

1994

Effect of dietary ethanol on atherosclerosis in rabbits

Edward F. Wilson
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Effect of dietary ethanol on atherosclerosis in rabbits

by

Edward F. Wilson

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Veterinary Physiology and Pharmacology
Major: Physiology**

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In Charge of Major Work

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**Iowa State University
Ames, Iowa**

1994

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION AND DISSERTATION ORGANIZATION	1
Dissertation Hypothesis	1
Dissertation Organization	1
CHAPTER 2. REVIEW OF THE LITERATURE	3
The Atherosclerotic Process and Development of Heart Disease	3
Atherosclerosis Results in Modified Vascular Vasoconstriction and Vasodilation	16
Evaluation of Atherosclerotic Regression in the Human and Rabbit	28
Potential of Dietary Ethanol as a Therapeutic Agent for the Treatment of Atherosclerosis	42
Dissertation Hypothesis	54
Literature Cited	55
CHAPTER 3. EFFECT OF DIETARY ETHANOL ON ATHEROSCLEROTIC RABBITS CONSUMING A CHOLESTEROL-FREE DIET	68
Abstract	68
Introduction	70
Materials and Methods	74
Results	81
Discussion	94
Literature Cited	101
CHAPTER 4. POTENTIATION OF VASOCONSTRICTIVE PROPERTIES IN AORTAS FROM ATHEROSCLEROTIC RABBITS FED A CHOLESTEROL-FREE DIET WITH AND WITHOUT DIETARY ETHANOL	106
Abstract	106
Introduction	108
Materials and Methods	110
Results	116
Discussion	126
Literature Cited	133
CHAPTER 6. GENERAL CONCLUSIONS	136
ACKNOWLEDGEMENTS	138
APPENDIX 1. STATISTICAL ANALYSIS OF TIME-DEPENDENT CHANGES IN PLASMA LIPID CONCENTRATIONS	140
APPENDIX 2. SHEEP UMBILICAL ARTERIES AND THE VASOCONSTRICTIVE DOSE-RESPONSE RELATIONSHIP FOR ETHANOL: PRELIMINARY DATA	146

CHAPTER 1. GENERAL INTRODUCTION**Dissertation Hypothesis**

This dissertation research evaluates the effect that dietary ethanol has on the health of the circulatory system. The effect of dietary ethanol on previously established atherosclerotic disease has never been addressed by either human or animal studies. Given that ethanol consumption promotes anti-atherogenic changes in cholesterol metabolism, enzyme activities and atherogenic stimuli, it is the proposal of this dissertation that the consumption of ethanol will promote the regression of previously established atherosclerotic lesions. Furthermore, because the atherosclerotic process is similar in the man and rabbit, this dissertation proposes to use atherosclerotic rabbits as a model for the effects of ethanol on atherosclerotic people.

Dissertation Organization

The chapters are organized after Chapter 1 in the following manner. Chapter 2 consists of a review of the literature which examines the development and regression of the atherosclerotic process, how ethanol has been shown to

affect atherosclerosis and concludes with the hypothesis for the dissertation. Chapter 3 evaluates the affects that a cholesterol-free diet, with or without ethanol, has on plasma lipoprotein-cholesterol and atherosclerotic severity. Chapter 4 evaluates the affects that a cholesterol-free diet, with or without ethanol, has on arterial contractile properties. Chapter 5 concludes the dissertation and discusses the general conclusions resulting from this dissertation. Appendix 1 presents a statistical analysis of the time-dependant changes observed in the plasma lipid concentrations described in Chapter 3. Appendix 2 evaluates how ethanol affects vasoconstriction in sheep umbilical arteries and how this may be a useful model for studying the causes of fetal alcohol syndrome in humans. The materials contained in Chapter 3 and 4 represents two papers that will be submitted to the Journal of Comparative Biochemistry and Physiology for publication; these two chapters are written according to the requirements for publication in this journal. The remaining materials are written according to the rules outlined in the Fifth Edition of the Council of Biology Editors Writers Guide.

CHAPTER 2. REVIEW OF THE LITERATURE

The Atherogenic Process and Development of Heart Disease

Atherosclerosis and its effect on the population

In 1990 some 486,000 Americans died of coronary heart disease. One of the most important and frequent post-mortem findings in these people were atherosclerotic deposits in the coronary arteries. These cholesterol-rich deposits cause a narrowing of arterial lumens and are frequently associated with sites of thrombosis or vasospasm, leading to occlusions and reduced blood flow to an effected portion of the myocardium. Due to the extremely high oxidative metabolic rate of the heart, a reduction in blood flow quickly results in hypoxia causing myocardial injury or necrosis. Thus, atherosclerosis is an essential part of what is termed heart disease and can result in myocardial infarction.

Atherosclerosis is unique when compared to other diseases because of the chronic course of its development, taking up to decades for clinical manifestation. The first signs of atherosclerosis are fatty streaks lining the arterial lumen. Fatty streaks are clinically benign lesions consisting of

cholesterol and fat deposits in the arterial wall. They become detectable in 65% of children by the age of 12 to 14 years of age (Stary 1989). Fatty streaks do not cause overt symptoms of disease, such as loss of cardiovascular function, but they do represent sites with the potential to progress into a more pathological atherosclerotic lesion, a process called atherogenesis. Atheromas represent areas where the fatty streaks have grown larger, with smooth muscle hypertrophy or proliferation, and a necrotic core resulting in lesion encroachment into the arterial lumen (Stary 1989). In the final stages of atherogenesis, the atheroma leads to the development of fibrous plaques, characterized by deposits of calcium and collagen in the arterial wall.

What is the etiology of factors promoting the development of the fatty streaks initiating the process of atherosclerosis and cardiovascular damage? Once atherosclerosis is present, how can progression be checked? If progression can be checked, are treatments available to reverse the process and return the vascular system to a more healthy status? Understanding the etiology of events which lead to atherosclerosis is the first step to addressing these issues.

Evolution of arterial tissue from a healthy to an atherosclerotic status

Fatty streaks form as focal sites underneath the endothelium where monocytes from the blood have infiltrated into the area in between the endothelium and the basement membrane. They then fill with lipid and cholesterol esters becoming swollen macrophages, loosely termed 'foam cells' (Stary 1989). The exact stimulus for the initial migration is not clear. Some suggested possibilities have included intimal infection by viruses (Span et al. 1991), local endothelial damage (Ross 1973) and circulatory turbulence (Zarins et al. 1983).

The heart tends to be one of the first organs affected by atherosclerosis, as well as one of the organs most severely affected because it has a large number of arterial bifurcations and a tremendous number of sites of circulatory turbulence (Zarins et al. 1983). Hence, the myocardium has a tremendous number of sites favoring fatty streak and atherosclerotic establishment. In contrast, the venous system is almost never affected by atherosclerosis because of the small amount of turbulence found there.

Monocytes and the macrophages derived from monocytes, directly mediate a series of events resulting in the promotion of fatty streaks. Beneath the endothelium,

macrophages release mediators including free radicals like O_2^- , chemoattractants, cytokines and growth factors (Ross 93). At the lesion site, these chemicals promote increased capillary permeability, additional monocyte and leukocyte recruitment and smooth muscle proliferation, resulting in a classic positive feedback loop promoting continued lesion growth.

Because macrophages mediate increased endothelial permeability, they also increase the permeability of the endothelium to low density lipoprotein (LDL) particles and increase the amount of LDL at the lesion site (Ross 1993). These micelle-like particles are rich in cholesterol, carry a single molecule of a protein called apolipoprotein B-100 (apo B-100) and are primarily responsible for cholesterol deposition in peripheral cells (Ross 1993).

Normally, LDL-particles are only slowly taken up by sources outside the liver after binding to apo B-100 receptors on cell membranes (Kreiger 1992). To promote high LDL uptake rates, LDL must first be chemically modified. LDL lipid and/or protein modifications may include attachment of acetyl or aldehyde groups, malondialdehyde or proteoglycans as well as direct lipid oxidation. Because oxidative modification has been proven to occur in vivo, oxidized-low density lipoproteins (ox-LDL) appear to represent the most

important modified LDL particles (Sparrow 1989; Palinska et al. 1989).

LDL may be oxidized by a variety of mechanisms. Oxidation can be facilitated by endothelially derived free radicals (Steinbrecher et al. 1984). Activated platelets may also oxidize LDL lipids through the action of a protein originating from the alpha granules during platelet aggregation (Fuhrman et al. 1991). However, the primary LDL oxidizer appears to be macrophages. The mechanism apparently utilizes free radicals released in association with the respiratory burst and/or released lipoxygenase (Carpenter et al. 1991).

In contrast to normal LDL particles, the ox-LDL particle binds with high affinity to scavenger receptors on cell membranes, is rapidly internalized (Kreiger et al. 1992) and preferentially accumulates at sites of atherosclerosis (Chang et al. 1993). Furthermore, ox-LDL particles serve as chemoattractants to monocytes in the circulation. (Parthasarathy et al. 1986). Thus, macrophages promote an increased rate of cellular cholesterol uptake by increasing LDL permeability, LDL oxidation and macrophage infiltration, thereby promoting increased cholesterol uptake by cells localized to the lesion site.

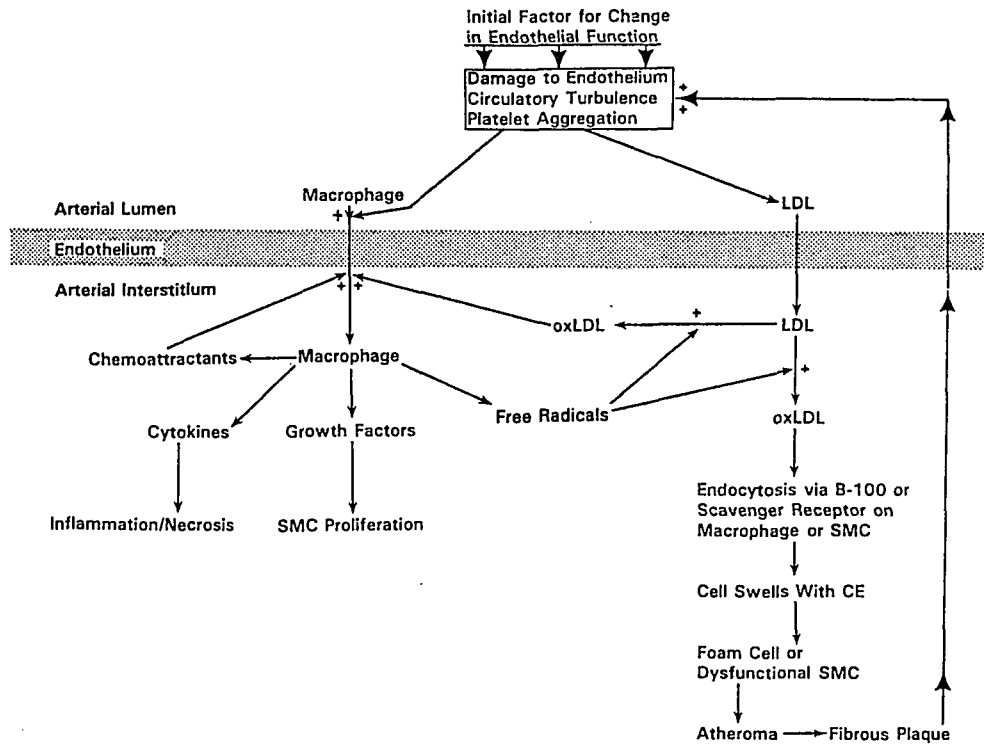
Cells, including macrophages, take up LDL and ox-LDL particles by endocytosis, leading to LDL degradation in a

lysosome. In macrophages the cholesterol esters are sequestered to a lipid pool in the cells center. As the content of cholesterol esters sequestered to the center of cells increase, the macrophages become swollen, growing larger and more spherical and ultimately lose their ability to leave the lesion area (Fielding 1992; Inoue et al. 1991; Brown et al. 1980). These macrophages become trapped between the endothelium and the basement membrane. Because they continue to signal inflammation and promote LDL oxidation, they continue to promote infiltration of macrophages and LDL at the lesion site (Badimon et al. 1993). As macrophages continue to enter and swell, the affected region begins to encroach upon the arterial lumen, creating circulatory turbulence within the lumen. This turbulence creates conditions that further promote the inflammatory response of the macrophages within the fatty streak. The factors involved in the establishment and development of the atherosclerotic process are reviewed in Figure 1.

Cholesterol transport to and from the periphery

The primary facilitators of cholesterol transport to peripheral targets are LDLs. Tissue like the ovaries, adrenal cortex and testis all have high metabolic demands for

Figure 1. Review of factors leading to the promotion of fatty streaks and atherosclerosis in the arterial wall.



sterols and hence express large numbers of apo B-100 receptors for LDL particles and an increased ability to take up LDL particles (Brown and Goldstein 1983). Although apo B-100 receptors are present in most tissues, the amount of LDL-cholesterol taken up by apo B-100 mediated uptake appears to be small, with prior LDL oxidation being required for high affinity uptake by a second LDL receptor class, the scavenger receptors. Scavenger receptors work in a very non-specific fashion to rapidly take up chemically modified LDL, like ox-LDL, at a rate much greater than that of unmodified LDL particles (Sparrow et al. 1989).

LDL particles represent the degradation product of a lipoprotein called the very low density lipoprotein (VLDL). Clinically, VLDL particles have a density range of 0.95 to 1.006 g/cm³ (Havel, Eder and Bragdon 1955). VLDLs are primarily secreted from the liver, are rich in triglycerides and contain only a small amount of cholesterol. While the entire VLDL particle can be endocytosed, typically only the triglycerides are removed by the action of lipoprotein lipase upon association of the VLDL particle with the endothelium.

When endocytosis does occur, it is mediated by a 550 kd protein called apo B-100 that serves as the receptor ligand of the lipoprotein, signalling endocytosis at the target cell. VLDL may be directly endocytosed when an additional apolipoprotein called apolipoprotein E (apo E) finds its

receptor. (Basu et al. 1983; Yamada et al. 1992; Nordestgaard et al. 1992)

VLDLs also carry a series of apolipoprotein C proteins termed apo CI, apo CII and apo CIII. Apo CII is the most important because it serves as a coenzyme for extrahepatic LPL activation (Gomez-Coronado et al. 1993). Lipoprotein lipase activation catalyzes the hydrolysis of triglycerides from the triglyceride rich VLDL particle, forming free fatty acids and facilitating fatty acid efflux into peripheral target cells.

