [255]. Similar experiments were done and the same results were obtained in rat tissue slices [256], and rat erythrocytes and plasma [257].

II-G-c. Natural Vitamin E and Synthetic Vitamin E

"Natural-source" and synthetic vitamin E do not have the same composition nor biological activities. Natural-source vitamin E is a single entity, while the synthetic form is a mixture of eight stereo isomers, among which only one is equivalent in molecular configuration to natural vitamin E.

Natural-source vitamin E was retained better than the synthetic form in rat tissues, with the exception of liver [258]. Similar experiments showed a preferential uptake of natural-source vitamin E by most tissues. For example, the greatest difference in the tissues was found in red blood cells of the rat; the accumulation of α -tocopherol with the natural α -tocopherol in the diet was 4 to 6 times that accumulated with the synthetic α -tocopherol diet [259]. The natural vitamin E concentration in the liver, kidney, spleen and heart of trout was 6 to 18 times higher than with synthetic vitamin E in the first 4 hours, and 2 to 3 times higher between 8 and 15 hours after oral administration, as compared to synthetic vitamin E [260]. This difference in retention is believed to be caused by the specific protein receptors for natural vitamin E [261]. The higher preferential enrichment of natural

vitamin E in very low density lipoproteins as compared to synthetic vitamin E was also demonstrated in humans [262].

II-H. Research on MAK

Since the worldwide introduction of MAK in 1986, numerous investigators have studied the antioxidant, antiatherogenic, anticarcinogenic, immunomodulatory, and general health-promoting effects of MAK on animals and humans. Research on MAK has been comprehensively reviewed by Sharma [47].

II-H-a. Antioxidant Effects of MAK

Fields et al. [50] reported that MAK-4, like SOD, completely scavenged SO generated by xanthine-xanthine oxidase and human neutrophils in vitro. SO was monitored by determining the reduction of ferricytochrome C using spectrophotometer at 550 nm. MAK-4 also did not compromise the viability or respiration (O_2 utilization) of polymorphonuclear cells.

Niwa [263] reported that MAK-4 and MAK-5 markedly decreased $O_2^{\circ-}$, H_2O_2 , and OH° generated by neutrophils and the xanthine/xanthine oxidase system in a dose-dependent manner. Niwa noted that the effects of MAK-4 and MAK-5 were comparable to or better than that of SOD.

Dwivedi et al. [52] examined the ability of MAK-4 and MAK-5 to inhibit rat liver microsomal lipid peroxidation in an NADPH-generating system or a system employing sodium ascorbate and an ADP-iron complex. Alcoholic and aqueous extracts of MAK-4 and MAK-5, when added to these incubation systems, inhibited hepatic microsomal lipid peroxidation in a

concentration-dependent manner. The aqueous extract of MAK-4 was the most effective antioxidant in these systems.

Sharma [264] demonstrated that both the aqueous and alcoholic extracts of MAK-4, and the aqueous extract of MAK-5 inhibited EC-induced and soybean lipoxygenase (SL)-induced LDL oxidation in a concentration-dependent manner.

Engineer, et al. [265], reported that alcoholic and aqueous extracts of MAK-4 and MAK-5, when added to an NADPH-generating system in the presence or absence of Adriamycin, inhibited rat liver microsomal lipid peroxidation in a concentration-dependent manner. The alcoholic extract of MAK-4 was the most effective antioxidant in these systems. A diet supplemented with 6.0% MAK-4 also significantly reduced Adriamycin-induced death in mice.

Sharma, et al. [266] found that both aqueous and alcoholic extracts of MAK-4 inhibited EC- and SL-induced LDL oxidation in a concentration-dependent manner. LDL was incubated with or without EC or SL in the presence of varying concentrations of MAK-4 or MAK-5 extracts. TBARS and diene conjugation were measured for the estimation of LDL oxidation.

Sharma et al. [54] reported that MAK-4 inhibited LDL oxidation and showed more antioxidant potency in preventing LDL oxidation than that of ascorbic acid, alpha-tocopherol, or probucol. MAK-4 also inhibited both the initiation and propagation of cupric ion-catalyzed LDL oxidation.

II-H-b. Antiatherogenic Effects of MAK

A relatively small number of studies have been performed on the antiatherogenic effects of MAK. Panganamala et al. [267] reported that rabbits fed Purina rabbit chow supplemented with 0.4% MAK-4 showed a reduction of plasma and hepatic lipid peroxidation and reduced platelet aggregation induced by collagen and ADP.

Sharma et al. [49] found that an aqueous extract of MAK-5 reduced platelet aggregation induced by arachidonic acid, collagen, and epinephrine. The aqueous extract of MAK-5 halted the second phase of aggregation caused by epinephrine, and deaggregated platelets that were aggregated by ADP.

Dogra et al. [268] showed that 30 patients with angina pectoris (21 males, 9 females; 11 smokers; ages 32-59) who received MAK-4 (10 g twice a day) and MAK-5 (600 mg tablet twice a day), extended their mean angina-free period from 3.5 to 18.5 days. Eighty percent of patients demonstrated significant improvement after 6 months. Five out of 11 hypertensive patients had reduced systolic blood pressure, and the HDL cholesterol concentration was increased.

Animal models, especially Watanabe heritable hyperlipidemic (WHHL) rabbits, that are a similar model to human familial hypercholesterolemia, would be the model of choice for the study of atherosclerosis prevention by MAK-4.

II-H-c. Other Miscellaneous Activities of MAK

1. Anticarcinogenic activities of MAK

Several experiments, as described below, have successfully demonstrated that MAK decreased the incidence, growth, and metastasis of cancer in animals.

Sharma, et al. [18] demonstrated that a 6% MAK-4-supplemented diet protected female rats from 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors, during both the initiation and promotion phases of cancer development. The control animals who developed tumors showed tumor regression in 60% of cases when supplemented with a MAK-4 diet for four weeks. In 50% of these rats, the tumors regressed completely.

Arnold et al. [269] found that an extract of MAK-4 inhibited benzo(a)pyrene (BP)-induced morphological transformation in rat tracheal epithelial (RTE) cells by 27% and inhibited growth of A427 human lung tumor cells by 51%. An aqueous extract of MAK-5 also inhibited transformation in RTE cultures containing BP by 53%.

Patel et al. [270] investigated the effects of MAK-4 on metastasis of Lewis Lung Carcinoma (LLC) in mice and found that the animals receiving MAK-4-containing Purina chow had significantly fewer and smaller metastatic lung nodules. Female mice were fed either chow supplemented with MAK-4 or normal chow, and inoculated subcutaneously with 5×10^5 tumor cells. Animals were sacrificed 1 to 3 days after the first animal died in either group. In the animals fed MAK-4, there was a 65% decrease in the total number of metastatic nodules and a 45% decrease in the size of the nodules.

Prasad et al. [271] showed that an ethanol extract of MAK-5 induced morphological differentiation (neurite formation) and biochemical differentiation (15-fold increase in tyrosine hydroxylase) in murine neuroblastoma cells in culture, whereas an aqueous extract of MAK-5 increased only the activity of tyrosine hydroxylase and to a much lesser extent than the alcoholic extract. Also, both an alcoholic and aqueous extract of MAK-5 increased the intracellular concentration of cAMP by about 4-fold in 3 days.

The anticarcinogenic effect of MAK-4 on animals has been demonstrated. The mechanism for preventing carcinogenesis can be hypothesized as MAK preventing damage from free radical attacks and alterations of DNA.

2. Immunomodulatory effects of MAK

The Ayur-Vedic system of medicine uses a variety of herbal compounds to enhance the body's resistance to infection and disease. Early experiments on MAK by Sharma [47] indicated that Maharishi Amrit Kalash stimulates the immune system [272].

Patel et al. [273] and Dileepan et al. [274] found that MAK markedly enhanced rat splenic lymphocyte proliferation caused by various mitogens. MAK (50mg/day) was gavaged for 10 or 20 days. After the splenic lymphocytes were removed, mitogen-induced lymphocyte proliferation, macrophage superoxide production, and phagocytosis were analyzed. The lymphoproliferative response was significantly enhanced even in animals treated for 10 days and persisted for at least 15 days after withdrawal of MAK.

Macrophage superoxide generation and phagocytosis were not affected by MAK.

Dileepan et al. [273] studied the effect of MAK on the lymphoproliferative response, macrophage-mediated tumor cell killing and generation of nitric oxide (NO^o) by macrophages, and found that MAK contained ingredients that enhanced in vivo priming of both T-cells and macrophages. After 6 weeks of a MAK-5-supplemented diet, splenic lymphocytes and peritoneal macrophages were isolated from the sacrificed mice. The lymphoproliferative response was measured by [³H] thymidine uptake after activation of the lymphocytes by phytohemagglutinin or anti-CD3 antibodies. Tumor cell killing and NO production were measured in the activated macrophages. The MAK-supplemented diet enhanced tumor cell killing and production of NO.

Niwa [263] demonstrated that MAK-4 inhibited chemotaxis significantly, and both MAK-4 and MAK-5 decreased the phagocytic activity slightly, but markedly decreased the generation of superoxide, hydrogen peroxide, and OH-generated both in neutrophil and xanthine-xanthine oxidase systems. Thus, MAK-4 and MAK-5 markedly inhibited neutrophil inflammatory mediators and scavenged ROS, therefore enhancing immunity.

The exact mechanisms of immunity enhancement by MAK have not been determined. More work is needed in this area. Immunity enhancement mechanisms of MAK can be hypothesized as MAK protecting cell membranes, including immune cells.

3. Effects of MAK on CNS

Sharma et al. [276] tested MAK for its effects on opioid receptors in the brain, neuropeptides, and psychological changes in human volunteers. MAK showed generalized inhibition of opioid receptor activity. The extent of inhibition differed depending upon the sub-type of receptor. Substance P, which is a neurotransmitter involved in the pain pathway, decreased in the MAK group to 36.0 pg/mL from 255.8 pg/mL over a three-month period. Decreased Substance P suggested that MAK-5 may relieve pain and may be helpful in preventing pulmonary and gastrointestinal inflammation. The self report scales suggested that MAK may reduce depression and psychiatric distress in general. No change in the volunteers was seen in hematological and biochemical tests, including liver enzymes.