After removal of the triglycerides from the VLDL core, apo E, apo CI, apo CII and apo CIII dissociate, leaving only apo B-100. The remnant is called the LDL lipoprotein and is composed of approximately 50% cholesterol esters, 30% phospholipids and a small amount of triglycerides and free fatty acids. Clinically, LDLs are identified as lipoproteins with a density of 1.019 to 1.063 g/cm³ (Havel et al. 1955). The Report of the National Cholesterol Education Program (NCEP; 1988) suggests that people with plasma LDL-cholesterol levels in excess of 160 mg/dl are at high risk for development of coronary artery disease and should receive follow up dietary or drug intervention. A variety of treatments are available and clinically used, some of which will be discussed later.

Endocytosis of LDL or ox-LDL particles in the periphery or the liver results in complete degradation of the lipoprotein contents within the lysosome where cholesterol esters are hydrolyzed to free cholesterol. After leaving the lysosome, free-cholesterol is re-esterified by cellular acyl-CoA:cholesterol acyltransferase (ACAT), which adds a fatty acid at the C-3 position of cholesterol. These neutrally charged cholesterol esters are then sequestered to the center cholesterol ester lipid pool of the cell (Fielding 1992). Hence, apoproteins on the VLDL particle may be reused, but the LDL particle and its contents are degraded within the target cell.

High density lipoprotein particles oppose the cholesterol depositing functions of LDL particles. Peripheral cholesterol is taken up and removed by HDL particles for redistribution in the body. HDL particles are the smallest and most dense lipoprotein particles with a density of between 1.063 and 1.21 g/cm³ (Havel et al. 1955). The NCEP (1988) has determined that plasma HDL cholesterol levels below 35 mg/dL are associated with an increased risk of coronary artery disease and that plasma levels above this result in reduced risk of coronary artery disease.

HDL particles are secreted by hepatocytes as discoidal particles that are rich in phospholipids and generally free of cholesterol (Tall 1990). Free cholesterol in the outer

membrane of peripheral cells is able to diffuse into the HDL particle along its concentration gradient and is rapidly esterified at C-3 with a fatty acid removed from a phospholipid like phosphatidylcholine by lecithin:cholesterol acyltransferase (LCAT). Because cholesterol esters are electrically neutral, the esters remain in the neutrally charged HDL core. Furthermore, once esterified, cholesterol is unable to leave the HDL particle without facilitated diffusion. As the discoidal particle continues to take up and esterify cholesterol, the particle becomes swollen and takes on its mature, cholesterol ester rich, spherical appearance (Tall 1990).

Several enzymes are associated with HDL function. LCAT and its co-factor apo AI function to esterify free cholesterol (Tall 1990; Rubanyi et al. 1990). Apo AII has been proposed to inhibit HDL uptake of free cholesterol from cells and promote atherogenesis (Fruchart et al. 1993; Warden et al. 1993). Cholesteryl ester transfer protein (CETP) facilitates the exchange of cholesterol esters from the HDL core, for triglycerides of apo B-100 containing lipoproteins like LDL and VLDL. In contrast to VLDL and LDL particles, HDL particles do not carry the apo B-100 protein. The apo C series become associated with HDL particles after their dissociation from VLDL particles, but appear to have little function while associated with HDL particles (Tall 1990).

Finally, apo E becomes associated with HDL and serves as a receptor for HDL uptake and endocytosis (Basu et al. 1983; Yamashita et al. 1990).

The cholesterol esters within HDL particles have a variety of potential destinations, with one being the peripheral tissues from which the cholesterol originated via apo E mediated uptake. However, the primary destination of HDL and its associated cholesterol is deposition in the liver. HDL associated apo E signals endocytosis via apo E receptors (Goldberg et al. 1991). Because macrophages within the atheroma may also secrete apo E and express apo E receptors, they can directly modify the removal of cholesterol laden cells of the atheroma.

The cholesterol esters in an HDL particle can also be transported to VLDL and LDL particles without HDL degradation (Brown et al. 1980). CETP catalyzes the exchange of cholesterol esters from the core of HDL particles for triglycerides of apo B-100 containing particles (Tall 90). Increased CETP activity is associated with increased sensitivity to dietary cholesterol. In terms of phylogenetic relationships, both rabbits and man have relatively high plasma CETP activities as well as a tendency to develop atherosclerosis when exposed to diets rich in cholesterol for prolonged periods. In contrast, animals like the mouse have

relatively low CETP activity and tend not to readily form atheromas when fed high cholesterol diets (Ha and Barter 1982).

In transclonal rodent experiments using mice expressing the human CETP gene, when CETP activity is increased the mice become sensitive to dietary cholesterol (Marotti et al. 1993). In contrast, transgenic mice expressing increased levels of the apo AI are less sensitive to dietary cholesterol and fatty streak development (Rubin et al. 1991). In contrast, mice become atherogenic when apo AII, which inhibits the action of apo AI, is over expressed (Warden et al. 1993). Finally, human studies of enhanced CETP activity associated with some familial hypercholesterolemics and persons without CETP activity due to a lack in the CETP gene, support the idea that CETP is important in diverting HDL cholesterol back into atheroma formation in the periphery (Inazu et al. 1992; Yamashita et al. 1990).

Atherosclerosis Results in Modified Vascular Vasoconstriction and Vasodilation

The endothelium separates plasma constituents from the tissues of the arterial wall

Before humoral materials can pass from the arterial lumen into foam cells or smooth muscle cells of the atherosclerotic arterial wall, the materials must first pass across a 0.3 to 0.4 μm thick layer of endothelial cells that line the inner surface of all healthy arteries (Crapo et al. 1983). These cells are central to determining the effect that plasma constituents will have on the physiology of the arterial wall. The primary purposes of endothelial cells are as follows: 1) To provide the vascular system with a endothelial surface facilitating laminar blood flow, 2) To prevent platelet aggregation, 3) To serve as a selectively permeable barrier regulating passage of plasma constituents to the underlying vascular smooth muscle, and 4) To directly regulate the contractile status of the vascular smooth muscle lying underneath the endothelium. All of these functions become suppressed during the progression of atherosclerosis.

The caliber of a vessel lumen is determined by the degree of constriction maintained by vascular smooth muscle and the

interplay of cellular and humoral factors on the vascular smooth muscle cells. At a focal site these factors, to be detailed later, may facilitate vasodilation or an increase in the arterial lumen diameter associated with increased blood flow to dependent sites and decreased vascular resistance. These factors can also promote vasoconstriction, or a decrease in the lumen diameter leading to decreased blood flow and increased resistance. As explained earlier, a vasospasm in the coronary arterial system represents a focal site of severe vasoconstriction that can cause reduced coronary blood flow leading to a myocardial infarction. The development of the atherosclerotic process alters endothelial function leading to conditions promoting vasospasm (Meredith et al. 1993).

The endothelium promotes vasodilation by releasing a compound called endothelium-derived relaxing factor

A healthy endothelium is critical for maintaining vascular smooth muscle in a relatively relaxed state of contraction. The endothelium does this by releasing endothelium-derived relaxing factor (EDRF), a chemical believed to be nitric oxide (NO) (Palmer et al. 1987). While basal EDRF production exists (Chester et al. 1990), EDRF production from

endothelial cells is stimulated by many factors including acetylcholine (Furchgott and Zawadski 1980), catecholamines, serotonin, thrombin and increased blood flow along the endothelium (Meredith et al. 1993).

EDRF is primarily generated by endothelial cells through the action of calmodulin-calcium dependent NO synthase (Bredt and Snyder 1990), which generates NO by removing the terminal guanidino-group of L-arginine (Palmer et al. 1988).

Secondary sources of EDRF have been found to include platelets (Radomski et al. 1990), macrophages (Yates et al. 1992) and smooth muscle cells themselves (Schini et al. 1991). Because EDRF is readily diffusible, it rapidly diffuses into both the arterial lumen and across the basement membrane to EDRF receptors on vascular smooth muscle cells.

At its target, the EDRF-receptor interaction results in activation of guanylcyclase, increasing the intracellular cyclic guanylnucleotide monophosphate concentration [cGMP]_i (Bossaller et al. 1987). The increase in the [cGMP]_i activates cGMP-dependent protein kinase and inhibits calcium entry into the target cell (Waldman and Murad 1987). Physiologically the elevated [cGMP]_i decreases the intracellular calcium concentration and promotes relaxation (vasodilation) of vascular smooth muscle as well as inhibiting calcium dependent platelet aggregation (Scott-Burden and Vanhoutte 1993).

The bioactivity of factors like epinephrine, norepinephrine, acetylcholine and serotonin is dependent upon whether a functional or dysfunctional endothelium overlays the smooth muscle. When these factors bind to their receptors on smooth muscle cells they cause smooth muscle cell contraction. However, when these factors bind to receptors on healthy endothelial cells, they cause the endothelium to release EDRF. Endothelium-derived relaxing factor serves as a potent physiological antagonist of the vasoconstrictor properties of the above factors that stimulated their release, such that vessels lined with a functional endothelium dilate in spite of the presence of constrictors. In contrast, vessels lacking a functional endothelium lack physiological antagonists to the vasoconstrictor properties of these compounds, predisposing a vessel to vasospasm (Meredith et al. 1993).

Hypercholesterolemia and atherosclerosis attenuate endothelially mediated vasodilation

The progression of the atherosclerotic process is linked to a loss in EDRF mediated vasodilation. In Watanabe heritable hyperlipidemic rabbits, changes in vascular reactivity during the atherogenic process appear to be

associated with a progressive loss of functional endothelial cells (Kolodigie et al. 1990). In an in vivo study of monkeys Macaca fascicularis, McLenachan et al. (1991) proved that EDRF mediated vasodilation and increased blood flow into femoral artery caused by intravenously administered acetylcholine is attenuated early in the development of atherosclerosis.

Similar effects have been observed for human atherosclerotic vessels. The atherogenic process leads to a loss of EDRF mediated vasorelaxation in human epicardial arteries when examined in vitro (Forstermann et al. 1988). Substance P and the thromboxane analog U46619 normally stimulate endothelial release of EDRF, but the release is attenuated when atherosclerotic human coronary arterial rings are used (Chester et al. 1990). Furthermore, the inhibitor of NO synthase N^G-monomethylarginine (NMMA) can also be used to show that human atherosclerotic vessels have reduced EDRF-mediated relaxation in the basal (non-stimulated) state (Chester et al. 1990).

Endothelium-mediated vasodilation has been shown, by using a bioassay, to be inhibited by atherosclerosis in rabbit aortas. Verbeuren et al. (1990) showed that exogenously administered NO produces less vasodilation in atherosclerotic abdominal and thoracic vessels segments than in non-atherosclerotic control rabbits. The abdominal and thoracic

aortas were also treated with acetylcholine to stimulate the release of EDRF. By collecting the tissue-bathing fluid and transferring the fluid to a detector tissue ring, it was shown that EDRF derived from atherosclerotic vessels were less able to promote endothelium-mediated vasodilation of paired EDRF detector vessels.

The observations of Verbeuren et al. (1990) were partially confirmed by Minor et al. (1990) who used a bioassay protocol coupled to a chemiluminescence detector set to determine the production of the primary NO metabolite NO_3^- . Minor et al (1990) studied rabbit aortas from control and atherosclerotic individuals, in addition to aortas removed from rabbits maintained on a short-term cholesterol-enriched diet such that hypercholesterolemia was developed (1448 ± 68 mg cholesterol/dl) and intimal lesions had not yet developed. To determine EDRF production, they used detector vessels, porcine arterial rings removed from the proximal left circumflex coronary arteries. They also observed that atherosclerotic vessels and vessels from hypercholesterolemic rabbits in which fatty streaks had not been established dilated less at the basal level and when stimulated to dilate with acetylcholine. In addition, they observed that vasodilation of the detector vessels was also decreased.

In the circulation, NO is oxidized to NO_3^- (Stamler et al. 1992). Minor et al. (1990) used chemiluminescence to

directly measure NO_3^- , as a measure of NO production by control, atherosclerotic and hypercholesterolemic vessels under basal and acetylcholine-stimulated conditions. Paradoxically, they observed that the hypercholesterolemic and atherosclerotic vessels actually generate more NO than did control arterial segments. This observation is supported by evidence for increased nitric oxide synthase induction in the aortas of cholesterol-fed rabbits (Verbeuren et al. 1993). Thus, in some poorly understood manner, diseased vessels are producing more EDRF, but are less able to dilate, possibly because NO is more rapidly converted into the inactive metabolite NO_3^- .

Hypercholesterolemia and increased levels of LDL-cholesterol are associated with increased amounts of LDL oxidation. In an experiment using coronary arterial rings removed from pigs, LDL particles were oxidized by reacting them with copper. These ox-LDL particles were found to prevent endothelium dependent vascular dilation in response to serotonin. In contrast, unoxidized or native LDL particles were not found to affect endothelially dependent relaxation of vessels by serotonin (Simon et al. 1990).

Degradation of EDRF by ox-LDL has been implicated from studies of fetal lung cultured fibroblasts (Chin et al. 1992). A scavenger of O_2^- radicals, O_2^- dismutase, was shown to promote vasodilation in aortic rings from cholesterol-fed

rabbits (Mugge et al. 1991). Their observations suggest that hypercholesterolemia results in excess production of O_2^- radicals and that EDRF degradation caused by O_2^- results in the attenuated endothelium-dependent vasodilation associated with hypercholesterolemia and atherosclerosis (Mugge et al. 1991).

Oxidized-LDL particles are produced by the reaction of O_2^- with LDL particles and have been found to be very important in determining why vessels from hypercholesterolemic and atherosclerotic individuals are less able to maintain their vasodilated state. In rabbit and human arteries, ox-LDL particles and the attenuation of arterial dilation are associated with a decrease in the tissue cGMP content (Galle et al. 1992). A more recent study of cholesterol-fed rabbits reported that, in addition to a reduction in tissue cGMP, ox-LDL reduces the activity of tissue guanlycylase in vessels from hypercholesterolemic rabbits (Schmidt et al. 1993). The reduced guanlycylase activity could result in reduced amounts of intracellular cGMP and reduced inhibition of intracellular calcium entry as well as reduced cGMP dependent protein kinase activation. As a result, smooth muscle cells should theoretically tend toward vasoconstriction promoted by the binding of vasoconstrictors to receptors on the smooth muscle cell membrane.