Curlee et al. [277] performed a clinical trial on patients with chronic neurological deficits. Twenty patients (age 22 to 37) who had sustained brain injuries 3 to 16 years previously (mean 8.8 years) were pair-matched in a double-blind, placebo-controlled, crossover study. After baseline studies of physical, neuropsychiatric, occupational, and physical therapy evaluations were performed, the same studies were performed at 6 months and at 12 months. MAK-4 and MAK-5 groups had significant improvement in total dominant and combined Jebsen hand function tests. Patients taking MAK-4 and MAK-5 showed a strong positive trend on the Weschler Memory Scale in serial addition, associative learning, and visual reproductions. Possible mechanisms for these findings are the protection of nerve and brain cells from free radical attacks by MAK-4 and 5.

4. Effects of MAK on other condition

Fields et al. [278] found that 58 male mice started on a MAK diet at 25 months of age for 8 weeks showed significantly ($p < 0.05$) more activity of locomotion (+85%), coordination (roto-rod, +23%), and lower heart weight (-30%). In a survival test with 18-month-old mice ($n=58$), 80% of mice in the MAK group were alive at 23 months vs 48% for the control group, which was a significantly ($p < 0.05$) finding. Survival rates of mice 7 days after injection of a cytotoxic drug, mitomycin-c at 3.25 mg/kg, were 100% (9 out of 9) for the MAK group and 33% (2 out of 6) for the control group.

II-I. WHHL Rabbits and Lipid Research

WHHL rabbits spontaneously develop massive hypercholesterolemia, which results from a single genetic defect of LDL receptors. This gene is the same as that of humans with familial hypercholesterolemia (FH). The pattern of atherosclerosis in WHHL rabbits is indistinguishable from the pattern in FH. In these rabbits, severe, full aortic atherosclerosis develops at 5 to 15 months.

II-I-a. WHHL Rabbits as the Experimental Model

WHHL rabbits offer important advantages over the cholesterol-fed rabbit model of atherosclerosis. Blood cholesterol concentrations of WHHL rabbits are similar to human FH, with concentrations of 350-1200 mg/dL [279]. The WHHL rabbits do not develop hepatic cholesterol toxicity or other organ damage, as happens in the cholesterol-fed rabbit. Cholesterol-fed rabbits develop aortic lesions that consist predominantly of foam cells

[280]. There are no sex preferences in the incidence and development of atherosclerosis and xanthomas in the WHHL rabbit [281].

II-I-b. Probucol as a Primary Drug of Choice in Antioxidant Research

Probucol, used for treatment of familial hypercholesterolemia, was shown to reduce LDL in WHHL rabbits by Natuszewicz [282]. LDL in the WHHL rabbits fed with probucol (1% w/w) was lowered by 36%, compared to a control group. Intrinsic changes in the structure and metabolism of plasma LDL in the probucol-treated rabbits was the cause of the lower LDL.

Carew et al. [107] found that probucol is an effective antioxidant, transports lipoproteins, including LDL, and prevents the oxidative modification of LDL in vitro. They also found that probucol significantly reduced the formation of fatty-streak lesions in WHHL rabbits compared to lovastatin-treated control rabbits, even though plasma cholesterol concentrations were no lower than in the control group. Probucol prevented atherosclerosis not by lowering cholesterol but by preventing LDL oxidation. This theory was confirmed by Steinberg, et al. [180] who stated that probucol, through antioxidant activity not necessarily related to its ability to lower plasma cholesterol concentration, can prevent the foam cell-rich fatty streak lesions in atherosclerosis.

Kita et al. [228, 283] demonstrated that probucol reduced the percentage of surface area of the total thoracic aorta with visible plaque to $7.0 \pm 6.3\%$ in the WHHL rabbits treated with 1% probucol for 6 months, compared to $41.1 \pm 18.8\%$ in the control WHHL rabbits. Average plasma cholesterol concentrations were 704 ± 121 mg/dL in the control group and

584±61 mg/dL in the test group. LDL was very resistant to oxidative modification by cupric ions and recognized minimally by macrophages in the test group. Nagano et al. [284] confirmed the antiatherogenic effect of probucol as an antioxidant. Probucol did not affect lipoprotein metabolism in macrophages of WHHL rabbits.

Probucol prevented formation of atheroma in young WHHL rabbits. Daugherty et al. [285] found that probucol did not promote regression, but retarded the continued deposition of cholesterol esters into atherosclerotic lesions of mature WHHL rabbits. WHHL rabbits fed with 1% (w/w) probucol for 6 months at age 9 months developed atheroma covering $78 \pm 7\%$ of the intimal area of the thoracic aorta, compared to $70 \pm 5\%$ in the control group fed normal chow until the age of 9 months, and $91 \pm 3\%$ in another control group fed normal chow for another 6 months. This finding was challenged by Nagano's group [181] who demonstrated the percentage area of aorta covered with plaque in the probucol-treated animals (1 g/day for 6 months at age of 8 months) was significantly ($p < 0.02$) smaller ($38.1 \pm 12.1\%$) than that of the control group (82.7 ± 22.65). Daugherty's group designed their experiment to observe the short-term effect of probucol and Nagano's group designed their experiment to observe the long-term antiatherogenic effect.

Mao et al. [286] compared probucol with its analogues to determine the effects on cholesterol lowering and antioxidant activities. Probucol was effective in lowering cholesterol in plasma, while the analogues were not. Both probucol and the analogues were effective in preventing cupric ion-

induced LDL oxidation. The antioxidant activity of probucol and its analogues is directly related to their concentration in LDL.

O'Brien et al. [287] used smooth muscle cell-specific (HHF-35), and macrophage-specific (RAM-11) antibodies in the WHHL rabbits and found that lesions from probucol-treated animals were significantly smaller ($p < 0.01$) and less cellular ($p < 0.05$) than those of the controls. Lesions from probucol-treated animals contained predominantly smooth muscle cell type, whereas lesions from the control consisted mainly of macrophages. The same magnitude of immunoreactivity was detected, even though probucol-treated animals had much less atheroma. These findings led to the conclusion that probucol-treated animals had a reduced monocyte/macrophage retention in lesions.

Vitamin E was studied for reduction of atheroma in WHHL rabbits by Williams and his colleagues [288]. A vitamin E-supplemented diet (0.5%, w/w) and regular chow were fed to the test and control groups, respectively. Vitamin E (d, L- α -tocopherol) not only reduced the plasma concentration of cholesterol, but also reduced the surface area of the lesions by 32%.

II-I-c. WHHL Rabbits Used for Other Experiments

Probucol (1%, w/w)-treated WHHL rabbits were compared with control WHHL rabbits and Japanese white rabbits for coagulation studies by Mori and coworkers [289]. They found that probucol attenuates atherosclerosis without decreasing the plasma cholesterol concentration. Primary findings showed blood clotting factor (factor VIII) and fibrinogen concentrations were statistically decreased in WHHL rabbits fed with

probucol ($p < 0.05$), therefore, increased factor VIII and fibrinogen in hyperlipidemia may contribute to the progression of thromboatherosclerosis. They did not discuss the idea that possible liver damage may lower fibrinogen and factor VIII.

A study on the effects of hypertension (created by the one-kidney, one-clip technique) and hyperlipidemia on the myocardium of WHHL rabbits was carried out by Nickerson et al. [290]. Hypertension caused left ventricular hypertrophy, medial thickening of the arteries, and hyaline arteriosclerosis. Since one of the complications of atherosclerotic plaque formation is an inflammatory process, Makheja and coworkers [291] investigated using an anti-inflammatory steroid (cortisone acetate, 5 mg/day for 3 months) on WHHL rabbits. The cortisone decreased plaque formation by 60% in the cortisone-fed animals.

II-J. Problem Statement

When excess amounts of endogenous and exogenous free radicals and ROS are produced, these highly reactive species and their oxidative byproducts cause cellular damage that leads to organ damage or dysfunction [19-44], inflammation, degenerative diseases, carcinoma [14-18], and atherosclerosis [6-13]. There is a strong association between atherosclerosis development and the concentration of oxidized LDL [6-13].

If our body maintains a balance between the production and scavenging of free radicals and ROS, with the help of endogenous antioxidants, exogenous antioxidants such as antioxidant-rich natural food supplements and antioxidant enzymes, free radical-induced degenerative diseases will be possibly prevented.

Many attempts to treat free radical-induced disorders by employing a single synthetic antioxidant, or a combination of two or three antioxidants, have been undertaken and have been successful for the most part. Antioxidants work best in combination with other antioxidants. These synergistic effects of antioxidants are documented in various articles [214, 249-257]. Among antioxidants, the naturally-occurring form of vitamin E works better than the synthetic form with regard to tissue retention and effectiveness [258-262].

MAK-4 and other Maharishi Ayur-Veda herbal mixtures have shown antioxidant effects [50-54, 263-266, 292], decreased platelet aggregation and improvement in angina pectoris symptoms in patients [49, 267-268], anticarcinogenic effects [18, 269-271], and immunomodulatory effects [47, 263, 272-275]. However, no in vivo study for the prevention or reduction of atherosclerosis has been done. Therefore, I would like to test the retardation of atherogenesis by the natural antioxidant mixture MAK-4 in Watanabe heritable hyperlipidemic rabbits.

II-K. Hypothesis

MAK-4 has antiatherogenic properties owing to its antioxidant effects.

Specific aims:

1. To test the effect of feeding WHHL rabbits a 6% (w/w) MAK- 4-supplemented diet on the endogenous antioxidant enzymes as assessed by measuring SOD in red blood cells and glutathione peroxidase in red blood cells and in serum.

2. To evaluate the effect of feeding WHHL rabbits a 6% (by weight) MAK-4-supplemented diet on lipid peroxides and diene conjugation in serum.
3. To test the effect of feeding WHHL rabbits a 6% MAK-4 supplemented diet on the susceptibility of LDL to oxidation by Cu^{++} ions as assessed by measuring TBARS.
4. To evaluate the effect of feeding WHHL rabbits a 6% MAK-4-supplemented diet on the susceptibility of LDL to oxidation by endothelial cells as assessed by determining TBARS.
5. To observe the effect of feeding WHHL rabbits a 6% MAK-4-supplemented diet on the development of atheroma.