Furthermore, EDRF has been shown to prevent the oxidative

modification of LDL particles by O_2^- from macrophages and leukocytes in vitro (Yates et al. 1992; Rubanyi et al. 1991). By sharing its unpaired electron, NO is able to neutralize O_2^- and prevent O_2^- -dependent modification of LDL particles (Yates et al. 1992), but by doing so EDRF becomes converted to NO_3^- and loses its dilating properties. Because macrophage-derived O_2^- represents a site of EDRF loss, a potential deficit of EDRF could be promoted in arterial regions affected by macrophage-rich fatty streaks or atherosclerosis. Exogenously administered L-arginine has been shown to partially reverse the loss in endothelium-dependent vasodilation in rabbits fed cholesterol, presumably by increasing the substrate for EDRF formation and compensating for the increased EDRF degradation by ox-LDL when the endothelium was stimulated with acetylcholine, though the effect of L-arginine administration in vivo is controversial (Girerd et al. 1990).

The preceding discussion has made it clear that the atherogenic process is linked to changes in vascular responsiveness. Because the vasodilatory message of compounds like catecholamines, acetylcholine and EDRF is lost during atherosclerosis, evaluation of the bioactivity of these compounds on arterial tissue will provide information as to the extent or severity of the disease present in an artery of interest.

Endothelin is a recently discovered protein whose functions in regards to atherosclerosis are rapidly being discovered

In contrast to the vasodilatory properties of EDRF, the endothelium has also been found to secrete a very potent vasoconstrictor. The existence of an endothelially originating vasoconstrictor was first suggested by Hickey et al. (1985) and later characterized as a small sized, short lived peptide by Yanagisawa et al. (1988) who called it endothelin. It was rapidly recognized that tissue and circulating levels of the peptide were positively correlated with the degree of atherosclerotic involvement (Lerman et al. 1991). Because endothelin potentiates coronary arterial contractile responsiveness to threshold levels of norepinephrine and serotonin, in addition to amplifying the force of contraction, endothelin may be important in promoting coronary arterial vasospasm (Yang et al. 1990).

Like EDRF, endothelin is secreted at both basal and stimulated levels (Boulanger and Luscher 1990). Stimulants of endothelin secretion have been proven to include thrombin, epinephrine, calcium ionophores and low shear stress (Luscher et al. 1992). EDRF has been shown to inhibit thrombin stimulated endothelin release from the endothelium (Boulanger and Luscher 1989). Because increased endothelin secretion is associated with sites of low shear stress and atherosclerosis

(Lerman et al. 1991), endothelin secretion may represent a signal of endothelial dysfunction to the underlying tissues.

The secretion of endothelin appears to promote the progression of the atherogenic process through a number of mechanisms. Endothelin promotes the atherogenic process by acting on smooth muscle cells. Endothelin serves as a co-mitogen to stimulate smooth muscle cell proliferation, a process believed to be especially important in the re-occlusion of arteries following angioplasty (Weissberg et al. 1990). Endothelin has also been found to have strong chemoattractant properties for promoting the migration of circulating monocytes into the arterial wall (Achmad and Rao 1992), thus increasing the potential for foam cell generation and macrophage mediated LDL oxidation.

The effects of ox-LDL on endothelin production are not well understood. Boulanger et al. (1992) have suggested that ox-LDL increases endothelial production of endothelin peptide and mRNA, and by this mechanism could provide a positive feedback loop, promoting local increases in endothelin at sites of atherosclerosis. However, negative controls may exist, Jougasaki et al. (1992) have suggested that lysophosphatidylcholine carried by ox-LDL can reduce the secretion of endothelin from endothelial cells. The

observations of these investigators appear contradictory and represent part of the immaturity of this rapidly changing field.

A link between atherosclerosis and endothelin clearly exists. Given that endothelin has only recently been discovered, a more complete understanding of how endothelin interacts with atherosclerosis and the complications of atherosclerosis will hopefully be forthcoming in the near future.

Evaluation of Atherosclerotic Regression in the Human and Rabbit

Evidence for regression of atherosclerotic lesions from clinical studies

Regression of atherosclerotic lesions is a goal in the treatment of established coronary heart disease. Clinical treatment of coronary heart disease typically involve some or all of the following: adherence to the American Heart Association Step 1 or Step 2 diets, therapy with lipid lowering drugs to decrease LDL-cholesterol and increase HDL-cholesterol, as well as moderate exercise (NCEP 1988). These

measures have variable levels of success. At a minimum, these measures typically slow down atherosclerotic progression and occasionally promote regression of established atherosclerotic lesions. Atherosclerotic regression in a diseased artery is indicated by any or all of the following changes: increased arterial lumen diameter, decreased atherosclerotic plaque thickness or decreased total arterial wall thickness.

Aggressive lipid lowering therapy has been found to promote atherosclerotic regression in humans. The Cholesterol Lowering Atherosclerosis Study (CLAS; Blankenhorn et al. 1987) administered a combination of the lipid lowering drugs colestipol and niacin. Two years later LDL- and HDL-cholesterol were modified by -43% and +37%, respectively, whereas the control group had changes of -5% and +2% for LDL- and HDL-cholesterol, respectively. Atherosclerotic regression was more likely to occur in the drug treated individuals (16.2% of individuals) than in the placebo treated individuals (2.4%), significance was $P=0.002$. Following the patients from their previous study, Blankenhorn et al. (1993) found that subjects receiving drug therapy had significant reductions in carotid arterial thickness after both 2 years ($P=0.0001$) and 4 years ($P=0.0001$) of drug therapy, in contrast placebo treated patients tended to show continued arterial wall thickening.

Atherosclerotic regression has also been validated in a two and one half year long study of coronary artery disease and lipid-lowering drug therapy with a combination of either lovastatin and colestipol or niacin and colestipol (Brown et al. 1990). Atherosclerotic regression was promoted in both drug treatment groups when compare to a placebo group receiving a cholesterol-lowering diet alone. While the lipid-lowering therapy promoted beneficial changes in the two drug treated groups, the plasma LDL- and HDL-cholesterol concentrations of the placebo treated participants were unchanged and little atherosclerotic regression was observed; ominously, mortality was also greatest within the placebo treated group.

Atherosclerotic regression can also be promoted by exercise and dietary modification (Schuler et al. 1992) and what are broadly termed 'Comprehensive Lifestyle Changes' (CLC) (Ornish et al. 1990). CLC consists of stress management training, moderate exercise and a strictly observed vegetarian diet that is very low in cholesterol and saturated fat (Ornish et al. 1990). After 12 months, LDL-cholesterol was significantly lower in the CLC group than in the control group, 2.46 ± 1.55 verses 4.07 ± 1.17 mmol/L, respectively ($P < 0.007$), furthermore, chest pain was least in the CLC group ($P < 0.0006$). Average lesion scores (12 month % lesion stenosis - initial % lesion stenosis) in the CLC group

changed in the direction of regression in 18 of 22 patients, with increased adherence to CLC being positively associated with increased atherosclerotic regression. In the control group atherosclerotic progression was observed in 10 of 19 participants and in only 8 of 19 was any regression observed. The study suggests that conventional recommendations for patients with coronary heart disease, such as adherence to a diet with 30% of calories as fat may not be sufficient to bring about regression in most patients and that aggressive CLC can promote atherosclerotic regression in most patients.

Schuler et al. (1992) evaluated atherosclerotic regression in patients with coronary heart disease induced stable angina patients assigned to a low-fat diet containing less than 20 percent of total calories with a polyunsaturated-to-saturated fatty acid ratio of less than one to one, and less than 200 milligrams of cholesterol per day, either by itself or with exercise. Assessment of coronary arterial morphology 12 months latter revealed that progression had occurred in 42% and 20% of individuals in the control and exercise group members respectively. Regression was observed in 4% and 30% of the control and intervention individuals, respectively. Furthermore, physical work capacity, myocardial oxygen consumption and stress-induced myocardial ischemia were all significantly improved in the exercise group. In conclusion, although exercise did not promote regression in all

participants, cardiovascular improvements were most likely to be occur in exercising individuals.

These studies of human atherosclerotic regression suggest four things: 1) That dramatic reductions in plasma LDL-cholesterol and dramatic increases in HDL-cholesterol are associated with the promotion of atherosclerotic regression, 2) Adherence to the American Heart Association Step 1 or Step 2 lipid lowering diets alone may not facilitate changes in plasma lipids conducive of atherosclerotic regression, 3) Although healthy changes in plasma lipids may occur, atherosclerosis in an individual may be resistant to regression and 4) These studies also show the complicated nature of human studies and how treatment compliance makes the human model of atherosclerotic regression particularly difficult to control and evaluate.

The rabbit as a non-human model for the evaluation of atherosclerotic regression

Atherosclerotic regression has been documented in a variety of non-human species including chickens, rats, dogs, pigeons, pigs, non-human primates and rabbits for a complete list see Manilow 1983. Of the non-primate models for human disease, the rabbit model is one of the most popular. The

popularity of rabbits stem from the relative ease by which the atherosclerotic process can be stimulated by dietary cholesterol, their relative low cost and because the lesions in the arterial lesions are similar to those developed in man (Duff 1936; Guyton and Klemp 1992).

It has long been recognized that rabbits develop atherosclerosis rapidly when cholesterol is included as a dietary supplement (Duff 1936). When hypercholesterolemia is induced by supplementing the diet with cholesterol, cholesterol esters accumulate in apo B-100 lipoproteins. In humans these cholesterol esters accumulate predominantly in the LDL fraction, but in rabbits dietary cholesterol tends to accumulate as cholesterol esters in both the VLDL and LDL fractions (Daugherty et al. 1986). Because both humans and rabbits tend to develop atherosclerotic lesions after inducement of hypercholesterolemia, the fact that different lipoproteins serve as the primary reservoir for circulating cholesterol appears to make little difference on the atherosclerotic lesions that develop.

The process of atherosclerotic regression in rabbits has been evaluated by many investigators. Comparison of these rabbit studies is difficult due to variations in dietary content, duration of dietary administration and the manner of atherosclerotic regression evaluation. However, some general statements can be made with respect to the effectiveness of

removing cholesterol from an atherogenic diet for the promotion of atherosclerotic regression: 1) Most studies have failed to find evidence for atherosclerotic regression, 2) The studies that have found evidence for atherosclerotic regression following consumption of an atherogenic diet typically had either short periods of cholesterol feeding or long periods of cholesterol-free feeding following administration of an atherogenic diet, and 3) Regression is more frequently observed when treatments are administered to supplement the effect of the consumption of a cholesterol-free diet.

Friedman and Byers (1958) showed that hypercholesterolemia persists after cessation of cholesterol feeding and suggest it is due to the discharge of previously accumulated tissue-cholesterol back into the circulation. They later observed (Friedman and Byers 1963) that normal cholesterol concentrations were not achieved in less than 5 months after discontinuance of dietary cholesterol supplementation, consisting of 2% cholesterol. In fact the atherosclerotic lesions continued to become more severe during the first 4 months of the cholesterol-free diet. The atherosclerotic process was found to be arrested at the 4 month level when rabbits had been fed the cholesterol-free diet for a total of 9 months. They concluded that regression could not be

promoted by dietary modification, but that arrest could be achieved when normal plasma cholesterol levels could be re-achieved.

The evaluation of regression by Albrect and Schuler (1965) is in agreement with the data of Friedman and Byer (1963). Albrect and Schuler (1965) promoted atherosclerosis by administering a diet supplemented with 3% cholesterol for 21 days and showed that, while plasma cholesterol levels dropped, the content of cholesterol in the aorta continued to rise during the 130-day regression period following the 21 days of cholesterol feeding.

Atherosclerotic regression was observed in two other early studies that used slightly smaller dietary cholesterol supplements. Prior and Ziegler (1965) evaluated regression after administration of an atherogenic diet with a slightly smaller cholesterol supplement of 1 gram of cholesterol per day for 6 months. They noted that approximately 4 months were required for rabbits to re-acquire normal plasma cholesterol levels. In contrast to both Friedman and Byers (1963) and Albrect and Schuler (1963), they observed that regression had occurred in aortas from rabbits killed 6, 10 and 16 months after cessation of cholesterol feeding, when compared to atherosclerotic lesions of rabbits killed 4 months after cholesterol-supplement cessation.

Bortz (1968) administered a 1% cholesterol diet for 14 or 21 days prior to administration of a cholesterol-free diet. Plasma cholesterol levels returned to normal in 5 to 6 weeks after a peak of 1,757 mg/dl at the end of the cholesterol feeding period. While the aortic cholesterol content initially increased after the dietary cholesterol was discontinued, rabbits sacrificed at between 50 and 300 days after discontinuance had progressively reduced aortic cholesterol contents suggesting efflux of tissue cholesterol out of aortic lesions. Similar results were observed by Gupta, Tandon and Ramalingaswmi (1970), who fed 2 grams cholesterol/day to rabbits for 6 months and found that regression was not observed until 6 months after cessation of cholesterol administration.

Additional recent studies have continued to evaluate atherosclerotic regression in rabbits (Adams et al. 1973; Vesselinovitch et al. 1974; Roach and Fletcher 1979; Dudrick 1987; Jayakody et al. 1989; Kappogoda et al. 1990; Badimon et al. 1990; Zhu et al. 1992) and provide additional examples of evidence both for and against atherosclerotic regression. These atherosclerotic regression studies are reviewed in Table 1. An additional review compiled from the data shown in Table 1 is compiled in Figure 2. Clearly many of the observations outlined in Table 1 are conflicting, but generally one observes that, in studies where the regression

Table 1. Comparison of rabbit cholesterol-feeding studies with respect to the evaluation of atherosclerotic regression.

Content of diet*	Days of CH Feeding	Days of Regression [#]	Regression Demonstrated? [®] (Yes or No)	Reference
Chow with 0.3% CH	70	70	No	Zhu et al. (1992)
Chow with 0.5% CH	60	30	No	Badimon, Badimon and Fuster (1990)
Chow with 1% CH	84	365	No	Adams, Morgan and Bayliss (1973)
Chow with 2% CH	126	42	No	Dudrick (1987)
Chow with 2% CH	84	112	No	Frideman and Byers (1963)
	84	252	No	
Chow with 2.5% CH	21	21	No	Kappagoda, Thomson and Senaratne (1990)
	21	49	No	
	21	115	Yes	
Chow with 3% CH	21	29	No	Albrecht and Schuler (1965)
	21	60	No	
	21	130	No	
Chow with 2% CH and 24% lipids	42	252	No	Jayakody et al. (1989)
Chow with 2% CH and 6% corn oil	28	14	No	Roach and Fletcher (1979)
	28	28	Yes	
	28	42	Yes	
0.4 grams CH/rabbit administered biweekly	98	70	No	Vesselinovitch et al. (1974)
1 gram CH/day	180	30	No	Prior and Ziegler (1965)
	180	60	No	
	180	90	No	
	180	120	No	
	180	180	Yes	
	180	300	Yes	
	180	480	Yes	
2 grams CH/day	56	70	No	Gupta, Tandon and Ramalingswami (1970)
	56	140	Yes	

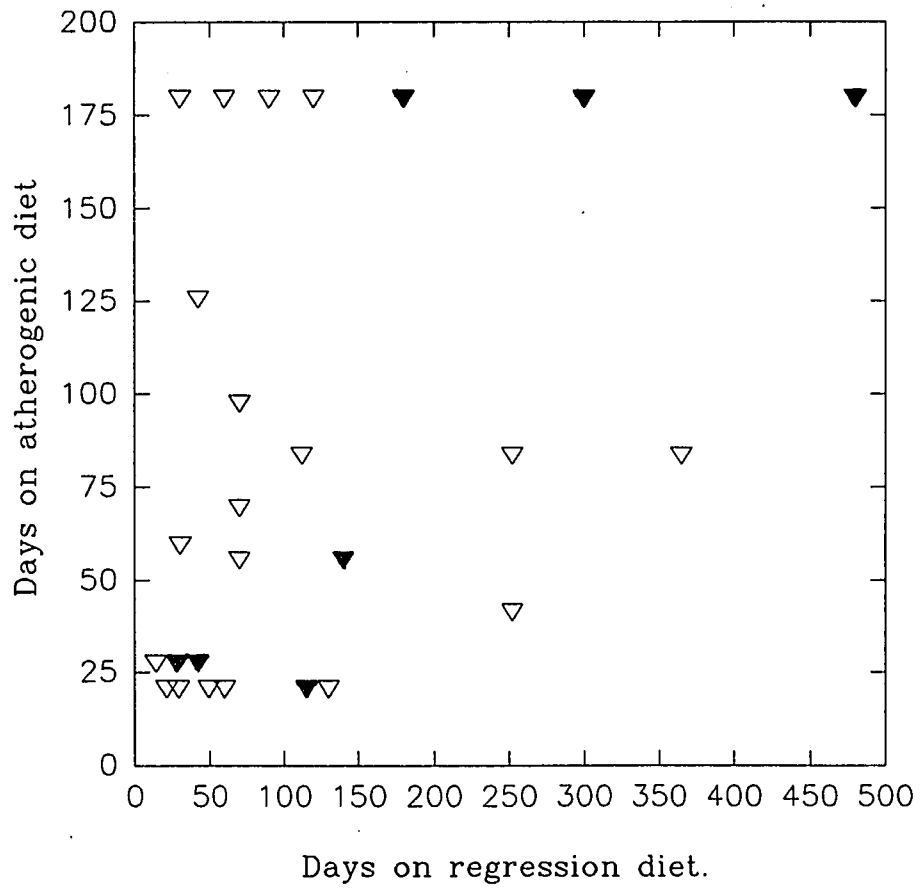
*Cholesterol is abbreviated as CH.

[#]Period during which cholesterol and/or lipid supplements were discontinued.

[®]Author(s) claimed that atherosclerotic regression had been observed (Yes).

Author(s) claimed that atherosclerotic regression was not observed (No).

Figure 2. Comparison of the rabbit atherosclerotic regression studies listed in (Table 1) with respect to cholesterol-feeding period, the duration of the following cholesterol free period and study outcome. Open triangles indicate that regression was not observed for the given combination of cholesterol-feeding days and cholesterol-free days; closed triangle indicates that regression was observed.



period was very long and/or where the cholesterol supplementation was at low levels or maintained for a short time, evidence for atherosclerotic regression is more frequently documented.

Hyperoxia has been shown to greatly stimulate atherosclerotic regression in rabbits placed onto a cholesterol-free diet. Vesselinovitch et al. (1974) fed a cholesterol-rich diet for 14 weeks before 10 weeks of a cholesterol-free diet. They observed that rabbit groups receiving just the cholesterol-free diet did not show evidence of regression. However, they observed that groups receiving 100% oxygen for 2 hours per day, as part of the regressive diet did show atherosclerotic regression in both the aorta and coronary arteries.

The process of atherosclerotic regression in rabbits was recently confirmed by Badimon et al. (1990) who administered a diet consisting of 0.5% cholesterol for 60 days when one group was sacrificed for reference and the remaining rabbits were divided into two groups for the next 30 days. Group one received the atherogenic diet alone. The other received the atherogenic diet and weekly administrations of a HDL-VHDL fraction collected from donor rabbits. After 30 days of HDL-VHDL administration, rabbits receiving the lipoprotein supplements had significantly fewer aortic fatty streaks than rabbits examined at 60 days or rabbits maintained on the

cholesterol diet for the full 90 days. The study proposed that the HDL-VHDL fraction was responsible for promoting cholesterol efflux away from arterial walls to the liver and out of the body in bile.

The lipid lowering agent lovastatin has also been shown to promote the regression of atherosclerotic lesions in rabbits (Zhu et al. 1992). A 0.3% cholesterol was administered to their rabbits for 10 weeks. For the next 10 weeks, the three study groups received a cholesterol-free diet, or a cholesterol-free diet in addition to a daily administration of either 10 or 20 mg lovastatin/day. Atherosclerotic severity progressed in rabbits receiving only the cholesterol-free diet but regressed in both groups receiving lovastatin.

Given the prevalence of heart disease as the number one killer of Americans today, there is tremendous interest in finding new agents that will decrease the rate at which atherosclerosis progresses or promote the regression of atherosclerosis in people. The preceding discussion will demonstrate how dietary ethanol represents an agent with potential to affect the atherosclerotic process. In humans, the risk of coronary heart disease is reduced in people who drink moderate amounts of alcoholic beverages. It has been proposed that ethanol does this by increasing the level of plasma HDL-cholesterol and by reducing plasma LDL-

cholesterol. However, studies of ethanol consumption of the regression of human atherosclerosis do not exist, in addition, the influence of ethanol on atherosclerotic regression in an animal model has not been evaluated.

**Potential of Dietary Ethanol as a Therapeutic Agent for
the Treatment of Atherosclerosis**

**Epidemiological studies have proven that moderate consumption
of ethanol reduces the risk of developing heart disease**

Ethanol has been a part of the human diet for thousands of years, yet only recently has it been recognized by the scientific community that drinkers develop heart disease less frequently. As Eberhard stated in 1936:

"There has been an impression among pathologists for many years that confirmed drunkards do not show as much arteriosclerosis for given age groups as do temperate persons".

This literature review will first seek to confirm that ethanol consumption is a protective agent against coronary

heart disease in humans and animal models, then survey the possible mechanisms providing protection and finally present a hypothesis about how ethanol consumption might also promote regression of atherosclerotic lesions.

The Framingham study of Friedman and Kimball (1986) represents one of the earliest studies of human ethanol consumption and coronary heart disease. The study utilized a population (n=4,745) from the Framingham Heart Study Cohort. Their study showed that coronary heart disease mortality followed a U-shaped curve with respect to non-smokers and ethanol consumption. For females coronary heart disease was lowest in those drinking 2-3 ounces/week and higher in non-drinkers and those drinking more than 2-3 ounces/week. In males coronary heart disease was lowest in those drinking 8 to 19 ounces of ethanol/week.

Friedman and Kimball's 1986 study has been validated by additional recent studies. The study of Rimm et al. (1991) used 51,529 male health professionals adjusted for people abstaining from ethanol because of pre-existing disease conditions. The study determined that "...increasing alcohol intake was inversely related to coronary disease incidence..." (P<0.001). Klatsky et al. (1992) studied 128,934 subjects and showed that people drinking 1 to 2 drinks per day had the lowest risk of coronary disease mortality.

Correlation of alcoholic beverage type to protection from coronary heart disease was evaluated by Friedman and Kimball (1986), who found that beer and wine provided approximately even and slightly better protection than hard liquor spirits. Their results contrast with those of Rimm et al. (1991) who found the strongest inverse correlation between ethanol consumption and coronary heart disease among drinkers of hard liquor spirits. In the study of Klatsky et al. (1992) heart disease mortality was found to be the greatest, intermediate and least in populations drinking beer, hard liquor and wine, respectively. When wine drinkers were evaluated, they found that those who drank red wine had the least risk.

The 'French Paradox' is a recently proposed theory (Renaud and De Lorgeril 1992) that supports the conclusions of Klatsky et al. (1992) in regards to red wine as the most effective agent for protection from coronary heart disease. The French people have a diet rich in coronary risk factors like cholesterol and saturated fats and tend to have relatively high plasma cholesterol levels, but when compared to Americans and the British, the French have less than half to a third the rate of mortality from coronary heart disease. Renaud and De Lorgeril (1992) suggest that the reason the French are protected is not just that they drink ethanol, but because they drink most of their ethanol in the form of red wine.

Animal studies of the relationship of ethanol consumption to atherosclerosis and coronary heart disease

Epidemiological studies suggest that dietary ethanol reduces the establishment of atherosclerosis and heart disease, but human experiments to test this hypothesis have been difficult because of the length of time needed to look for changes in a slow disease process like atherosclerosis and because of ethical as well as legal implications surrounding ethanol consumption and alcoholism. However, the long-term effects of dietary ethanol on non-human primate-atherosclerosis have been examined by the studies of Rudel et al. (1981) and Karsensty et al. (1985).

Rudel et al. (1981) fed monkeys (Macaca nemestrina) liquid diets containing 0.3 or 1.0 mg cholesterol/kcal and 36% of the daily caloric intake as either ethanol or a carbohydrate substitute in a 2X2 factorial design for 18 months before the atherosclerotic severity was evaluated. It was determined that dietary ethanol inhibited atherosclerotic development. In a similar study by Karsenty et al. (1985), baboons were fed cholesterol-enriched diets for one to nine years, receiving 50% of dietary calories as ethanol or carbohydrate. Although atherosclerotic severity was not directly evaluated, they concluded that ethanol consumption promoted an increase in cholesterol excretion by the liver as well as changes in

the plasma lipoproteins, consistent with providing the protection from atherosclerotic development observed by Rudel et al. (1981).

The effects of ethanol administration on experimental atherosclerosis in rabbits have been evaluated by several studies. Among the earliest was that of Eberhard (1936) who administered 0.5 grams of cholesterol/day to rabbits receiving either water or ethanol (25% v/v) in drinking water and evaluated atherosclerotic progression at periods of up to 120 days. It was demonstrated that ethanol administration resulted in reduced deposition of cholesterol in the aorta.

In contrast, Kritchevsky and Moyer (1960) were unable to show that administration of a 9.5% ethanol solution in drinking water could inhibit atherosclerotic development in rabbits fed a diet containing 3% cholesterol and 6% corn oil. However, their negative result could be due to the extremely large amount of cholesterol administered.

More recently the ethanol-induced inhibition of atherosclerotic development observed by Eberhard (1936) was confirmed by the rabbit studies of Goto et al. (1974) and Klurfeld and Kritchevsky (1981). Goto et al. (1974) fed rabbits a diet consisting of 0.5% cholesterol and 5% lard for between 134 and 139 days; during this time, rabbits received water or water containing either 5% or 10% ethanol. The rabbits receiving water had aortas with $24.4 \pm 5.4\%$ of their

aortas covered by atherosclerotic lesions, aortas from rabbits receiving 5% ethanol had aortas covered by $9.9 \pm 1.9\%$ covered by lesions; this was significantly less than the water fed rabbits ($P < 0.05$). Rabbits consuming 10% ethanol were even less affected, having aorta covered by only $5.5 \pm 2.4\%$ ($P < 0.05$).

Correlation of beverage type to protection from atherosclerotic development to inhibition was evaluated by Klurfeld and Kritchevsky (1981). An earlier investigation by Kritchevsky and Moyer (1960) used a diet with 3% cholesterol with 6% corn oil with or without 9.5% ethanol and concluded that ethanol did not inhibit atherosclerotic progression. Klurfeld and Kritchevsky (1981) used a smaller dietary cholesterol supplement (0.5%) and compared several beverage types (ethanol in water, red wine, white wine, beer, whiskey) adjusted with ethanol to a final ethanol content of 9.5% against water with a isocaloric glucose supplement for the inhibition of atherosclerotic development. In their study atherosclerotic severity was expressed as percent Sudan-IV staining area as a percentage of the total aortic surface area. Red wine significantly inhibited atherosclerotic development when compared to rabbits receiving just glucose in water ($P < 0.05$). Where as rabbits consuming ethanol, white wine, whiskey and beer did not developed statistically significant differences in atherosclerotic development.

Because red wine was found to be the most effective inhibitor of atherosclerosis, the results of Klurfeld and Kritchevsky (1981) confirm the French Paradox suggested by Renaud and De Lorgeril (1992).

The influence of ethanol on pathways of cholesterol transport and atherosclerotic development

When compared to men who do not drink alcoholic beverages, consumption of dietary ethanol (5-6 ounces/week) by men has been found to promote a 10 percent increase in plasma HDL-cholesterol and a 10 percent decrease in plasma LDL-cholesterol (Castelli et al. 1977). The increase in HDL-cholesterol and the decrease in LDL-cholesterol are responsible for approximately 50% (HDL) and 17% (LDL) of the protection from coronary heart disease associated with ethanol consumption (Langer et al. 1992). After cessation of ethanol consumption for two to three weeks the healthy changes in the lipoprotein profile disappear (Taskinen et al. 1982). How ethanol causes HDL-cholesterol to increase and LDL-cholesterol to decrease is still not understood.