CHAPTER III

THE ANTIOXIDANT AND ANTIATHEROGENIC EFFECTS OF MAK-4 IN WHHL RABBITS

III-A. Introduction

More than half a million Americans die from heart attacks annually. Three times that number will have heart attacks that mainly are caused by atherosclerosis. Free radicals and ROS are believed to be linked to cause atherosclerosis which might be prevented by antioxidants. Therefore, I would like to demonstrate that antioxidant-rich MAK-4 reduces the atheroma formation in the WHHL rabbits, the best animal model for familial hyperlipidemia in human.

There is an increasing accumulation of evidence that the pathogenesis of atherosclerosis is linked to free radicals and their by-product, lipid peroxide [1, 2]. Atherosclerosis in humans and animals is characterized by accumulation of foam cells in the intima [6,151,153]. The sources of foam cells are monocytes and macrophages which enter the arterial wall and take up oxidatively-modified low density lipoprotein (ox-LDL) through scavenger receptors [7, 9-11, 180]. The following explains how oxidized LDL may contribute to atherogenesis [180]. LDL enters the artery wall and is oxidized by the endothelial cells. Monocytes, which are chemoattracted to the cell-modified LDL in the subendothelial space, become macrophages.

On contact with ox-LDL in the subendothelial space, macrophages are inhibited from exit. The trapped macrophages accumulate ox-LDL and become foam cells. There are 4 ways in which ox-LDL could contribute to the development of atheromatous plaque [180]: 1) Ox-LDL attracts circulating monocytes into the subendothelial space. 2) It is rapidly taken up by monocytes which converted to macrophages, generating foam cells. 3) It inhibits the mobility of macrophages, trapping them into lesion areas. 4) Ox-LDL is toxic to endothelial cells and cause endothelial cell injury.

The involvement of ox-LDL in atherogenesis is based on: 1) the presence of ox-LDL and lipid peroxides in the areas of the atherosclerotic plaque [105]; 2) the inhibition of macrophage uptake of native LDL by the antioxidant probucol [107] and the anti-atherogenecity of antioxidant therapy [108]; 3) the increased lipid peroxidation of LDL isolated from atherosclerotic patients [109]; 4) the antibodies developed against ox-LDL, and MDA-conjugated LDL on the aortic lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits [109]; 5) the finding from the Western blot analysis that reveals the reactivity of LDL extracted from human and rabbit lesions to antibodies to ox-LDL [106]; and 6) the presence of autoantibodies that cross-react with MDA-conjugated LDL in the sera of humans and WHHL rabbits [106, 293].

If LDL is oxidized by free radicals *in vivo*, atherosclerosis can theoretically be prevented by reducing ox-LDL and foam cells using antioxidants. Antioxidants have been studied for their free radical-scavenging properties, and have been suggested as a means to prevent free radical-induced damage. Probucol [228, 229, 294], vitamin E [295, 296],

vitamin A [222, 223], vitamin C [216-218], carotenoids [208, 295], flavonoids [235, 236], and many other biological antioxidants have shown promising results. The potent antioxidant probucol [224, 225] has been shown to reduce atheroma in WHHL rabbits [107, 228, 282-285]. Another antioxidant, vitamin E, was reviewed for its anti-atherogenic effects by Ferns et al. [203]. Vitamin E, together with other antioxidants, showed strong synergistic effects in preventing tissue damage [255]. The low concentration of superoxide dismutase (SOD) was correlated with the increasing of lipid peroxide in the systemic lupus erythematous [297]. Glutathione peroxidase plays an important role in the reduction of lipid peroxide in glial cells [298].

MAK-4 is a complex herbal mixture from the natural system of health care known as Maharishi Ayur-Veda [47]. In Ayurvedic texts, the herbs in MAK-4 have been characterized as effective in the treatment of carcinoma, gastrointestinal disorders, anxiety, and atherosclerosis [48]. In the last eight years, MAK-4 has been researched extensively and has been found effective in treating and preventing various disorders, as well as enhancing the immune system and overall well-being [47, 48]. In the area of atherosclerosis, MAK-4 was effective in inhibiting platelet aggregation in whole blood caused by collagen and ADP [49]. MAK-4 has also shown powerful antioxidant activity, inhibiting microsomal lipid peroxidation in rat liver [52] and inhibiting human LDL oxidation [54].

In this study we investigated the effects of the herbal mixture MAK-4 on the reduction of atheroma formation in the aorta, the prevention of cupric ion-catalyzed and endothelial cell (EC)-induced oxidation of LDL, and the

concentration of SOD, glutathione peroxide, lipid peroxide, and diene conjugation in WHHL rabbits.

III-B. Materials and Methods

III-B-a. Chemicals and Instruments

Chemicals used in these experiments were purchased from Sigma Chemical Co. (St. Louis, MO 63178). Normal rabbit chow was purchased from Agway Inc., Syracuse, NY 13221. MAK-4 was obtained from Maharishi Ayur-Veda Products International (Colorado Springs, CO 80949). Malonaldehyde bis(dimethyl acetal) and thiobarbituric acid were obtained from Aldrich Chemical Co., Inc (Milwaukee, WI 53233). Dialysis membrane (12,000-15,000 Da cutoff) was obtained from Fisher Scientific Co. (Pittsburgh, PA 15219). Ultracentrifuge tubes were purchased from Seton Scientific (Sunnyvale, CA 94088). An ultracentrifuge (Beckman L5-50, Beckman Instruments, Palo Alto, CA 94306) and a fluorescence spectrophotometer (Hitachi F-2000, Hitachi Ltd., Tokyo, Japan) were used in the analyses.

III-B-b. Animals and Diets

Eleven 8-weeks-old female homozygous WHHL rabbits weighing 1.0 to 1.6 (1.4 ± 0.2 , Mean $\pm$ SD) kg were purchased from Camm Research Lab Animals, Wayne, NJ 07470. The WHHL rabbits were randomly divided into two groups: a control group of 5 (CONT); a test group of 6 (MAK-4) which is similar numbers ($n=4$ to 6) used by other researchers in evaluating the antioxidant on preventing atheroma formation [160, 161, 208]. After a 3

days (control) and 10 days (MAK-4) of adjustment to the surroundings (conditioning period), the control rabbits were still fed regular chow, the MAK-4 group was fed chow supplemented with 6.0% (by weight) MAK-4. This 6% dose of MAK-4 was extrapolated from the recommended dose of MAK-4 given to human. Both groups received their diets for 6 months. Each animal was restricted to 120 g of food per day during the study period and water was given ad libitum. Each rabbit was housed in a stainless steel cage in a temperature and moisture-controlled room. The water supply lines and food consumption were monitored daily.

Food containing 6% MAK-4 (w/w) was prepared by simply mixing chow and MAK-4. Six months later (at the age of 8 months) the rabbits were sacrificed. The blood, organs and aortas were obtained for analysis or observation.

III-B-c. Blood Collection

After an overnight fast, about 4 mL of blood was drawn from the ear vein into plain glass tubes for baseline assays for cholesterol, triglycerides, HDL, lipid peroxide, and glutathione peroxidase; 2.7 mL of blood was immediately transferred into citrate (0.3 mL of 0.129 mol/L sodium citrate) tubes and mixed well for red cell and plasma harvesting. Thereafter, blood was taken every 2 months from the rabbit ear, after injecting 0.1 mL of PromAce (Acepromazine maleate) into the ear for vasodilation. Weights were measured every two weeks. At the time of sacrifice, during which 1.0 mL of sodium pentobarbital (300 mg/mL) was injected into an ear vein, about 30 mL or more of blood was taken by cardiac puncture; 20 mL of

blood was combined with 600 μ L of 50 mg/ml EDTA for LDL and VLDL isolation, and 5 mL was saved for lipid profiles and other tests.

III-B-d. Maharish Amrit Kalash -4 (MAK-4)

MAK-4 consists of a mixture of natural herbs in carrier substances [299-311]. On analysis, MAK-4 was found to contain multiple antioxidants. Ghee, a constituent of MAK-4, contains lactose(12.0%), protein (36.0%), fat (32.4%, of which 27% is oleic acid), moisture (14.4%) , and ash (5.2%) [312].

II-B-e. Ingredients and Chemical Constituents of MAK-4

INGREDIENTS

Raw sugar, ghee (clarified butter), Indian gall nut, Indian gooseberry, dried catkins, honey, nutgrass, Indian pennywort, shoeflower, aloeweed, licorice, cardamom, cinnamon, Indian cyprus, turmeric, butterfly pea, and white sandalwood [18].

CHEMICAL CONSTITUENTS

Tannic acid, flavonoids, catechin, alpha-tocopherol, polyphenols, ascorbate, riboflavin, and beta-carotene. These components have antioxidant activities [54].

III-B-f. Quality Control of MAK-4

According to the manufacturer, the following steps are taken for the quality control of the herbal mixtures:

1. Harvesting the herbs at the proper time according to Ayur-Veda.
2. Identification of herbs by an expert botanist.
3. Testing the herbs for microbiological and heavy metal contamination.
4. Testing the herbs for the proper chemical composition by thin layer chromatography and high pressure liquid chromatography.

In Dr. Sharma's laboratory, the following additional tests were performed on MAK-4.

1. Different batches of MAK-4 were tested for preventing human LDL oxidation induced by Cu^{++} ions, they gave similar results.
2. Prevention of DMBA-induced breast carcinoma in rats by different batches of MAK-4 done at different times gave similar result [313].
3. Since the molecular weights of herbal mixtures cannot be determined, phenol groups were measured as a standard for comparing different herbs and herbal mixtures. Phenol groups are also associated with antioxidant properties; therefore, the estimation of phenol groups in the herbal mixtures provides an added parameter to evaluate their antioxidant properties.

III-B-g. Measurement of Phenol Groups in MAK-4

1. Preparation of aqueous or alcoholic extracts

One g of MAK-4 was mixed with 9.0 mL of Ham's F-10 medium (ICN Biomedicals, Inc., Irvine CA 92713-9921) for the aqueous

extract, and 9.0 mL of 95% ethanol for the alcoholic extract.