HDL particles are responsible for transporting cholesterol away from the periphery and to the liver, where it may be removed from the body in bile or recycled into VLDL

particles. Cholesterol ester transfer protein provides cholesterol esters within HDL particles with an opportunity to return to atherogenic LDL and VLDL particles before the HDL particle and the cholesterol esters within it can be removed by the liver (Tall 1990). It has been observed that CETP activity in alcoholics is very low (Savolainen et al. 1990) and that the increase in HDL-cholesterol in alcoholics could be due to a decrease in both the plasma CETP activity and concentration, effects that are reversible with abstinence from ethanol (Hannuksela et al. 1992).

Ethanol consumption may also affect the HDL-cholesterol concentration by acting on apolipoprotein A-I and LCAT. Apo A-I is the primary activator of LCAT in the plasma, and LCAT serves to esterify free cholesterol in HDL particles (Jonas 1991). The activity of apo A-I and LCAT maintains a concentration gradient for diffusion of free cholesterol from the cell membranes of peripheral cells into HDL particles (Jonas 1991). Because ethanol consumption increases both the activity and the rate of Apo A-I (Camargo et al. 1985; Amarasuriya et al. 1992), ethanol apparently increases the rate of cholesterol esterification within the HDL particle and promotes the maintenance of a concentration gradient responsible for removal of free-cholesterol from peripheral cell membranes to the HDL particle. Ethanol consumption appears to promote the uptake of peripheral cholesterol from

sites like an atherosclerotic plaque and prevent the cholesterol from being redeposited into VLDL and LDL particles, and ultimately into atherosclerotic plaques.

The reduction in plasma LDL-cholesterol associated with ethanol consumption has been difficult to explain. It is possible that by inhibiting CETP, ethanol inhibits the entry of HDL-bound cholesterol esters into LDL particles. Evaluation of this problem is further complicated by the fact that ethanol consumption increases both the rate of VLDL synthesis by the liver (Savolainen et al. 1986) and the content of triglycerides in VLDL particles, and to a lesser extent LDL and HDL particles as well (Schneider et al. 1985).

Paradoxically, the concentration of the catabolic product of VLDL metabolism, the LDL particle is decreased by ethanol consumption. This paradox has been recently examined by Wehr et al. (1993), who suggest that acetaldehyde, the metabolic product of ethanol, forms adducts with apo-B containing particles, increasing the rate of their uptake from the circulation. By this mechanism, even though VLDL synthesis is increased and more LDL particles are produced as a result of VLDL degradation, an increase in LDL uptake due to the modification is great enough to lower the total LDL-cholesterol concentration. This hypothesis is intriguing and awaits confirmation, but is potentially flawed because factors which increase LDL uptake, like oxidative

modification promote the atherogenic process, where as ethanol consumption inhibits the process.

In contrast to the previously described beneficial effects of ethanol, the effects of ethanol on the formation of EDRF represent an example of how ethanol may not be beneficial in cases of heart disease. It has recently been shown that ethanol inhibits the formation of endothelially-dependent relaxing factor and arterial relaxation (Persson and Gustafsson 1992; Hatake et al. 1993). These recent observations may help explain why ethanol infusion results in contraction of canine epicardial arteries (Hayes and Bove 1988; Rogers and Bove 1989). Furthermore, a reduction in EDRF formation can promote conditions in the arterial wall favoring promotion of the atherosclerotic process.

Clearly, ethanol has both potential health benefits and risks in regards to heart disease and atherosclerosis. But ultimately clinical studies have overwhelmingly shown that ethanol consumption is correlated with a reduction in both the incidence and mortality of coronary heart disease. Given that heart disease is the greatest single killer of people of industrialized nations, ethanol must be considered beneficial in regards to its effect on human health.

Can ethanol consumption promote the regression of previously established atherosclerotic lesions?

As previously reviewed in this literature review, investigators have determined that consumption of ethanol inhibits the development of atherosclerosis in humans, non-human primates and rabbits. Whether ethanol consumption could promote the regression of atherosclerotic lesions established prior to inclusion of ethanol in the diet has not been examined at either the clinical or the experimental level on humans or animals. There are several reasons to believe that ethanol could have potential as a therapeutic agent promoting regression of previously established atherosclerotic disease.

Ethanol-induced changes in the lipoprotein profile favor reverse cholesterol transport out of peripheral sites, like atherosclerotic plaques. Epidemiologically, individuals with high HDL-cholesterol have a very low incidence of atherosclerosis and the infusion of additional HDL particles promote the regression of atherosclerotic plaques in rabbits (Badimon et al. 1990). Because HDL-cholesterol concentrations increase as a result of ethanol consumption, ethanol may promote increased transport from places like atherosclerotic plaques to the liver for removal from the body, thus promoting atherosclerotic regression.

Ethanol consumption might also promote atherosclerotic regression by reducing the delivery of cholesterol to peripheral sites like atherosclerotic plaques and the LDL-cholesterol concentration. Given that cholesterol is in a constant state of flux in and out of macrophage derived foam cells (Brown et al. 1980), any reduction in the transport of cholesterol into a lesion would have potential to increase the net effect of cholesterol efflux and promote a reduction in the cholesterol mass of the plaque and the ultimate lesion size.

The activity and secretion of several enzymes from the liver is also modified by ethanol in a manner that should promote regression of atherosclerotic plaques. Ethanol associated increases in apo A-I secretion and activity would also help prevent cholesterol recirculation into VLDL and LDL particles by promoting faster esterification of free-cholesterol associated with HDL particles and sequestration of cholesterol esters to the center of HDL particles. By inhibiting CETP, ethanol prevents cholesterol from being recycled into lipoproteins that deposit cholesterol in the periphery, reducing the rate of cholesterol deposition into the periphery.

Ethanol might also decrease the stimulus for continued plaque growth by acting on platelets. Activated platelets release factors like platelet derived growth factor and

serotonin that increase LDL permeability, LDL oxidation, LDL uptake and smooth muscle proliferation. Because ethanol inhibits platelet aggregation, ethanol consumption might reduce the stimuli for additional atherosclerotic plaque growth, thereby increasing the relative influence of factors promoting efflux of cholesterol out of atherosclerotic plaques.

Dissertation Hypothesis

The effect of dietary ethanol on previously established atherosclerotic disease has never been addressed by either human or animal studies. Given that ethanol consumption promotes anti-atherogenic changes in cholesterol metabolism, enzyme activities and atherogenic stimuli, it is the proposal of this dissertation that the consumption of ethanol will promote the regression of previously established atherosclerotic lesions. Furthermore, because the atherosclerotic process is similar in the man and rabbit, this dissertation proposes to use atherosclerotic rabbits as a model for the effects of ethanol on atherosclerotic people.

The research described in this dissertation asks whether ethanol consumption can reduce the severity of atherosclerotic disease in rabbits by:

- 1) Promoting a healthier lipoprotein-cholesterol profile,
- 2) Enhancing cholesterol transport out of atherosclerotic lesions,
- 3) Reducing the arterial surface area affected by atherosclerosis,
- 4) Reducing the degree of foam cell infiltration in the arterial wall, and
- 5) Reducing the vascular constrictive responses to several known vasoconstrictive compounds.

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**CHAPTER 3. EFFECT OF DIETARY ETHANOL ON ATHEROSCLEROTIC
RABBITS CONSUMING A CHOLESTEROL-FREE DIET**

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Comparative Biochemistry and Physiology.

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Abstract

The effect of dietary ethanol on plasma lipids and atherosclerotic regression in atherosclerotic rabbits was addressed by feeding 19 male New Zealand white rabbits a diet enriched with 0.8% cholesterol for 45 days, followed by randomization to one of three diets for the next 45 days: 1) Continued consumption of a cholesterol-rich diet (CC; n=6), 2) Consumption of a cholesterol-free diet (CF; n=6), and 3) Consumption of a cholesterol-free diet supplemented with ethanol in the drinking water (10% v/v; ECF; n=7). Data are expressed as mean \pm SEM, means with the same letter are not significantly ($P < 0.05$) different. On day 0, the plasma

triglyceride, total-, very low density lipoprotein (VLDL)-, low density lipoprotein (LDL)- and high density lipoprotein (HDL)-cholesterol concentration (n=19) was 54.93 ± 7.75 , 77.04 ± 15.51 , 23.06 ± 5.21 , 32.22 ± 10.67 and 21.73 ± 2.3 mg/dl, respectively. On day 45, the triglyceride, total-, VLDL-, LDL-, and HDL-cholesterol concentration was 437.90 ± 49.17 , 2281.7 ± 133.96 , 1316.36 ± 56.10 , 731.34 ± 63.44 and 238.62 ± 42.80 mg/dl, respectively. On day 90, the total plasma cholesterol concentration of the CC, CF and ECF groups was 1532 ± 107.01^A , 440.50 ± 44.08^B and 957.79 ± 157.21 mg/dl^C, respectively. On day 90, the plasma very low density lipoprotein (VLDL)-cholesterol concentration of the CC, CF and ECF groups was 1018.40 ± 79.29^A , 217.40 ± 24.05^B and 597.50 ± 115.36^C mg/dl, respectively. On day 90, the plasma low density lipoprotein (LDL)-cholesterol concentration of the CC, CF and ECF groups was 483.63 ± 79.29^A , 205.18 ± 39.51^B and 312.77 ± 49.93^B mg/dl, respectively. On day 90, the plasma high density lipoprotein (HDL)-cholesterol concentrations of the CC, CF and ECF groups were 46.46 ± 5.16^A , 17.92 ± 3.54^A and 47.51 ± 15.95 mg/dl^A, respectively. On day 90, the plasma triglyceride concentration of the CC, CF and ECF groups was 238.97 ± 64.28^A , 79.97 ± 11.77^A and 433.32 ± 122.07^B mg/dl, respectively. Between days 45 and 90, the resolution of the developed hyperlipidemia in the CC, CF and ECF groups is graded as least, moderate and greatest,

respectively. On day 90, no significant differences were noted in regards to the percent of aortic surface covered by Sudan-IV staining lesions, fatty streak areas in aortic cross-sections, smooth muscle area or aortic cholesterol content. On day 90, the total liver cholesterol content of the CC, CF and ECF groups was 3.98 ± 0.33^A , 2.36 ± 0.30^B and 1.97 ± 0.31^B grams cholesterol/liver, respectively. This investigation demonstrated that neither the CF nor ECF diets promoted atherosclerotic regression, and that plasma lipid resolution was only moderate in the ECF group.

Introduction

Cholesterol-rich diets are associated with increased plasma VLDL- and LDL-cholesterol concentrations which are positively correlated with increased atherosclerotic severity (Nordesgaard, Tybærg-Hansen and Lewis, 1992; Adams et al., 1982). In contrast, increased HDL-cholesterol concentrations are negatively correlated with atherosclerotic development (Johansson et al., 1991; Gaziano et al., 1993). Atherosclerotic severity is a product of increased cholesterol deposition into the arterial wall, resulting in the formation of cholesterol-rich fatty streaks and plaques along the surface of the arterial lumen. As the disease

progresses, these lesions cover increased percentages of arterial surface area and encroach upon the arterial lumen, leading to potential reductions in blood flow (Ross, 1993). Primary clinical treatment of high plasma cholesterol and atherosclerosis includes a lipid-lowering diet with reduced cholesterol content (National Cholesterol Education Program, 1988).

Atherosclerotic regression refers to a reduction in the severity of the previously mentioned factors associated with increased atherosclerotic severity. Atherosclerotic regression in humans and rabbits has been documented in association with decreases in VLDL- and LDL-cholesterol (Brown et al., 1990; Blankenhorn et al., 1987; Daugherty et al., 1986). Furthermore, it has been shown that intravenous administration of HDL particles to rabbits can also promote atherosclerotic regression by promoting HDL-mediated cholesterol efflux (Badimon et al., 1990).

Eberhard (1936) fed rabbits a crude cholesterol purified from gallstones to rabbits with or without a 25% (v/v) solution of ethanol and determined that the cholesterol content of the rabbits receiving dietary cholesterol was 2.00 grams of cholesterol per 100 grams dry weight and that the aortas removed from rabbits receiving both dietary ethanol and cholesterol was less at 1.26 grams of cholesterol per 100 grams dry weight; however, statistical comparisons were not

performed. Goto et al. (1970) maintained rabbits on a diet containing 0.5% cholesterol and 5.0 % lard for 13 weeks with fluid in the form of water, 5% (v/v) ethanol or 10% (v/v) ethanol. After 13 weeks rabbits fed water, 5% ethanol and 10% ethanol, had Sudan-IV stained lesions covering 24.4%, 9.9% and 5.5% of the aortic surface areas, respectively. The 5% ethanol and 10% ethanol groups had significantly less lesion area at the $P < 0.05$ and $P < 0.01$ levels, respectively. More recently Klurfeld and Kritchevsky (1981) fed rabbits a diet supplemented with cholesterol (0.5% w/w) for 3 months with either water or red wine (10% ethanol v/v). Klurfeld and Kritchevsky (1981) found that 34% and 10% of the aortic arch was covered with Sudan-IV staining lesions in the water and red wine groups, respectively, significance was $P < 0.05$ with a t-test.

The studies of rabbits confirm that atherogenesis in rabbits is inhibited by ethanol consumption, the same observations have been made in humans. The epidemiological study of Friedman and Kimball (1986) demonstrated that in human non-smoking males consuming 4 to 7 ounces of ethanol per week, over all mortality and mortality from coronary heart disease was 48% and 10% less, respectively, than that observed in non-smoking males who did not consume ethanol.

The primary mechanism for the protective effect of ethanol consumption is an increase in plasma HDL-cholesterol and

cholesterol efflux out of atherosclerotic lesions (Castelli et al., 1977; Langer et al., 1992). The mechanisms for protection may also include the following: 1) Ethanol associated inhibition of cholesteryl ester transfer protein (CETP) and decreased transfer of cholesterol from HDL to LDL particles (Hannuksela et al., 1992; Savolainen et al., 1990), 2) Ethanol associated increases in Apo AI secretion and activity, resulting in improved cholesterol efflux (Amarasuriya et al., 1992; Camargo et al., 1985), and 3) Ethanol associated decreases in plasma LDL-cholesterol concentration (Castelli et al., 1977; Langer et al., 1992).

The effect of dietary ethanol on cholesterol metabolism and atherosclerosis during administration of a cholesterol-free diet is unknown. Dietary ethanol has the potential to affect atherosclerotic severity because of its effect on HDL-cholesterol, LDL-cholesterol, CETP and Apo AI. This investigation evaluated the effect of dietary ethanol administration on atherosclerotic rabbits consuming a cholesterol-lowering diet in regards to the promotion of atherosclerotic regression.