2. The mixtures were vortexed for 5 min and centrifuged at 3000 rpm (1800 x g) for 5 min.
3. The supernatants were filtered prior to use.
4. The phenol group concentration of Trolox (vitamin C analog) and the alcoholic and aqueous extracts of MAK-4 were determined by a modification of the method of Barnes et al. [314]. Aliquots (1 mL) of various concentrations of the agents were added to 1 mL Folin-Ciocalteu reagent and 0.2 mL 10% Na₂CO₃. The mixtures were placed in a water bath at 90-100°C for 1 minute, then cooled. The concentration of phenol groups was measured spectrophotometrically at the wavelength of 700 nm, using catechin as the standard. The concentration of phenolic groups in the alcoholic and aqueous extracts of MAK-4 were $111.8 \pm 7.9 \mu\text{mol/L}$ and $101 \pm 2.8 \mu\text{mol/L}$, respectively, n=9.

III-B-h. Test for In Vivo Interference of MAK-4 on Biochemical Assays

The biochemical tests were run and compared on serum obtained from the albino rabbits before feeding MAK-4 and after feeding the MAK-4-supplemented diet for one month to check for any in-vivo interferences of MAK-4 on the biochemical tests. There were no statistical differences between groups during the one month period.

III-B-i. Quality Control of Methodologies

1. All reagents were the best grade available for each procedure.

2. All instruments were calibrated for human blood and were confirmed to be suitable for animal blood testing. All instruments were maintained for 24 hr operating requirements.
3. Commercial quality controls were used in most cases.

III-B-j. Weighing of Rabbits

Each rabbit was weighed biweekly.

III-B-k. Lipid Profiles on Kodak Ektachem 700-XR

All reagents (slides, electrolyte reference solution, quality controls, and calibration solution) were purchased from Eastman Kodak Company (Rochester, NY 14650).

Lipid profiles included cholesterol, triglyceride, and HDL. The Kodak Ektachem 700 XR Analyzer is a fully automated instrument that generates very accurate and precise results. This instrument was well maintained and well accepted in terms of quality control during the testing period. The Kodak Ektachem employs dry-slide technology, a unique process that has replaced traditional wet-reagent testing. In dry-slide technology, multilayered reagents are applied to a clear polyester support base. When the sample to be measured comes into contact with these dry chemical layers, colorimetric and potentiometric reactions occur. Analytes are measured by end-point colorimetry, rate reflectance spectrophotometry, or potentiometry. Serum cholesterol and triglyceride in human and rabbit are the same in the chemical properties in which chemical analysis is based on.

The quality control data of cholesterol and triglyceride for the months during which rabbit's sera were analyzed on the Kodak 700XR are shown in the following chart:

Between Run QC Data

Tests	Target Values		Observed Values			
	Mean	2SD	Mean	2SD	CV	n
Chol (mg/dL)	159	6.5	158	3.7	2.3	61
Trig (mg/dL)	108	6.5	104	1.6	1.3	60

III-B-1. Lipid Peroxide (LP)

LP was analyzed by methods of Yagi's [74] and Satoh's [315].

1. Principle

Lipid peroxides are derived from the oxidation of polyunsaturated fatty acids and their esters; they are capable of further lipoperoxide production via a free radical chain reaction.

The principle of this method is to isolate lipids, precipitating the plasma protein with phosphotungstic acid-sulfuric acid system, then indirectly measuring the plasma peroxide concentration by measuring thiobarbituric acid-reactive substances (TBARS) using a thiobarbituric acid reaction in an acetic acid solution. The reaction product, which is fluorescent, is extracted with n-butanol and the relative fluorescence intensity is measured using 515 nm as the excitation wavelength and 553 nm as the emission wavelength.

Malondialdehyde (MDA) is used as the standard for this procedure.

2. Reagents

All reagents listed below were purchased from Sigma Chemical Co.(St. Louis, MO 63178), except thiobarbituric acid and malonaldehyde-bis(dimethyl acetal), which were purchased from Aldrich Chemical Co., Inc., (Milwaukee, WI 53233).

a. Phosphotungstic acid (100 g/L)

10 g phosphotungstic acid is dissolved in enough dist. water to make 100 mL of solution.

b. Sulfuric acid (0.08 mol/L)

1 mL of 4.08 mol/L H_2SO_4 added to 50 mL dist. water.

c. Thiobarbituric acid (TBA) (0.0385%)

0.67 g of TBA, QS to 100 mL with dist. water. Dissolve TBA with the aid of heat. When TBA is dissolved and cooled, add 100 mL glacial acetic acid.

d. Malonaldehyde-bis(dimethyl acetal) (MDA)

(1,1,3,3 tetramethoxypropane)

Purchased from Sigma Chemical Co., kept in desicator and stored in the refrigerator.

e. n-Butanol

Purchased from Sigma Chemical Co., kept in the anti-flammable cabinet.

f. MDA stock solution (5 $\mu\text{mol/L}$)

10 μL 1.0 mmol/L MDA added to 1990 μL dist. water.

g. Standard solution

nmol/L MDA	Amount of stock MDA	Amount of DDH ₂ O
0.0	0 μL	4000 μL
2.5	2	3998
7.5	6	3994
12.5	10	3990
17.5	14	3986
25.0	20	3980
75.0	60	3940
125.0	100	3900

3. Equipment

- a. Spectrofluorometer
- b. Vortex mixer
- c. Centrifuge
- d. Boiling water bath
- e. Ice water bath
- f. Test tubes (16 x 125 mm)

4. Quality control

MDA, 25 nmol/L, in 0.05 mol/L sulfuric acid can be prepared by making 1 : 200 dilution of the stock solution with 0.05

mol/L sulfuric acid, and aliquoting into small tubes.

5. Procedure

- a. Pipette 20 μL of standard solution, distilled water as a blank, control, and platelet-free plasma in 16 x 125 mm test tubes in duplicate.
- b. Add 4.0 mL of 0.08 mol/L H_2SO_4 into sample tubes only, mix with vortex.
- c. Add 0.5 mL phosphotungstic acid (100 g/L) and vortex.
- d. Allow tubes to stand at room temperature for 5 minutes.
- e. Centrifuge samples at 3000 rpm (1800 x g) for 12 minutes at room temperature.
- f. Decant and discard supernatant.
- g. Add 2.0 mL of 0.08 mol/L sulfuric acid and 0.3 mL of phosphotungstic acid (100 g/L) to pellet and vortex.
- h. Centrifuge at 3000 rpm (1800 x g) for 12 minutes at room temperature.
- i. Decant and discard supernatant.
- j. Add 4.0 mL of dist. water and 1.0 mL of TBA into all tubes, and vortex.
- k. Heat in a boiling water bath for 60 min., then cool in an ice water bath.
- l. Add 5.0 mL of n-butanol, mix with vortex, and centrifuge as above.
- m. Transfer each supernatant into a micro cuvette and measure the

fluorescence of the organic layer on a spectrofluorometer, using 515 nm as the excitation wavelength and 553 nm as the emission wavelength. Use the "0" standard as the blank.

- n. Plot a standard curve on graph paper and obtain the concentration of the control and samples.

III-B-m. Superoxide Dismutase (SOD)

SOD was analyzed by a modification of Sun's [316, 317] and Spitz's methods [318].

1. Principle

Superoxide dismutase (E.C.1.15.1.1) catalyzes the dismutation of superoxide radicals:

The function of this enzyme is to protect oxygen-metabolizing cells against the harmful effects of superoxide ions formed by reduction of oxygen during enzymatic activity in the mitochondria and cytosol. The enzyme must be determined indirectly since the above reaction of the free radical proceeds spontaneously in aqueous solution. The free radical $\text{O}_2^{\circ-}$ is generated enzymatically by xanthine and xanthine oxidase that in turn reduces nitroblue tetrazolium (NBT). SOD will compete with NBT for superoxide radicals and thereby decreases the

amount of reduced NBT. One unit of activity is defined as the amount of enzyme required to inhibit the standard rate of NBT formation by 50%.

2. Reagents

SOD, xanthine oxidase, xanthine, fatty acid-free bovine albumin, NBT, sodium carbonate, CuCl_2 , diethylenetriamine pentaacetic acid (DETAPAC), disodium bathocuproine disulfonate (BCS), catalase, ammonium sulfate, chloroform, and ethanol were purchased from Sigma Chemical Co.

a. Reagent mixture

1. 0.3 mmol/L xanthine

Dissolve 91.26 mg of xanthine in 2000 mL dist. water.

2. 1.0 g/L bovine albumin (fatty acid free)

Dissolve 0.5 g of bovine albumin in 500 mL dist. water.

3. 150 $\mu\text{mol/L}$ NBT

Dissolve 122.6 mg of NBT in 1000 mL dist. water.

4. 400 mmol/L Na_2CO_3 (pH 10.2)

Dissolve 42.4 g of Na_2CO_3 in enough dist. water to make 1000 mL solution.

5. 6 mmol/L Diethylenetriamine pentaacetic Acid (DETAPAC)

Dissolve 2.36 g of DETAPAC in enough dist. water.

6. 4 mmol/L Bathocuproine disulfonate (BCS)

Dissolve 0.88 g of BCS in 500 mL dist. water.

7. 717 U/L catalase (prepare fresh just before use)

Prepare by diluting with dist. water according to the bottle value (ca. 2600 U/mg) to obtain 717 U/L.

b. 208 U/L xanthine oxidase

1. 2.0 mol/L ammonium sulfate

Dissolve 66.1 g of ammonium sulfate in 500 mL dist. water.

2. 208 U/L xanthine oxidase (prepare fresh just before use).

Dilute 20 μ L xanthine oxidase (25 U/0.7 mL) with 2.0 mL of 2.0 mol/L ammonium sulfate.

c. 0.8 mmol/L CuCl_2

Dissolve 68.2 mg of CuCl_2 in 500 mL dist. water.

d. SOD standard solutions (0 to 250 ng/tube or 3.1 to 25.3

U/dL) using 1.3 mg/mL or 15,000 U/3.7 mL. Prepare fresh stock solution (1300 ng/mL) just before use by diluting 50 μ L of SOD (15,000 U/3.7 mL) in 50 mL of dist. water. Prepare working standard solutions as follows:

Dil factor	μL of SOD stock	mL of H_2O	U/mL
1 : 16	100	1.5	0.253
1 : 21	100	2.0	0.193
1 : 26	100	2.5	0.156
1 : 36	100	3.5	0.113
1 : 46	100	4.5	0.088
1 : 71	50	3.5	0.057
1 :131	50	6.5	0.031

3. Instruments

- a. Beckman AccuSpin FR Centrifuge
- b. Beckman DU-7 spectrophotometer
- c. Dispensers, automatic pipettes and tips, and test tubes.

4. Sample preparation

- a. Draw venous blood from rabbit's ear into 5 mL evacuated glass tube containing 7.5 mg of tripotassium EDTA.
- b. Centrifuge the whole blood at 3000 rpm (1800 x g) for 10 min. at 4 °C.
- c. Separate the plasma from the cells (plasma will be used for other tests).
- d. Lyse 0.1 mL of packed red cells with 0.9 mL of ice-cold dist. water.
- e. Remove hemoglobin by adding 0.3 mL of chloroform and 0.5 mL of ethanol and vortex vigorously for 1 min.

- f. Centrifuge the mixture at 18,000 x g for 60 min.
- g. Separate the supernatant, transfer to small plastic tubes and freeze at -70 °C.
- h. Make a final RBC dilution of 1:800 before testing.

5. Quality Control (QC)

Add 5 mL of human red blood cells to 195 mL of cold dist. water and 50 mL of chloroform/ethanol (3/5 by vol.), followed by vigorous shaking. Aliquot the supernatant into small tubes and freeze at -70 °C.

6. Procedure

- a. Prepare SOD working reagent mixture by combining 40.0 mL of xanthine, 20.0 mL of DETAPAC, 6.0 mL of BCS, 6.0 mL of serum albumin, 20.0 mL of NBT, 2.0 mL of catalase, and 12.0 mL of sodium carbonate.
- b. Pipette 2.45 mL of the SOD working reagent into properly marked test tubes.
- c. Add 0.5 mL of dist. water (for blank), standards, QC, and rabbit samples into corresponding test tubes, and mix with vortex.
- d. Add 50 µL of xanthine oxidase to each tube at 15 sec. intervals.
- e. Let stand at room temperature (25 °C) for 20 min.
- f. Terminate the reaction by adding 1.0 mL of 0.8 mmol/L

CuCl₂ to each tube at 15 sec. intervals.

- g. Read the absorbance (A) of each sample at 560 nm against a water blank. The blank absorbance is about 0.25.
- h. Calculate % inhibition:

$$(15) \quad \% \text{ inhib.} = [A \text{ blank} - A \text{ sample} / A \text{ blank}] \times 100$$

- i. Draw the standard inhibition curve with the X-axis being the protein concentration or unit of activity and the Y-axis being the absorbance.

III-B-n. Glutathione Peroxidase (GSH-Px)

1. Principle

GSH-Px was determined using the method of Wheeler [319].

Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and hydroperoxides formed from fatty acids with reduced glutathione (GSH) donating the hydrogen. The reactions are as follows:

Tert-butyl hydroperoxide (t-BuOOH) is used as the peroxidase substrate. The formation of GSSG catalyzed by GSH-Px is coupled

to the oxidation of NADPH. The change in absorbance of NADPH at 340 nm vs. a water blank was measured on a Roche Cobas Mira automated analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ 07042).

2. Reagents and equipment

All reagents were purchased from Sigma Chemical Co. The Roche Cobas Mira Analyzer was purchased from Roche Diagnostic Systems, Inc. (Montclair, N J 07042).

a. Phosphate buffer, 100 mmol/L, 1 mmol/L EDTA, pH 7.0

183 mL of 0.2 mol/L K_2HPO_4

117 mL of 0.2 mol/L KH_2PO_4

228.12 mg of EDTA

300 mL of dist. water.

b. Prepare NADPH, 2.25 mmol/L in 1 g/L sodium bicarbonate by dissolving 9.38 mg of NADPH in 5.0 mL 1 g/L $NaHCO_3$.

Prepare fresh before each experiment.

c. Glutathione reductase (GSSG-R), 25 U/0.25 mL phosphate buffer. Prepare fresh before each experiment.

d. GSH, 37.5 mmol/L in phosphate buffer. Prepare fresh before each experiment.

e. t-BuOOH, 15 nmol/L in dist. water.

f. Working reagent

Mix-together 9.25 mL of phosphate buffer, 1.25 mL of NADPH, 0.25 mL of glutathione reductase, and 0.5 mL of glutathione

reduced form (GSH).

3. Specimen

Use serum , or plasma obtained from citrated blood, or RBC hemolysate prepared by mixing 100 μ L packed red cells with 1.9 ml cold dist. water.

4. Automated procedure

The working reagent, stored in a 20 mL size container, and a start reagent, t-BuOOH, in a 5 mL size container, are placed in the designated compartments on the reagent rack of the Mira. About 100 μ L of sample is added to a 600 μ L disposable plastic tube and placed in a sample rack. The instrument is programmed to deliver 225 μ L of reagent and 40 μ L of specimen with a 30 μ L rinse, and 30 μ L of t-BuOOH with a 30 μ L rinse. The instrument is programmed to incubate for 2.5 min for temperature equilibration to 37 °C and to read the absorbance at 340 nm every 10 sec for 2 min. The enzyme activity is calculated automatically in U/mL. Multiply by a dilution factor of 20 for the hemolysate. Manual calculation of the enzyme activity in U/mL can be carried out using the following equation.

$$(17) \quad U/L = \Delta A / \text{minute} \times F$$

where ΔA : Change in absorbance

F : $1/e \times tv/sv \times 1/d \times 1/10^{-6}$

e : millimolar absorbance coefficient

tv : total volume in mL

sv : sample volume in mL

d : light path in cm

$1/10^{-6}$: converts to micromoles

An example of manual calculation:

if $\Delta A = 0.2$, $e = 6.22 \times 1000$, $tv = 0.395$ mL, $sv = 0.1$ mL,

$d = 0.6$ cm

then, $F = 1/6200 \times 0.395/0.1 \times 1/0.6 \times 1/10^{-6}$

$= 1061$

so, $U/mL = 0.2/2 \times 1061$

$= 106$ U/L

III-B-o. Measurement of Diene Conjugation

Serum diene conjugates were determined by the methods of Esterbauer [72] and Lezerovich [320] on a Beckman DU-7 spectrophotometer at 234 nm. The autoxidation of polyunsaturated fatty acids results in an increase in UV absorbance with a maximum at 234 nm. Measurement of this absorbance is a useful method to estimate the degree of lipid oxidation. A Beckman DU-7 spectrophotometer was turned on in the UV mode for 30 min before samples were read vs. a water blank. Cuvettes were well washed and rinsed with dist. water between specimens.

III-B-p. LDL Isolation

Rabbit blood specimens (20 mL) were collected in plastic tubes containing 600 μ L of EDTA (50 mg/mL), immediately mixed by inversion, and kept at 4°C for 3 hr. Plasma was separated by centrifugation at 2,000 g for 20 min at room temperature. Gentamycin sulfate (1 mg/20 mL plasma) was added to the plasma sample. LDL (d 1.019-1.063) was isolated by sequential isopycnic ultracentrifugation [321]. LDL was dialyzed at 4°C for 16-20 hr against 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl and 0.01% EDTA. The protein concentration of the LDL was determined by the Lowry method [322-323]. The purity of LDL was confirmed by the finding of one band on electrophoregrams stained for either lipid (fat red 7B) or proteins (amido black).

III-B-q. Endothelial Cell (EC) Isolation

EC isolation was performed by Laura Lantry in Dr. Stephen's laboratory.

Capillary human endothelial cells (EC) were obtained from the human eye from autopsy case. The choroid was briefly cross-black minced, followed by digestion in a buffer containing collagenase type I and type II for 30 min at 37°C. The digestion was centrifuged at 800 xg for 5 min at 4°C. The process of centrifugation and suspension was done twice, then the pellet was suspended in M-199 medium (ICN Biomedicals, Inc., Irvine CA 92713-991), plated onto fibronectin-coated tissue culture plates (ICN Biomedicals, Inc., Irvine CA 92713-991), and incubated at 37°C in humidified 95% air and 5% CO₂.

III-B-r. Thiobarbituric Acid Reactive Substances in LDL

The methods of Steinberg [9] and Steinbrecher [293] were used.

1. Isolation of LDL by method of Havel [321]

a. Preparation of salt solutions

NaCl (0.196 M), with EDTA (0.1 mg/L) was prepared by dissolving 11.4542 g of NaCl and 0.1 g EDTA in one liter of dist. water. The theoretical density of this solution is 1.063.

The density of this solution was verified using a pycnometer.

b. NaBr (4.570 mol/L) was prepared to obtain a density of about 1.346.

2. Blood collection

Thirty mL of rabbit blood was taken from the heart at necropsy and mixed with EDTA (1 mg/mL). Plasma was separated by centrifugation (2000 x g) for 20 min at room temperature.

3. Density adjustment and ultracentrifugation

The plasma density was measured and adjusted to 1.019 using 0.196 mol/L NaCl-0.01% EDTA solution. The volume of the NaCl-EDTA solution was determined using the following formula:

$$(18) \quad VP \times DP + VS \times DS = (VP + VS) \times 1.019$$

where: VP is the volume of plasma, DP is the density of plasma, DS is the density of salt solution (NaCl, EDTA) , VS is the volume of salt solution (NaCl-EDTA) needed to be added to the plasma to obtain a density of 1.019. The plasma adjusted

to a density of 1.019 was distributed into two centrifugation tubes equally and centrifuged at 10°C for 22 hr. at 38,000 rpm (100,000 x g). After centrifugation, the middle of the clear layer was cut using a tube slicer. The supernatants containing VLDL and LDL were transferred into a tube using pasteur pipettes. The subnatants were collected and adjusted to density of 1.063 using NaBr solution, followed by centrifugation at 38,000 rpm at 10 °C for 22 hr. The top portion containing LDL was collected and dialyzed for 16-20 hr. against 0.01 mol/L sodium phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl and 0.01% EDTA. The purity of the LDL was confirmed by electrophoresis.