Materials and Methods

Experimental design: Male New Zealand White rabbits weighing 1.9 to 3.0 kg were obtained from LSR Inc. Union Grove, Wisconsin. Rabbits were obtained as a set of 18, followed by an additional set of 5, staggered 45 days behind the set of 18. Rabbits were fed from a single manufactured lot of Teklad #0533 (Madison, Wisconsin) rabbit chow (140 grams/day). The chow was made atherogenic by adding a cholesterol-supplement prepared by dissolving cholesterol in butylmethylphenol-free ethyl ether (1 gram/10 ml) and evenly dispersing the solution onto the rabbit chow. The ether was evaporated from the chow under a fume hood, leaving chow enriched with 0.8% cholesterol.

This atherogenic chow was fed to all rabbits from days 0 to 45. On day 45, the rabbits were randomized to one of three treatments for the next 45 days: 1) Rabbits received the same atherogenic rabbit chow (Cholesterol consuming; CC), 2) Rabbits received chow with no cholesterol supplement (Cholesterol-free; CF), and 3) Rabbits received dietary ethanol in their drinking fluid (10% ethanol v/v) in addition to the cholesterol-free diet (ECF).

During the course of the experiment the rabbits had free access to the following drinking solutions. Water was administered between days 5 to 45 to all rabbits. Antibiotic

treatment of pneumonia was repeated for all rabbits (days 0 to 4, days 50 to 58 and days 71 to 80) and consisted of tetracycline (2 mg/ml) dissolved in Kool-Aide (0.16% Kool-Aide; 5% sugar) for improved palatability. The Kool-Aide solution was also administered to all rabbits between days 45 and 90; this solution was supplemented with ethanol (10% v/v) for rabbits assigned to the ECF treatment group. Three rabbits died of pneumonia during the course of the experiment, and one rabbit was euthanized as a result of a handling injury; these rabbits were excluded from this experiment.

Blood collection and analysis. During the experiment, blood was collected after a 12-hour fast from the central ear artery at three different times: 1) On day 0 prior to administration of the CC diet, 2) Just prior to dietary reassignment on day 45, and 3) On day 90. To promote animal sedation, acepromazine (Fort Dodge Laboratories, Fort Dodge, Iowa; 0.4 mg/kg) was administered intramuscularly 5 minutes prior to collection. Blood was collected from the central ear artery with a 21 gauge butterfly catheter and mixed with disodium EDTA (2 mg/ml) to prevent clotting. After being placed on ice, the packed cell volume of the raw blood was determined by centrifugation. The remaining blood was then centrifuged for 15 minutes (650 x g) and the plasma removed.

A 2.5-ml portion of the plasma was used for lipoprotein determination, and the remainder was frozen (-20°C) for plasma triglyceride determination at a later time.

Plasma lipoproteins were separated with ultracentrifugation on the basis of density by using the method of Havel et al. (1955). Lipoprotein separation was performed on 2.5 ml of plasma mixed thoroughly with 0.5 ml of 0.9% NaCl. Lipoproteins were separated into density gradients of less than 1.005 g/ml, 1.005 to 1.063 g/ml and 1.063 to 1.210 g/ml, representing the VLDL, LDL and HDL fractions, respectively. The lipoprotein fractions were collected into cryovials and frozen at -20°C.

After the lipoprotein and raw plasma samples had been thawed, the plasma triglyceride concentration and the cholesterol content of each lipoprotein fraction was determined by using enzymatic test kits from Stanbio Inc. (San Antonio, Texas). The samples were diluted as needed to bring the sample concentrations into the concentration range of 1 to 200 mg/dl, a concentration range in which the colorimetric assay was known to function in a linear fashion. All samples were referenced to a 200 mg/dl certified reference standard (Stanbio Inc., San Antonio Texas).

Evaluation of atherosclerosis. On day 90, and after being bled, the rabbits were returned to their cages preserving

their respective diet for at least 3 hours. Then, following a 2- to 3-hour secondary fast from food and fluids, pentobarbital (40 mg/kg) was administered into the ear vein. Anesthetized rabbits were decapitated and bled to facilitate organ removal. The aorta was gently dissected starting 3 mm distal to the aortic valve and running distally to the iliac bifurcation.

Lipophilic deposits on the surface of the arterial lumen of two aortic segments from each rabbit were stained by using the Sudan-IV staining method of Holman et al. (1958). The first segment consisted of tissue that started 37 mm distal from the aortic valve and stopped at the renal artery; the second segment started 25 mm distal of the renal artery and ended at the iliac bifurcation. The fresh segments were slit lengthwise, opened flat and pinned in this position prior to immersion in 10% formalin (pH 7.3) and fixed for 24 hours. Fixed tissues were removed, rinsed briefly with 70% (v/v) ethanol and immersed in the staining solution (5 g Sudan-IV, 500 ml acetone and 500 ml ethanol) for 15 minutes prior to rinsing with 80% ethanol. After differentiating the stain in a 80% ethanol bath for 20 minutes, the segments were washed in cold tap water and stored in 5% formalin at 4°C until color photographed.

Computer images of the aortic segments were acquired from the photographs by using a Zeiss SEM-IPS image analysis

system (Roche Inc., Elan College, North Carolina; IBAS version 2.00). Images were captured with a Sony DXC-3000A 3 CCD color video camera (Sony Inc., San Diego, California) calibrated to a scale measuring in millimeters, normalized and shade corrected. The stained area on each segment was discriminated from a computer generated color plane and the total area of Sudan-IV staining area was calculated as the percent of the total surface area by using a computer program (Image Analysis Facility, Iowa State University). All aortic segments were processed in one session by one person with the tissues re-numbered so that the operator would not know how the tissues were assigned to treatments.

Histological features of the aorta were evaluated by using aortic rings (2 mm long) removed at a location starting 3 mm distal from the aortic valve and at a position starting another 30 mm distally. The rings were washed and immediately placed in a 10% formalin solution (pH 7.3), prior to embedding in paraffin wax. Thin sections from the center of the embedded segments were cut and stained with hematoxylin and eosin (Histology Laboratory, Department of Pathology, Iowa State University).

Images of the aortic cross-sections were captured with a Sony DXC-3000A3 CCD color video camera and digitalized with a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00) and then computer-scaled to measure in

millimeters. Captured images were then shade corrected and normalized. The tissues were given new identification numbers so that the operator would not know how the tissues were assigned to treatments, the area of vascular smooth muscle and fatty streak visible in each section was then outlined and calculated by using computer software prepared by the Image Analysis Facility of Iowa State University. The areas of smooth muscle and fatty streak in the two rings were then added together and divided by two, generating average cross-sectional values for each animal.

A section of aortic tissue starting at the renal arteries and ending 25 mm distally was used in a study described separately in Chapter 4.

Tissue cholesterol determination. The cholesterol content of each liver was determined by first using the lipid extraction method of Bligh and Dyer (1959). The livers were combined with 50 ml H₂O and homogenized with Tekmar tissue homogenizer. A 250- μ l sample from the homogenate was vortexed for 60 seconds in 8.32 ml of a chloroform:methanol:water solution (1:2:0.8; v:v:v) and shaken overnight. Then, 2.5 ml of chloroform and 2.5 ml of KCl (0.37%) was added prior to 10 seconds of vortexing. The sample was centrifuged for 20 minutes (650 X g) and the top layer removed. Then, 3.33 ml KCl (0.37%) was added and the

solution was vortexed for 10 seconds and centrifuged (650 x g) for 15 minutes. The top layer was removed and 3.33 ml KCl (0.37%) was added, prior to 10 seconds of vortexing and 15 minutes of centrifugation (650 x g). The top layer was removed and the extract was filtered through glass wool into a scintillation vial. Then N₂ gas was blown over the extract until dry. After N₂ gas treatment, isopropanol was added to re-solubilized the lipids for enzymatic determination of tissue cholesterol content as previously described for the plasma samples. Liver cholesterol was expressed as grams of cholesterol per liver and milligrams of cholesterol per gram of wet weight liver.

The 30-mm long segment located at the arch of the aorta was used for a tissue cholesterol assay. After collection, the adventitial tissue was removed. Then the segment was cut lengthwise and visually inspected for atherosclerotic deposits. After visual inspection, each segment was weighed and frozen at -20°C until lipid extraction could be performed. The aortic segments were finely minced prior to vortexing for 60 seconds in 8.32 ml of a chloroform:methanol:water solution (1:2:0.8; v:v:v) and shaken for six hours for lipid extraction. Extraction was then completed exactly as for the liver. Aortic cholesterol content was then expressed on a per segment basis and on a per gram of wet weight tissue.

Statistical Analysis. Data are presented as the mean \pm the standard error of the mean (SEM). When group differences were detected by the analysis of variance, Duncan's Multiple Comparison test was used to test for significant differences ($P < 0.05$) between individual group means.

The Student's t-test was used to assess the significance of time dependent changes in the plasma lipid concentrations, the result of these tests are found and discussed separately in Appendix 1.

Results

Weight gain by the three groups of rabbits was similar throughout the 90-day period (Table 1). The rate of food consumption (Table 2) after day 45 was similar for the three groups. The rate of fluid consumption after dietary reassignment is shown in Table 3. The rabbits in the CF group drank significantly more fluid at all intervals than did the rabbits in the CC and ECF groups (t-test; $P < 0.05$). On day 90, significant differences between the three groups were not detected in regards to the percent packed cell volume, aortic arch segment weight, liver weight and lung weight (Table 4); in addition, signs of pneumonia were not observed in any lungs on day 90.

Table 1. Body weights (kg) recorded at five-day intervals during the 90 day experimental observation period. Data are represented as mean \pm SEM.

Group*	Animal Weight				
	Day 0	Day 5	Day 10	Day 15	Day 20
CC	2.30 \pm 0.10	2.55 \pm 0.09	2.81 \pm 0.11	2.77 \pm 0.04	2.85 \pm 0.04
CF	2.57 \pm 0.16	2.86 \pm 0.10	2.96 \pm 0.11	3.01 \pm 0.08	3.07 \pm 0.09
ECF	2.55 \pm 0.12	2.87 \pm 0.09	3.07 \pm 0.08	3.02 \pm 0.08	3.09 \pm 0.08
	Day 25	Day 30	Day 35	Day 40	Day 45
CC	3.13 \pm 0.07	3.24 \pm 0.06	3.27 \pm 0.07	3.34 \pm 0.07	3.44 \pm 0.09
CF	3.25 \pm 0.07	3.43 \pm 0.06	3.38 \pm 0.09	3.49 \pm 0.06	3.44 \pm 0.06
ECF	3.32 \pm 0.05	3.38 \pm 0.16	3.48 \pm 0.06	3.56 \pm 0.05	3.62 \pm 0.10
	Day 50	Day 55	Day 60	Day 65	Day 70
CC	no data	3.48 \pm 0.07	3.39 \pm 0.08	3.49 \pm 0.05	3.51 \pm 0.07
CF	no data	3.60 \pm 0.13	3.65 \pm 0.09	3.65 \pm 0.08	3.56 \pm 0.08
ECF	no data	3.58 \pm 0.12	3.69 \pm 0.14	3.70 \pm 0.09	3.82 \pm 0.10
	Day 75	Day 80	Day 85	Day 90	
CC	3.44 \pm 0.13	3.43 \pm 0.11	3.48 \pm 0.11	3.52 \pm 0.14	
CF	3.57 \pm 0.12	3.57 \pm 0.12	3.66 \pm 0.11	3.74 \pm 0.14	
ECF	3.67 \pm 0.12	3.81 \pm 0.13	3.77 \pm 0.10	3.82 \pm 0.11	

*Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF; n=6) and Ethanol-consuming and cholesterol-free (ECF; n=7).

Table 2. Food consumption measured as the average of five-day intervals between days 55 and 85. Data are represented as mean \pm SEM.

Day	Food Consumption (g/kg · day)		
	Treatment Group*		
	CC (n=6)	CF (n=6)	ECF (n=7)
55	28.2 \pm 2.7	27.7 \pm 2.3	21.1 \pm 2.0
60	27.5 \pm 4.6	24.1 \pm 2.0	21.6 \pm 1.1
65	26.0 \pm 1.4	26.3 \pm 2.8	23.6 \pm 1.1
70	23.9 \pm 2.4	25.7 \pm 1.5	22.0 \pm 1.9
75	20.9 \pm 2.2	25.3 \pm 1.7	21.1 \pm 1.9
80	22.4 \pm 2.8	25.8 \pm 1.2	19.5 \pm 1.4
85	25.8 \pm 1.1	27.2 \pm 2.4	19.1 \pm 1.3

*Treatment Groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF).

Table 3. Fluid consumption measured as the average of five-day intervals between days 55 and 85. Data are represented as mean \pm SEM.

Day	<u>Fluid Consumption (ml/kg · day)</u>		
	Treatment Group*		
	CC (n=6)	CF (n=6)	ECF (n=7)
55	48.0 \pm 6.7	66.4 \pm 1.0	38.7 \pm 3.5
60	48.7 \pm 7.3	67.4 \pm 3.7	44.8 \pm 3.5
65	41.9 \pm 5.0	65.7 \pm 1.7	44.9 \pm 2.3
70	44.8 \pm 4.5	71.9 \pm 4.5	44.4 \pm 2.0
75	37.5 \pm 5.1	63.0 \pm 2.1	44.2 \pm 3.1
80	33.9 \pm 2.2	63.6 \pm 4.2	37.7 \pm 2.4
85	42.5 \pm 8.9	65.7 \pm 2.5	33.2 \pm 3.7

*Treatment Groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF). At all times rabbits in the CF group drank significantly more fluid/kg · day than did animals in the CC and ECF groups (Student's t-test; $P < 0.05$).

Table 4. Hematocrit and organ weights collected on day 90.

Variable [§]	Treatment Group [*]		
	CC (n=6)	CF (n=6)	ECF (n=7)
Hematocrit (%)	30.42 ± 1.32	37.55 ± 1.28	38.76 ± 3.26
Aorta Wt (mg)	393.33 ± 50.02	359.33 ± 18.87	332.43 ± 18.87
Liver Wt (g)	123.55 ± 2.52	139.33 ± 13.71	132.47 ± 7.86
Lung Wt (g)	14.55 ± 1.12	16.12 ± 1.47	17.86 ± 1.52

^{*}Treatment Groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF).