4. Measurement of protein in LDL by methods of Bilheime [322] and Weinstein [323]
 - a. Stock reagents are:

Sodium hydroxide (1 mol/L), 20 mg/L copper sulfate, 40 mg/L sodium potassium tartrate and 0.25 mg/L Triton X 100.
 - b. Working solutions
 1. Prepare standard bovine serum albumin (50 mg/100 mL double dist. water).
 2. Prepare Folin phenol reagent by adding 5 mL of Folin reagent (Sigma Chemical Co.) to 5 mL of double dist. water.
 3. Prepare alkaline copper tartrate solution by dissolving 2.0 g of anhydrous sodium carbonate in 100 mL of double dist. water containing 10 mL NaOH (1.0 mol/L), and mix

with 0.5 mL of 40 g/L sodium potassium tartrate and 0.5 mL of 20 g/L copper sulfate.

4. Prepare standard solutions containing 0 to 12 µg/mL of bovine serum albumin.

c. Procedure

1. Pipette 2.0 mL of standard solution, diluted (1:20) LDL, and quality control specimens into properly marked test tubes.
 2. Add 2.0 mL of the alkaline copper tartrate solution to each tube.
 3. Incubate for 10 min at room temperature.
 4. Add 0.2 mL of Folin phenol reagent to all tubes and mix. with the vortex mixer.
 5. Incubate for 45 min. at room temperature in the dark.
 6. Read absorbance at 650 nm on spectrophotometer against a water blank and obtain protein concentration from the standard curve plotted on graph paper.
 7. Note: If any sample is lipemic, add 0.1 mL of 2.5% aqueous Triton X-100 to all tubes, including the standard solutions.
5. Measurement of TBARS by method of Steinbrecher [293]
- The principle, equipment, and reagents are the same as for the lipid peroxide determination (Section III-B-1) except a fluorescence spectrophotometer (Hitachi F-2000, Hitachi, Ltd., Tokyo, Japan) is used.

- a. Prepare standard solutions containing 0.1 to 5 nmoles/mL MDA.
- b. Procedure
 1. Pipette 0.5 mL of standard, quality control specimens, and LDL solution containing 50 μ g LDL protein into test tubes.
 2. Add 1.5 mL of 200 g/L trichloroacetic acid into all test tubes.
 3. Add 1.5 mL of thiobarbituric acid (6.7 g/L in 0.05 mol/L NaOH) into all tubes.
 4. After mixing, incubate for 45 min. in an 80-90 °C water bath.
 5. After cooling for 2 hr. at room temperature, read the fluorescence using excitation and emission wavelengths of 515 and 553 nm, respectively.
 6. Results are expressed as nmoles MDA/mg LDL protein.

III-B-s. Resistance of LDL to Cupric Ion-Catalyzed Oxidation

Incubation mixtures were prepared using 100 μ g LDL protein/mL in Ham's F-10 medium in a total volume of 2 mL, with or without 1 μ M Cu^{++} ions. The incubation was carried out at 37°C in 95% humidified air and 5% CO_2 . All mixtures were taken out at 0, 1, 2, 4, 6, 8, 10, 12, 14, and 24 hr, and stored with 1.0 mmole EDTA at -80°C. The incubated mixtures were assayed using TBARS for the degree of LDL oxidation [324].

III-B-t. Resistance of LDL to Endothelial Cell-Induced Oxidation

LDL (0.2 mg protein) was incubated in Ham's F-10 medium in a total volume of 2 mL, with or without EC, at 37°C in humidified 95% air and 5% CO₂. Samples were taken out at 0, 3, 6, 8, 10, 12, 14, and 24 hr and stored at -80°C with 1 mmole EDTA. The LDL oxidation was assessed by measuring TBARS.

III-B-u. Measurements of Areas of Atheromatous Plaque

1. Measurement of plaque on total thoracic aorta using Nagano's [181] and Kita's [283] methods.

The entire thoracic aorta was isolated from the rabbits at necropsy, opened longitudinally after removal of adherent tissues, and fixed in 10% neutral buffered formalin. Photographs of each inner surface were taken at the same magnification on slide film with a ruler for comparison alongside. The photographs of the inner surface were developed from the slides. I measured the percentages of intimal surface area involved with atheromatous plaque by the method described by Nagano [181] and Kita [283]. Photographs of aorta were copied to graph paper with 2-times magnification, and the total and lesioned area was measured. This technique was confirmed by modification of the original techniques. Briefly, the slides, magnified 2 x, were projected on transparent graph paper to measure the areas of plaque for each rabbit. The number of squares covered by the plaque were counted on the transparent graphs. The percentages of the areas of atheromatous plaque which developed on total thoracic aorta were calculated.

2. Measurement of plaque on arch of thoracic aorta using methods of Nagano [181] and Kita [283]

The percentage of surface area of plaque was calculated by measuring the area covered by plaque on the arch of aorta, 2 cm from the origin of the aorta from the heart. This was also confirmed by the modified technique described above.

III-C. Results

III-C-a. Weights of Rabbits

The WHHL rabbits used in this study weighed 1.4 ± 0.2 kg at the beginning of the study and 2.9 ± 0.1 kg at the end of the study. Statistically significant differences were not seen between the body weights of the two groups (Fig. 5).

III-C-b. Chemical Analysis of Lipid Profiles, Lipid Peroxide, SOD, Glutathione Peroxidase, and Diene Conjugation

Chemical analyses for cholesterol, triglyceride and lipid peroxide on sera are given in Table 11. HDL was too low to be measured accurately (<8 mg/dL) on the Kodak instrument. There were no significant differences in the cholesterol concentrations between the MAK-4 and control groups during the entire experimental period, except for the baseline concentration (Fig. 6). The triglycerides decreased with age without significant differences between the two groups (Fig. 7). The triglyceride concentration may be decreased with age due to the limited food given and the requirement of more energy for a growing rabbit.

The lipid peroxide did not show any change in the control group, however it showed significant decrease ($p=0.05$) in the MAK-4 group after 2 months of treatment and continued till the end of the experiment (Fig. 8). No significant differences were found in the red blood cell glutathione peroxidase, however, there was a significant decrease in both groups at month 6 as compared to the baseline (Fig. 9). There was a significant increase in serum glutathione peroxidase in the MAK-4 group at months 2 ($p<0.03$), 4 ($p<0.05$), and 6 ($p<0.03$) compared to the control group (Fig. 10). At the same time, there was a significant ($p<0.05$) decrease in glutathione peroxidase at month 2 from the baseline in the control group and continued throughout the experiment. Red blood cell SOD was significantly ($p<0.05$) increased at month 4 in the MAK-4 group compared to the control group (Fig. 11). Diene conjugation (diene C) was same in the control group throughout the experiment. In the MAK-4 group, diene C was similar to control group till month 4, however at 6th month, there was a significant drop ($p<0.05$) of diene C compared to both group at 4 months and before. Also there was a significant difference ($p<0.05$) in diene C between two groups at month 6 (Fig. 12).

III-C-c. Resistance of LDL to Cupric Ion-catalyzed and EC-induced Oxidation

LDL isolated from the MAK-4 group showed an increase in the lag phase (6 hours) for both the cupric ion-catalyzed and EC-induced LDL oxidation when compared to the control group (<1 hr and 3 hr, respectively). There was a delay in the start of the propagation phase for the MAK-4

group, by 6 hours for both the cupric ion-catalyzed and EC-induced LDL oxidation, compared to the control group which was delayed by less than 1 hour for cupric ion-catalyzed and 3 hours for the EC-induced LDL oxidation. Both the lag phase and the delay in the start of the propagation were significantly different ($p < 0.05$) in the cupric ion-catalyzed (Fig. 13) and EC-induced (Fig. 14) LDL oxidation in the MAK-4 group as compared with the control group.

III-C-d. Atheroma Formation

Plate I shows photographs of the inner surface of the aorta of WHHL rabbits in the MAK-4 and control groups. Percentages of aortic lesions in the two groups are summarized in Tables 12 and 13. The percentages of total surface area with visible plaques in the MAK-4 vs control groups were $17.2\% \pm 3.1\%$ vs $41.0\% \pm 10.6\%$ ($p < 0.05$) (Table 12, Fig. 15). The percentage of surface area of plaques in the aortic arch of the MAK-4 group was $22.5\% \pm 4.2\%$, which was reduced significantly ($p < 0.01$) compared to the control group ($47.6\% \pm 6.8\%$) (Table 13, Fig. 16).

III-D. Discussion

The purpose of this study was to investigate the antioxidant and antiatherogenic effects of MAK-4, an herbal mixture, in WHHL rabbits. My experiments show that MAK-4 has antioxidant properties and reduces atheroma formation in WHHL rabbits. The antioxidant properties of MAK-4 are demonstrated by its effects on endogenous antioxidant enzymes,

such as the increase in glutathione peroxidase (GSH-Px) concentration and the increased SOD activity, in the MAK-4 group. The production rate of serum GSH-Px, which protects glial cells against lipid peroxidation [298], was significantly ($p<0.05$) higher in the MAK-4 group for the entire 6-month period. There was a significant ($p<0.05$) decrease in GSH-Px which is associated with increase production of thromboxin and lipid peroxide [328] from 2 month from the baseline in the control group. The decrease in the serum GSH-Px in the control group suggests exhaustion of enzyme activity due to excessive free radical load. However, the sustained increase of GSH-Px in the MAK-4 group indicates that MAK-4 is protecting against free radical load and sparring the enzyme and or MAK-4 increases the enzyme activity. Further experiments are needed to clarify this point. The RBC GSH-Px showed significant decrease in both groups at 6th month. The reason is not clear. The contribution of RBC GSH-Px in protecting atheroma formation, if any, is minimal. RBC GSH-Px protects the RBC membrane from free radical load. However, serum GSH-Px protects the circulating lipids from oxidation and is more relevant in reducing atheroma formation by preventing LDL and other lipids from being oxidized.

The antioxidant enzyme, SOD, was significantly ($p<0.05$) higher in the MAK-4 group at month 4 compared to the control group. The increase of SOD in the MAK-4 group may be attributed to the copper, zinc, and manganese [297] in MAK-4. Since the SOD was tested in red blood cells, whose life span is 70 days, I speculate that the increase in SOD was detected in the sample collected at the 4th month in the MAK-4 group. The decrease of SOD at 6th month is not significant. MAK-4 scavenges superoxide in

the same way as SOD [50], and it scavenges H_2O_2 and the hydroxyl radical in a manner comparable to or better than that of SOD [263].

Other evidence for the antioxidant properties of MAK-4 is the fact that the lipid peroxidation and diene conjugation in serum are lower in the MAK-4 group compared to the control group. Similar to my results, other investigators have shown that probucol, an antioxidant drug, reduces atheroma in WHHL rabbits [107, 181, 208, 283, 285, 286, 325] without significant reduction of cholesterol concentration. The only exception was the Daugherty [285] and Kleinveld [325] experiments where it was demonstrated that probucol reduced cholesterol concentration. The evidence suggests that lipid peroxidation is linked to the initiation of atherosclerosis [1, 2, 323, 324, 326, 327] and MAK-4 reduces serum lipid peroxides and diene conjugation, which represents the degree of lipid peroxidation [72].

In the present studies, MAK-4-fed animals showed an increased resistance of their LDL to EC-induced and cupric ion-catalyzed LDL oxidation, as demonstrated by the increase in the lag phase and delay in the start of the propagation phase. In the cupric ion-catalyzed LDL oxidation, the concentration of TBARS at 1, 2, 3, 4, and 6 hours in the LDL isolated from the MAK-4 group was significantly lower ($p < 0.05$) than that of the control group. In the EC-induced LDL oxidation, the concentration of TBARS at 6 hours in the MAK-4 group was significantly lower ($p < 0.05$) when compared to the control group. Although the mechanism by which MAK-4 inhibits LDL oxidation is not known, the increase in resistance to EC-induced and cupric ion-catalyzed LDL oxidation may be due to the scavenging of free radicals by the incorporated antioxidants from MAK-4, or

to the alteration of the structure of LDL constituents that promote resistance to oxidation. The antioxidant effect of MAK-4 is more likely the mechanism, since MAK-4 was proven to have antioxidant effects but MAK-4 has not been studied regarding change in LDL structure.

In this study, we showed that MAK-4 reduced the formation of atheroma in homozygous WHHL rabbits in the absence of a significant reduction in serum cholesterol concentration. The basal cholesterol concentration in the control group was higher than that of the MAK-4 group. The blood samples in the control group were drawn 3 days after their arrival, and in the MAK-4 group 10 days after their arrival at OSU. I speculate that the stress of transportation and new environment increased the catecholamines in the animals. The increased catecholamines may increase cholesterol by activating lipoprotein lipase [329]. Since the blood samples of the MAK-4 group were taken 10 days after their arrival as compared to 3 days for the control group, it is possible that these animals were more adjusted to the new environment and thus their catecholamine levels were back to normal. Thus, this may be the reason their cholesterol concentrations were lower than the control group at the baseline.

MAK-4 reduced the average thoracic aorta atheroma by 49% ($p < 0.05$) and aortic arch atheroma by 53% ($p < 0.01$) as compared to the control group. My findings of the reduction of aortic atheroma correlated with those found by using the antioxidant probucol and the cholesterol-lowering agent lovastatin, by Carew [107] (% development of plaques in probucol group,

4.3 ± 2.1%, lovastatin group, 27.5 ± 4.6%, and control group, 40.6 ± 5.1%).

The antioxidant effects of MAK-4 have been demonstrated previously [50-54, 263-266, 292]. The reduction of atherogenic factors by MAK-4 have been studied by Panganamala [267] in rabbits. Alcoholic and aqueous extracts of MAK-4 inhibited microsomal lipid peroxidation in a concentration-dependent manner [52, 292, 265], inhibited EC- and soybean lipoxygenase-induced LDL oxidation [264], and also showed more antioxidant potency in preventing LDL oxidation than ascorbic acid, alpha-tocopherol, or probucol in vitro [54].

Since there was no significant decrease in cholesterol and triglyceride concentration, my data indicates that the reduction of atheroma may be attributed to the antioxidant effects of MAK-4 through its inhibition of LDL oxidation. The strong antioxidant effect may be due to the synergistic activities of the multiple antioxidants that are present in MAK-4. Several animal studies on the synergism of different antioxidants demonstrated that combinations of two or more antioxidants protect organs against oxidative damage better than an individual antioxidant [255-257]. Another possible advantage of MAK-4 is that it is a natural mixture instead of synthetic. Natural-source vitamin E was retained better than the synthetic form in various tissues, except liver [258]. Similar experiments showed a preferential uptake of natural-source vitamin E by most tissues [259].

III-E. Conclusions

This study has demonstrated that MAK-4, a natural herbal mixture comprised of multiple antioxidants, reduced LDL oxidation and increased

resistances of LDL to oxidation caused by EC-induced and cupric ion-catalyzed reactions, and reduced atheroma formation in WHHL rabbits, an animal model for human familial hypercholesterolemic patients. The action of MAK-4 may be an antioxidant activity that retards the oxidation of LDL.

III-F. Suggestions for Further Studies

I recommend that the different antioxidants in MAK-4 be tested singly and in combination for synergistic activity and toxicity in vitro and in vivo. The analysis of dose response of MAK-4 in vivo is also desirable. A standard procedure for the aqueous and alcoholic extraction of MAK-4 is also needed. Clinical trials of MAK-4 for the investigation of antiatherogenic effects are recommended. Ongoing human studies (protocol #93H-0067) on preventing human LDL oxidation in hyperlipidemic subjects are being undertaken by the Clinical Research Center (CRC) at The Ohio State University Medical Center. The total antioxidant capacity and free radical concentration present in the MAK-4-treated rabbit blood may also be compared to that of the control group.

III-G. Statistical Analyses

With this experimental design, the appropriate statistical treatment was analysis of variance (ANOVA) with repeated measures to assess the statistically significant differences between the groups. In cases of $p < 0.05$ a Fisher PLSD test was used to compare each group. Values are presented as $\text{MEAN} \pm \text{SE}$.

Table 1. Possible Sources of Free Radicals [47, 56]**Toxins**

Carbon tetrachloride	Toluene
Diquat	Aniline dyes
Bromobenzene	Benzo(a)pyrene
Paraquat	

Drugs

Adriamycin	Primaquine
Bleomycin	Pamaquine
Mitomycin C	Streptozotocin
Nitrofurantoin	Nitrofurazone
Chlorpromazine	Anthracyclines
Acetaminophen	Rifamycins
Cocaine	Polyene antifungal antibiotics
Alloxan	

Air Pollution**Primary sources**

Carbon monoxide	Unburned hydrocarbons
Nitric oxide	Sulfur dioxide

Secondary sources

Ozone	Aldehydes
Nitrogen dioxide	Alkyl nitrates

Metals

Excess of iron, copper, manganese, nickel,
cadmium, aluminum, chromium, and lead

Others

Alcohol	Tobacco smoke
Radiation	Ultraviolet radiation
Inhaling pure oxygen	
Smoked and barbecued food	
Peroxidized fats in meat and aged cheeses	
Exposure to hyperbaric oxygen	

Table 2. List of Known Products of Oxygen Free Radicals That Attack Biomolecules and Their Roles in Pathophysiology [15, 55]

<u>Oxidative process</u>	<u>Some products</u>	<u>Influence on pathophysiology</u>
Lipid peroxidation	Membrane structure change	Changes in membrane enzyme action, change in ion pumping capacity, etc.
	Lipofuscin	May contribute to aging
	Aldehydes including malondialdehyde	React with proteins and nucleic acids, may be mutagenic
	4-Hydroxynonenal	Reacts with LDL, may contribute to atherosclerosis
Protein oxidation	Oxidized protein	May accumulate in aging
	Oxidized inactive enzymes	May contribute to build-up of substrates
	Glutamine synthetase	glutamate in stroke
	Oxidized LDL	Contributes to atherosclerosis
	Oxidized α 1-protease inhibitor	May allow protease to be active, thus causing emphysema
Nucleic acid oxidation	Strand breaks and Altered bases	Repair necessary, may help produce errors in mutagenesis
	8-Hydroxyguanine	Mutagenic, may contribute to carcinogenesis
	5-Hydroxymethyl uracil	
	Thymine glycol	
Carbohydrate oxidation	Depolarization (Hyaluronic acid, elastin, collagen)	Loss of structural and functional capabilities of molecules
		Damaged to carbohydrate portion of cell receptors can lead to the loss of various cellular functions (T-cells)

Table 3. Diseases and Conditions That May Be Caused by Free Radicals and Reactive Oxygen Species.

Aging [19]	Keloid formation [19]
Atherosclerotic heart disease [19]	Liver damage [34, 35]
Autism [47]	McArdle's disease [332]
Behcet's disease [330]	Mental disorders [47]
Brain / central nervous system disorders [32, 33]	Mucocutaneous syndrome [19]
Carcinogenesis [19]	Muscular injury [333]
Cataracts [19]	Organtransplantation rejection [19]
Cerebral ischemia (stroke) [19]	Osteoporosis [47]
Chemotherapeutic agent-related toxicities [19]	Pancreas injury [36, 37, 46]
Chronic bronchitis [19]	Paraquat toxicity [19]
Crohn's disease [19]	Parkinson's disease [47]
Dermatitis herpetiformis [28]	Progressive systemic sclerosis [19]
Diabetes mellitus [21, 22]	Pulmonary fibrosis [19]
Eclampsia [19]	Release of enzymes [36, 45]
Emphysema [19]	Release of histamine [45]
Facial lines and aging spots [94]	Renal disease [41, 42]
Heart injury [38, 39]	Respiratory distress syndrom [19]
Hemolytic anemia [43]	Retrolental fibroplasia [19]
Hepatitis [19]	Rheumatoid arthritis [19, 20]
Hormone starvation [47]	Schizophrenia [47]
Immune disorders [44, 47]	Sunburn [19]
Intestinal dysfunction [331]	Systemic lupus erythematosus [19]
Intestinal ischemia [19]	Ulcerative colitis [94]
Ionizing radiation side-effects [19]	Ulcers in burns or wounds [94]

Table 4. Half-Lives of Free Radicals [63]