[§]Data expressed as mean ± SEM; no significant differences (P < 0.05) detected between groups.

On day 0, the plasma triglyceride, total-, VLDL-, low density lipoprotein (LDL)- and HDL-cholesterol concentration (n=19) was 54.93 ± 7.75 , 77.04 ± 15.51 , 23.06 ± 5.21 , 32.22 ± 10.67 and 21.73 ± 2.3 mg/dl, respectively. On day 45, the plasma triglyceride, total-, VLDL-, LDL-, and HDL-cholesterol concentration was 437.90 ± 49.17 , 2281.7 ± 133.96 , 1316.36 ± 56.10 , 731.34 ± 63.44 and 238.62 ± 42.80 mg/dl, respectively.

Plasma triglyceride concentration was modified by dietary assignment (Table 5). Compared to day 0 values, all three groups had greater amounts of plasma triglycerides after consuming the cholesterol-rich diet for 45 days. During the period of 46 to 90 days, hypertriglyceridemia was maintained in the CC and ECF animals; but by day 90, the triglyceride concentration of the CF group had returned to near the day 0 concentration. Statistical comparison of the three groups on day 90 showed that the ECF group had a significantly greater plasma triglyceride concentration ($P < 0.05$) than did the CF group and that the CC group also had significantly more ($P < 0.05$) plasma triglycerides than did the CF group.

On day 90, the CC group had significantly ($P < 0.05$) more total cholesterol than did any other group and the CF group had significantly less ($P < 0.05$) total cholesterol than did any other group (Table 6). Diet effects on the VLDL-cholesterol concentration (Table 7) and the LDL-cholesterol

Table 5. Plasma triglyceride concentrations on day 0, 45 and 90.

Day	Triglycerides (mg/dl; mean \pm SEM)		
	Treatment Group*		
	CC [#]	CF	ECF
0	46.81 \pm 13.12	66.45 \pm 18.43	50.87 \pm 9.15
45	367.54 \pm 67.25	402.52 \pm 76.52	528.56 \pm 100.08
90 [§]	238.97 \pm 64.28 ^{AB}	79.97 \pm 11.77 ^A	433.32 \pm 122.07 ^B

*Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF, n=6) and Ethanol-consuming and cholesterol-free (ECF, n=7).

[#]For the CC group, n=5 at day 0.

[§]Statistically significant ($P < 0.05$) differences between groups, means with same letter are not significantly different.

Table 6. Total plasma cholesterol on day 0, 45 and 90, expressed as the sum of the VLDL-, LDL- and HDL-cholesterol concentrations.

Day	Total cholesterol (mg/dl; mean \pm SEM)		
	Treatment Group*		
	CC	CF	ECF
0	125.14 \pm 32.90 ^f	43.00 \pm 4.23	71.77 \pm 27.90
45	2307.88 \pm 295.49	2353.56 \pm 232.89	2288.56 \pm 231.98
90 ^g	1532.82 \pm 107.01 ^A	440.50 \pm 44.08 ^B	957.79 \pm 157.21 ^C

*Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF, n=6) and Ethanol-consuming and cholesterol-free (ECF, n=7).

^fFor the CC group, n=5 at day 0.

^gStatistically significant ($P < 0.05$) differences between groups, means with same letter are not significantly different.

Table 7. Plasma VLDL-cholesterol concentration on day 0, 45 and 90.

Day	Cholesterol (mg/dl; mean \pm SEM)		
	Treatment Group [*]		
	CC	CF	ECF
0	31.90 \pm 15.84 [#]	18.68 \pm 5.18	20.49 \pm 6.78
45	1301.43 \pm 79.36	1339.18 \pm 108.60	1308.80 \pm 87.15
90 ^s	1018.40 \pm 79.29 [^]	217.40 \pm 24.05 ^{^B}	597.50 \pm 115.36 ^{^C}

^{*}Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF, n=6) and Ethanol-consuming and cholesterol-free (ECF, n=7).

[#]For the CC group, n=5 at day 0.

^sStatistically significant (P < 0.05) differences between groups, means with same letter are not significantly different.

concentration (Table 8) were similar to that observed for the total cholesterol concentration at days 0, 45 and 90.

The HDL-cholesterol concentration (Table 9) was increased in all groups after 45 days of cholesterol feeding. However, the high HDL values observed at day 45 may have reflected cholesterol contamination from the LDL fraction because the density contrast between lipoprotein fractions on day 45 were poorly delineated after ultracentrifugation, making separation difficult. This situation was not encountered when lipoproteins from the day 90 plasma samples were separated. On day 90, the HDL-cholesterol concentration of the CC and ECF animals was greater than that of the CF animals, but no significant differences ($P < 0.05$) were detected between the groups.

Atherosclerotic lesion severity was evaluated on day 90 by the use of Sudan-IV staining and histological evaluation. Sudan-IV stain was used to delineate the percentage of surface area covered by atherosclerotic lesions on proximal segments, distal segments and the total area of the two segments. No significant differences ($P < 0.05$) were detected in the amount of Sudan-IV stained surface areas between the three groups (Table 10). When the aortic cross-sections were evaluated, no significant differences ($P < 0.05$) between the groups were detected in regards to the arterial outer perimeter, the area of intact smooth muscle

Table 8. Plasma LDL-cholesterol concentration on day 0, 45 and 90.

Day	mg total /dl (mean \pm SEM)		
	Treatment Group*		
	CC	CF	ECF
0	6.34 \pm 21.80 ^f	8.68 \pm 1.50	30.17 \pm 20.15
45	754.02 \pm 143.58	806.60 \pm 143.58	647.39 \pm 97.64
90 ^s	483.63 \pm 79.29 ^A	205.18 \pm 39.51 ^B	312.77 \pm 49.93 ^B

*Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF, n=6) and Ethanol-consuming and cholesterol-free (ECF, n=7).

^fFor the CC group, n=5 at day 0.

^sStatistically significant ($P < 0.05$) differences between groups, means with same letter are not significantly different.

Table 9. Plasma HDL-cholesterol concentration on day 0, 45 and 90.

Day	Cholesterol (mg/dl; mean \pm SEM)		
	Treatment Group*		
	CC	CF	ECF
0	29.90 \pm 6.31 [#]	15.65 \pm 1.13	21.11 \pm 2.42
45	252.43 \pm 99.48	209.37 \pm 40.22	253.92 \pm 83.39
90 [§]	46.46 \pm 5.16	17.92 \pm 3.54	47.51 \pm 15.95

*Treatment Groups: Cholesterol consuming (CC; n=6), Cholesterol-free (CF, n=6) and Ethanol-consuming and cholesterol-free (ECF, n=7).

[#]For the CC group, n=5 at day 0.

[§]Statistically significant ($P < 0.05$) differences were not detected between groups at day 90.

Table 10. Atherosclerotic severity on day 90 expressed as the percentage of total aortic surface area staining positive with Sudan-IV stain.

Segment Location	Aortic surface area stained in each treatment group [*] (%; mean \pm SEM)		
	CC	CF	ECF
Proximal	63.03 \pm 8.44	85.07 \pm 7.19	64.10 \pm 14.38
Distal	63.72 \pm 7.77	66.15 \pm 12.23	42.09 \pm 11.71
Total [Ⓢ]	63.10 \pm 8.26	81.84 \pm 7.68	60.88 \pm 13.57

^{*}Treatment Groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF); no statistically significant ($P < 0.05$) differences between the groups were detected.

[Ⓢ]Total was derived from the sum of the proximal and distal areas staining positive divided by the sum of the two total areas.

and area of fatty streak involvement (Table 11). In addition, no significant differences ($P < 0.05$) between the groups were detected in regards to the cholesterol content of the aortic arch segments (Table 12).

Data on hepatic cholesterol content (Table 12) expressed as mg cholesterol per gram of liver (wet weight), demonstrate that the CC group had significantly ($P < 0.05$) more cholesterol than did the ECF group. Furthermore, the total hepatic cholesterol content of the CC rabbits was also significantly ($P < 0.05$) greater than that of the CF and ECF groups.

Discussion

Continued cholesterol deposition and atherosclerotic development during administration of a cholesterol-lowering diet to rabbits has been observed by several investigators (Daugherty et al., 1986; Kappogoda et al., 1989; Jayakody et al., 1990; Albrecht and Schuler, 1965; Freidman and Byers, 1963). Due to the high CETP activity in rabbit plasma, cholesterol is also readily transferred from the HDL fraction to the VLDL and LDL fractions (Ha and Barter, 1982). In addition, cholesterol continues to be recycled back into VLDL and LDL particles as a result of continued hepatic

Table 11. Evaluation of aortic cross-sections collected on day 90 for total perimeter, vascular smooth muscle area and the area infiltrated by fatty streaks.

Measurement [#]	Treatment Group [*]		
	CC	CF	ECF
Outer Per (mm)	14.46 ± 0.72	15.14 ± 0.71	15.17 ± 0.63
SMCA (mm ²)	4.42 ± 0.65	4.25 ± 0.23	4.01 ± 0.23
FSA (mm ²)	3.35 ± 0.41	3.30 ± 0.97	2.93 ± 0.54

*Treatment Groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF); no statistically significant ($P < 0.05$) differences between the groups were detected.

[#]Measurements expressed as mean ± SEM; Abbreviations: Outer Per, outer perimeter of the artery (mm); SMCA, area (mm²) of smooth muscle in the artery; FSA, area (mm²) of artery containing lipid filled cells.

Table 12. Cholesterol content of the aortic arch and liver on day 90.

Treatment Group*	Cholesterol Content (mean \pm SEM)			
	Segment from Aortic Arch		Liver	
	mg/g wet weight [§]	mg/segment [§]	mg/g wet weight [§]	g/liver [§]
CC	37.29 \pm 2.00 ^A	14.81 \pm 2.31 ^A	32.14 \pm 2.40 ^B	3.98 \pm 0.33 ^B
CF	29.65 \pm 1.00 ^A	10.70 \pm 0.77 ^A	17.41 \pm 0.99 ^{AB}	2.36 \pm 0.30 ^A
ECF	36.90 \pm 4.02 ^A	12.78 \pm 2.10 ^A	15.10 \pm 2.65 ^A	1.97 \pm 0.31 ^A

*Treatment groups: Cholesterol consuming (CC), Cholesterol-free (CF) and Ethanol-consuming and cholesterol-free (ECF).

[§]Statistically significant differences ($P < 0.05$), means with the same letter are not significantly different.

lipoprotein synthesis (Daugherty et al., 1986). In this investigation, cholesterol apparently remained in the circulation after the cholesterol supplement was stopped and presumably continued to be deposited in the VLDL and LDL lipoprotein fractions as well as in atherosclerotic lesions. A similar theory to explain continued atherosclerotic development after cessation of cholesterol feeding has been suggested by Friedman and Byers (1963).

Consumption of the CF diet between days 45 and 90 promoted a marked reduction of plasma triglyceride, VLDL-, LDL- and HDL- cholesterol concentrations from the day 45 values toward the day 0 values. However, the aortic arch cholesterol content, Sudan-IV stained area, and fatty streak cross-sectional areas of the CC and CF groups were not different. Based on these observations, there is no evidence to suggest that the CF diet alone modified the development of atherosclerotic lesion development or promoted atherosclerotic regression.

Comparison of the baseline plasma cholesterol concentration on day 0 (Table 6) reveals that a wide range of cholesterol concentrations were present in the total population. By comparison, base line total plasma cholesterol concentrations of between 84 ± 10 (mean \pm SEM) and 43.6 ± 3.3 (mean \pm SEM) were observed for two groups of similar sized male New Zealand White rabbits used in a study

by Jayakody et al. (1989). In contrast, the base line plasma cholesterol concentration of our CC group is clearly very high, and the values for the CF and ECF groups are similar to those obtained by Jayakody et al. (1989).

It is possible that the high plasma cholesterol concentration in the CC group on day 0 could have predisposed this group to respond to their dietary cholesterol supplement in a manner different from that of the CF and ECF groups. However, because all three groups showed similar dramatic increases in total cholesterol to around 2300 mg/dl at day 45, the initially high plasma cholesterol observed in the CC group was probably not an important factor during days 45 to 90. Furthermore, baseline cholesterol concentrations have been suggested to be unimportant for determining whether New Zealand white rabbits will be resistant to hypercholesterolemia (Loose-Mitchell et al., 1991). In future experiments, this problem could be resolved by assigning the rabbits into the three groups with consideration of their baseline plasma cholesterol concentrations, such that rabbits with high, medium and low plasma cholesterol concentrations were equally distributed into the three groups.

Atherosclerotic regression resulting from the removal of cholesterol from the diet of rabbits is correlated with the degree to which normal plasma cholesterol concentrations can

be re-achieved, the duration of cholesterol feeding and the severity of the atherosclerotic lesions established prior to administration of the regression diet (Adams, Morgan and Bayliss, 1973). In the present study, the diet-induced hypercholesterolemia was not entirely resolved in any group at the end of 90 days, and net cholesterol deposition and atherosclerotic development probably continued between day 45 and 90 in all three groups. Continued deposition is probable because atherosclerosis in rabbits can be promoted by plasma cholesterol levels as low as 200 to 300 mg/dl (Adams et al., 1982).

The CC group had a greater amount of hepatic cholesterol per gram of tissue than did either the CF and ECF groups. This observation suggests that rabbits in the CC group continued to absorb dietary cholesterol, but that the rate of cholesterol deposition into the arterial wall and the rate of atherosclerotic progression was similar for the three groups; an effect observed in previous investigations (Albrecht and Schuler, 1965; Bortz, 1968).

Direct intravenous administration of HDL particles has been shown to induce atherosclerotic regression in spite of persistent hypercholesterolemia (Badimon et al., 1990). In their study, regression was presumably promoted by increased cholesterol efflux from atherosclerotic lesions into the added HDL particles, reducing the cholesterol content and the

severity of atherosclerotic lesions. Ethanol consumption by humans and rabbits is associated with increased HDL-cholesterol concentrations and the inhibition of atherosclerotic development (Gaziano et al., 1993; Castelli et al., 1977; Klurfeld and Kritchevsky, 1981). The effect of ethanol-stimulated increases in HDL-cholesterol during administration of a cholesterol-free diet have not been evaluated.