Species	Common Name	Half-life (37° C)
$O_2^{\circ-}$	Superoxide radical	Unstable, Not known
HO_2°	Hydroperoxyl radical	Not known
HO°	Hydroxyl radical	1 nanosecond
RO°	Alkoxyl radical	1 microsecond
ROO°	Peroxyl radical	7 seconds
RNO°	Nitroxyl radical	5 seconds

Table 5. Biological Antioxidants [7]**A. Enzymes**

Superoxide dismutase : Cu/Zn-SOD and Mn-SOD

Catalase

Glutathione peroxidase: Se-GSH-Px and PLOOH-GSH-Px

Glutathione reductase

B. Lipid-soluble vitamins and small molecules

Carotenoids (α & β -carotene, lutein, zeaxanthin, lycopene)

Vitamin E (α -tocopherol, r-tocopherol, d-tocopherol)

Vitamin A

Ubiquinol-10 (reduced coenzyme-Q)

Food additives (butylated hydroxyanisole & butylated hydroxytoluene)

C. Water-soluble vitamins and small molecules

Ascorbate

Bilirubin

Glutathione

Glucose

Pyruvate

Urate

Table 6. Plasma Proteins Acting as Antioxidants (Metalloenzymes)

[7]

PROTEINS	METALS BOUND
Ceruloplasmin	Copper, iron
Ferritin	Iron
Transferrin	Iron
Myoglobin	Iron
Lactoferrin	Iron
Albumin	Copper, iron, zinc, cadmium
Metallothioneines	Copper, zinc, cadmium, mercury
Haptoglobin	Iron in free hemoglobin, met-hgb
Hemopexin	Iron in heme

Table 7. Other Chemicals Used as Antioxidants and Scavenger Agents

CHEMICAL	USED FOR	Ref #
Allopurinol		[137]
Captopril		[137]
Carminic acid	lipid/carbohydrate damage	[334]
Cystine/creatinine		[185]
Desferrioxamine		[137]
Dihydrolipoic acid	membrane protection	[335]
Dimercaptosuccinic acid	red blood cells	[336]
Dimethylsulphoxide &-thiourea	lipid peroxidation	[337]
Dimethylthiourea	retinal light damage	[338]
Ebselen		[137]
Ferulic acid	impinging UV radiation	[339]
Flavonoids	inhibition of cell proliferation	[340]
2-hydroxy estrone & -estradiol		[185]
5-hydroxytryptophan	microsomal lipid peroxidation	[341]
Lazaroids		[137]
L-carnosine	crystalline lens	[342]
Lipoxygenase inhibitors		[137]
Manitol, glucocorticoid	ischaemic brain injury	[343]
Methimazole	kidney damage	[344]
Methimazole/propylthiouracil	heat shock protein	[345]
Methylene blue	ischemia/reperfusion injury	[346]
Monohydroxamates		[137]
N-acetylcysteine	pulmonary O ₂ toxicity	[347]
Phytic acid		[348]
Probucol	LDL oxidation	[108]
Pyrroloquinoline quinone	liver injury	[349]

Table 8. Extracellular Antioxidants: Their Locations and Actions [185]

ANTIOXIDANT	LOCATION	MECHANISM OF ACTION
Albumin	Plasma	Binds copper ions and bilirubin
Ascorbic acid	Plasma, synovial fluid, cerebrospinal fluid	Scavenges free radicals and $O_2^{\circ-}$; reduces α -tocopheroxyl radical to α -tocopherol
Ceruloplasmin	Plasma	Binds copper ions and prevents reinitiation reactions: oxidizes Fe^{++} to Fe^{+++} , thereby inhibiting ion-dependent lipid peroxidation
Haptoglobin	Plasma	Binds free hemoglobin, preventing it from reacting with H_2O_2 to accelerate lipid peroxidation
Hemopexin	Plasma	Binds free heme and functions similarly to haptoglobin
Lactoferrin	Plasma (leukocytes)	Similar to transferrin
Transferrin	Plasma	Removes free iron from solution, thereby preventing reinitiation reactions
Uric acid	Plasma	Free radical scavenger

Table 9. Possible Anticarcinogenic Mechanisms of Vitamin A, β -carotene, Vitamin E, and Vitamin C [186]

Vitamin	Major Mechanisms	Minor Mechanisms
Vitamin A (retinoids)	Cell differentiation Antipromotion of cancer Cytotoxicity DNA, RNA, proteins Gene expression; proteins Reversion to normal phenotype	Stimulates growth factor Immune function Collagen synthesis Apoptosis
β -Carotene (provitamin A)	Antioxidation Inhibition of cell growth Inhibition of mutagenesis	Enhances the immune response
Vitamin E (α -tocopherol)	Antioxidation Free radical scavenger Membrane biogenesis Cytotoxicity Cell differentiation DNA, RNA, proteins	Immune function Inhibits nitrosamines Mutagenicity Synergism with selenium and vitamin C
Vitamin C (ascorbic acid)	Antioxidation Inhibition of nitrosamines Enhance collagen synth. Cytotoxicity DNA, RNA, proteins Oncogene expression Increased phagocytic & chemotactic activity	Inhibits hyaluronidase Decreases vitamin E degradation Antibacterial Antiviral Hormone synthesis Reverses cell to normal type Apoptosis

Table 10. Immunologic Responses to Vitamin A Status [223]

<u>Subjects or Model</u>	<u>Response</u>
VITAMIN A SUPPLEMENTATION	
Children	Enhanced cellular immunity to bacille Calmette Guérin
Humans	Reversal of abnormalities in T-lymphocyte subsets
Humans	Increased number of T-lymphocytes
Humans	Enhanced delayed-type hypersensitivity
Human lymphocytes	Enhanced mitogenic response
Human macrophages	Improved phagocytic activity
Mice	Increased tumor-specific immunity
Mice	Increased number of T-lymphocytes
Mice	Increased interleukin-2 production
Mice	Augmentation of allograft responses
Mice	Increased number of plaque-forming cells
Mice	Activation of nonspecific immunity
Rats	Activation of nonspecific immunity
Rats	Regulation of macrophage function
VITAMIN A DEFICIENCY	
Humans	Decreased CD4/CD8 ratio in T-lymphocytes: decreased production of CD4 T-lymphocytes
Human lymphocytes	Abnormal interferon secretion
Mice	Decreased antibody production
Mice	Decreased levels of immunoglobulins G1 and G3
Mice	Decreased delayed-type hypersensitivity
Mice	Abnormal interferon secretion
Rats	Reduced mitogenic response
Rats	Decreased natural killer-cell activity
Rats	Decreased antibody production
Rats	Low levels of antibody to tetanus toxoid
Rats	Impaired bacterial clearance and phagocytosis
Various	Atrophy of the thymus and spleen

Table 11. Comparison of Cholesterol, triglyceride, and Lipid Peroxide Concentration at Different Ages of WHHL Rabbits

	Cholesterol (mg/dL)		Triglyceride (mg/dL)		Lipid Peroxide (nmole MDA/L)	
	MAK-4	CONT	MAK-4	CONT	MAK-4	CONT
0 m	476±41	772±44	383±31	326±27	20.5±8.2	17.2±2.2
2 m	677±43	705±31	350±23	240±14	13.11±1.0	18.0±1.5 *
4 m	717±47	738±22	333±32	224±15	13.3±1.3	16.0±1.7
6 m	622±48	672±51	341±39	247±12	14.0±1.4	16.7±0.8

Data are reported as MEAN ± SE

m: month

* p<0.05

n=5 for control, and n=6 for MAK-4

Table 12. Percent Visible Intimal Surface Area of Total Aortic Lesions in Control and MAK-4-Treated WHHL Rabbits

Groups	Total % atheroma area of each rabbit aorta by methods of [181, 283] MEAN ±SE
MAK-4	17.2% ± 3.1%
Control	41.0% ± 10.6%
p value	p<0.05

Table 13. Percent Visible Intimal Surface Area of Aortic Arch Lesions in Control and MAK-4-Treated WHHL Rabbits

GROUPS	% ATHEROMA AREA OF AORTIC ARCH						MEAN % ±SE
ANIMAL NO.	1	2	3	4	5	6	
MAK-4 (n=6)	12.2	29.1	22.1	22.0	38.2	11.6	22.5 ± 4.2 *
CONTROL (n=5)	73.1	38.4	48.4	34.1	43.9		47.6 ± 6.8 *

* P < 0.01

Figure. 2. Major Intracellular Antioxidants

Figure. 3. Structures of Common Lipid-Soluble Antioxidants

Figure 4. Structures of Water-Soluble Biological Antioxidants and Naturally Occurring Flavonoids

Figure 5. Body Weight Changes in WHHL Rabbits During 6 Months

Figure 6. Effect of MAK-4 on Cholesterol Concentration in WHHL Rabbits *p<0.05

Figure 7. Effect of MAK-4 on Triglyceride Concentration in WHHL Rabbits

Figure 8. Effect of MAK-4 on Serum Lipid Peroxide in WHHL Rabbits *p<0.05 MDA: Malonedialdehyde

Figure 9. Effect of MAK-4 on Red Blood Cell Glutathione Peroxidase **Hgb: Hemoglobin**

Figure 10. Effect of MAK-4 on Serum Glutathione Peroxidase in WHHL Rabbits * : $p < 0.05$
#: Glutathione peroxidase (U/gm protein) ±SE

Figure 11. Effect of MAK-4 on Superoxide Dismutase *:p<0.05

Figure 12. Effect of MAK-4 on Diene Conjugation in WHHL Rabbits *:p<0.05 O.D. : Optical Density

Figure 13. Effect of MAK-4 on Propagation and Lag Phase of Cupric Ion-Induced LDL Oxidation
***p<0.05**

Figure 14. Effect of MAK-4 on Propagation and Lag Phase of EC-Induced LDL Oxidation
***p<0.05**

Figure 15. Effect of MAK-4 on Plaque Formation on Total Thoracic Aorta in WHHL Rabbits
***p<0.05**

Figure 16. Effect of MAK-4 on Plaque Formation on Aortic Arch in WHHL Rabbits *p<0.01

Plate I. Aortic Specimens Showing the Extent of Atherosclerotic Plaques. The top two (MAK-4-A and MAK-4-B) are from MAK-4 group and the bottom two (Cont-C and Cont-D) are from control group.

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