Administration of an ECF diet in this study was associated with a day 90 HDL-cholesterol concentration that was not significantly greater than that in rabbits receiving the CF and CC diets. In addition, atherosclerotic severity, defined in terms of aortic surface area, aortic fatty streak cross-sectional area and aortic cholesterol content, was not significantly different among the CC, CF and ECF groups. Thus, the inclusion of dietary ethanol as supplement to a CF diet did not affect the atherosclerotic disease process.

In future studies, it would be useful to evaluate the effects of ethanol on atherosclerotic regression when plasma cholesterol concentrations have been returned to normal levels for longer periods of time. It is under these conditions that one would expect dietary ethanol to have the greatest impact for stimulating net cholesterol efflux from atherosclerotic deposits as well as minimizing the amount of VLDL- and LDL-cholesterol available for deposition into

atherosclerotic lesions. However, it is also important to recognize that long treatment times may not be available to human patients suffering from coronary artery disease because of angina or disabilities related to their disease.

This study used a rabbit model to evaluate the effects of dietary ethanol on human cholesterol-lowering diets. The data indicate that benefits are not associated with the inclusion of dietary ethanol in a cholesterol lowering diet. Previous studies had shown that dietary ethanol is associated with increased HDL-cholesterol and the suppression of atherosclerotic development in both humans and rabbits. In this study, dietary ethanol was associated with the maintenance of several risk factors for continued atherosclerotic development, including hypertriglyceridemia and impaired resolution of plasma hypercholesterolemia. The data suggest that consumption of dietary ethanol, during the administration of a cholesterol-free diet would not be of benefit to humans undergoing cholesterol-lowering dietary therapy.

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CHAPTER 4. POTENTIATION OF VASOCONSTRICTIVE PROPERTIES IN
AORTAS FROM ATHEROSCLEROTIC RABBITS FED A CHOLESTEROL-FREE
DIET WITH AND WITHOUT DIETARY ETHANOL

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Abstract

The effect of dietary ethanol on atherosclerotic regression and vasoconstriction in atherosclerotic rabbits was addressed by feeding male New Zealand white rabbits (n=19) a diet enriched with 0.8% cholesterol for 45 days, followed by randomization to one of three diets for the next 45 days: 1) Continued consumption of a cholesterol-rich diet (CC; n=6), 2) Consumption of a cholesterol-free diet (CF; n=6), and 3) Consumption of a cholesterol-free diet supplemented with ethanol in the drinking water (10% v/v; ECF; n=7). On day 90 the rabbits were killed and abdominal aortic rings (4 rings/rabbit) were evaluated for constrictive force

generation. Data are expressed as mean \pm SEM, means with the same letter are not significantly ($P < 0.05$) different. Atherosclerotic deposits were visually observed in 73 of the 76 arterial rings evaluated. Norepinephrine (NE; 10^{-7} M) promoted maximal contractile forces in the CC, CF and ECF groups of 3.69 ± 0.32^A , 5.09 ± 0.39^B and 5.31 ± 0.30^B grams, respectively. Potassium chloride (KCl; 80 mM) promoted maximal contractile forces in the CC, CF and ECF groups of 4.01 ± 0.35^A , 5.04 ± 0.38^{AB} and 5.34 ± 0.47 grams^B, respectively. The contractile forces developed in response to 200 mg/dl ethanol by the CC, CF and ECF groups were 51.4 ± 7.5^A , 71.2 ± 12.6^A and 104.9 ± 31.5^A , respectively. Contractions in the ECF group were more variable in terms of their response to ethanol and while statistical significance from the CC groups was not observed, the ECF diet does appear to promote increased contractile sensitivity in some arterial rings. Serotonin (10^{-7} M) developed contractile forces in the CC, CF and ECF groups of 2.35 ± 0.24^A , 2.13 ± 0.21^A and 2.89 ± 0.59^A grams, respectively. In conclusion: 1) Switching from a cholesterol-rich to an ECF diet promotes increased vasocontractile responses to NE, KCl and possible ethanol, and 2) Switching from a cholesterol-rich to a CF diet promotes increased vasoconstrictive responses to KCl.

Introduction

Increased vascular sensitivity of human arteries to the vasoconstrictive properties of NE and 5-HT is a hallmark of atherosclerosis (Mudge et al., 1976; Golino et al., 1991). Sources of NE include release from nerve endings in the vessel or release directly into the blood from the adrenal glands. A primary source of 5-HT at atherosclerotic lesion sites includes platelet aggregation and degranulation (Golino et al., 1991).

The cause of increased vascular sensitivity during atherosclerosis has not been conclusively determined, but a decrease in NE-mediated and 5-HT-mediated endothelium-dependent relaxation has been implicated (Förstermann et al., 1988; Minor et al., 1990). Increases in the number of 5-HT receptors and increases in the number of α_1 -adrenocetors have also been implicated (Nanda and Henry, 1980). Because the atherosclerotic process also involves changes in the morphology of vascular smooth muscle cells (Ross, 1993), receptor-independent changes in smooth muscle contractility may also be responsible.

Ethanol promotes arterial vasoconstriction (Altura, Altura and Carella, 1983; Savoy-Moore et al., 1989), increased blood pressure (Maheswaran et al., 1991) and has been implicated with producing both vasospasm (Altura, Altura and Carella,

1983) and variant angina (Takizawa et al., 1984). In contrast to these health risks, moderate consumption of dietary ethanol by humans has been associated with a decreased incidence of coronary heart disease and overall mortality (Friedman and Kimball, 1986). Thus, dietary ethanol is associated with both health benefits and potential risks in regards to the coronary vasculature.

Public interest in ethanol consumption has increased because of the association of ethanol consumption with improved vascular health by preventing atherosclerotic development (Klatsky and Armstrong, 1993). At the same time, public interest in lowering plasma-cholesterol concentrations by dietary modification has also increased (Patsch, Patsch and Gotto, 1989). The effect of dietary ethanol on vascular reactivity in atherosclerotic arteries during administration of a cholesterol-lowering diet has not been determined. This investigation protocol required the production of atherosclerosis in rabbits with a cholesterol-rich diet. Development of atherosclerosis was followed by the inclusion of dietary ethanol as part of a plasma-cholesterol lowering diet and the evaluation of differences in vascular reactivity to potassium chloride, ethanol, norepinephrine and serotonin resulting from the dietary change.

Materials and Methods

Animal and Tissue Handling: Male New Zealand White rabbits weighing 1.9 to 3.0 kg were obtained from LSR Inc. Union Grove, Wisconsin. Rabbits were obtained as a set of 18, followed by an additional set of 5, staggered 45 days behind the set of 18. Rabbits were fed from a single manufactured lot of Teklad #0533 (Madison, Wisconsin) rabbit chow (140 grams/day). The chow was made atherogenic by adding a cholesterol-supplement prepared by dissolving cholesterol in butylmethylphenol-free ethyl ether (1 gram/10 ml) and evenly dispersing the solution onto the rabbit chow. The ether was evaporated from the chow under a fume hood, leaving chow enriched with 0.8% cholesterol.

Atherogenic chow was fed to all rabbits from days 0 to 45. On day 45, the rabbits were randomized to one of three treatments for the next 45 days: 1) Rabbits received the same atherogenic rabbit chow (Cholesterol-Consuming; CC; n=6), 2) Rabbits received chow with no cholesterol supplement (Cholesterol-free; CF; n=6), and 3) Rabbits received 10% dietary ethanol in their drinking fluid in addition to the cholesterol-free diet (ECF; n=7).

During the course of the experiment all rabbits had free access to the following drinking solutions. Water was administered between days 5 to 45 to all rabbits. Antibiotic

treatment of pneumonia was repeated for all rabbits at three different time (days 0 to 4, days 50 to 58 and days 71 to 80) and consisted of tetracycline (2 mg/ml) dissolved in Kool-Aide (0.16% Kool-Aide; 5% sugar) for improved palatability. The Kool-Aide solution was also administered to all rabbits between days 45 and 90; this solution was supplemented with ethanol (10% v/v) for rabbits assigned to the ECF treatment group. Three rabbits died of pneumonia during the course of the experiment, and one rabbit was euthanized as a result of a handling injury; these rabbits were excluded from this experiment.

For the determination of the in vitro vascular responsiveness on day 90, rabbits were anesthetized with sodium pentobarbital (40 mg/kg) administered into the ear vein prior to decapitation and removal of the aorta. Immediately upon removal of the aorta, a segment of abdominal aorta starting at the renal artery bifurcations and extending 2.5 cm distally was cut free and placed in a modified-Krebs solution (115.21 mM NaCl, 4.70 mM KCl, 1.80 mM CaCl₂, 1.16 mM MgSO₄, 1.18 mM KH₂PO₄, 22.14 mM NaHCO₃, 7.88 mM glucose and 0.027 mM EDTA) and oxygenated with a gas mixture of 95% oxygen and 5% CO₂ at 20°C. Adventitial tissues were then removed from the segment. Starting 2 mm distal to the renal arterial bifurcation and no more than 1 mm from any vertebral arterial bifurcation, four aortic cross-sectional rings (3 mm

long) were sequentially cut and numbered (#1 (most anterior), #2, #3 and #4 (most posterior)). At all times the tissues were handled with extreme care to ensure that the endothelial layer of these segments was not damaged.

Evaluation of Aortic Rings: Evaluations of the four rings were made in vitro by using Grass FT03 force-displacement transducers (Grass Inc., Quincy, Massachusetts) and a Beckman R611 recorder (Beckman Instruments Inc., Schiller Park, Illinois) to monitor changes in the grams of tension maintained by the arterial rings. For the evaluation of vascular responses to KCl, NE and 5-HT the Beckman recorder was calibrated to measure tension in the range of 1-9 grams. For the evaluation of contractile responses to ethanol, the Beckman recorder was calibrated to measure tension in the range of 1 to 3 grams.

Tissue baths were perfused with modified Krebs solution, oxygenated and maintained at 37°C. Baths were thoroughly flushed with modified Krebs solution at least every 30 minutes. Arterial rings were mounted in triangular wire tissue holders, placed into the baths, attached to the force-transducers and allowed to rest for 10 minutes. Then, 6 grams of tension was gradually applied over a 20-second period. Ring tension was adjusted until a stable 2 gram resting tension could be achieved for 60 minutes or more.

Potassium chloride was then added at a final bath concentration of 80 mM and the gram scale set at 1-9 grams to develop a maximal tissue contractile force (Kishi and Numano, 1984). The maximal contraction was defined by measuring the gram difference between the tension maintained just before KCl was applied and the peak developed tension. After the contractile maximum had been reached, the bath was flushed thoroughly at least three times.

The dose of ethanol (200 mg/dl) was chosen to represent a level clinically observed in the human circulation and at a level that would generate contractile forces that were large enough to be confidently measured. By comparison in humans the lethal plasma concentration for ethanol is in the range of 350 to 900 mg/dl, with a plasma level of 200 mg/dl resulting in intoxication (Tierney, McPhee and Papadakis, 1994). Preliminary trials with non-atherosclerotic rabbits showed that a dose of 200 mg/dl produced consistently observable contractile forces at a plasma ethanol level attainable by humans. As a result, the 200 mg/dl ethanol dose was chosen for evaluation of arterial contraction in this study.

A period of at least 45 minutes was allowed to pass after KCl had been flushed. This period of time was found to be necessary for re-establishment of the 2 gram resting tension prior to administration of ethanol at a bath concentration of

200 mg/dl. Because contractile responses were occasionally absent and because the rate of contractile generation was occasionally slow, the bath was maintained with ethanol for a standard 20 minutes in all cases instead of flushing immediately after the contractile maximum had been reached. Tissue responses were measured on a 1 to 3 gram scale, and the contractile response was measured as before.

For purposes of comparison with other studies, the effects of NE and 5-HT on arterial vasoconstriction were studied at a concentration near their ED_{50} values. In studies of atherosclerotic rabbit aortas (Henry and Yokoyama, 1980; Kishi and Numano, 1984; Kappagoda, Thomson and Senaratne, 1990; Du and Woodman, 1992), the ED_{50} of NE in rabbit aortas has been determined to be in the range of 10^{-7} to 10^{-6} M. The ED_{50} for 5-HT for rabbit aortas has also been suggested to lie in the range of 10^{-7} to 10^{-6} M (Henry and Yokoyama, 1980; Kishi and Numano, 1984). In this study, an agonist concentration of 10^{-7} M was chosen to represent the ED_{50} for NE and 5-HT.

A final 45-minute period followed after the ethanol was flushed, during which a 2 gram tension was re-established for the rings. An ED_{50} dose of NE was administered to the baths containing segments #1 and #3, and an ED_{50} dose of 5-HT was administered to the baths containing segments #2 and #4. After the maximal response was reached, the tissues were removed and stored at 4°C in a separate container of Krebs

solution with 95% O₂-5%CO₂ aeration. Within 12 hours of removal from the bath, the weight of each ring was determined and the inner arterial lumen was examined for the presence of raised white atherosclerotic lesions.

Chemical Preparation: Potassium chloride at a concentration of 4.00 M was used for dilution throughout the study. Ethanol at a concentration of 20% (v/v) was used for dilution throughout the study. Norepinephrine in the bitartrate salt was prepared in deionized water containing 0.1% ascorbate as a preservative and frozen at -20°C, then thawed and diluted to 10⁻⁵ M in deionized water and stored for 1-4 hours at 4°C until final dilution in the bath at 10⁻⁷ M. Serotonin (10⁻⁵ M) was prepared daily by dissolving the 5-HT in deionized water and stored at 4°C prior to final dilution in the bath at 1 X 10⁻⁷ M.

Statistical Analysis: Data are presented as the mean ± the standard error of the mean (SEM). Analysis of variance was performed by using SAS to detect the presence of group differences (P < F 0.05). When group differences were detected by the analysis of variance, Duncan's Multiple Comparison test was used to test for significant differences (P<0.05) between individual group means.

Results

On day 90, the rabbits in the CF, ECF and CC groups weighed 3.74 ± 0.14 , 3.82 ± 0.11 and 3.52 ± 0.14 kg, respectively, with no statistically significant differences observed. Arterial rings of the CF, ECF and CC groups weighed 9.83 ± 0.42 , 10.21 ± 0.44 and 9.78 ± 0.28 milligrams, respectively, with no significant differences observed. After being weighed, the inner lumen of the arterial rings were examined and raised white atherosclerotic lesions were observed in 73 of the 76 total arterial rings.

The contractile responses to 80 mM KCl (Figure 1) revealed that rings from rabbits in the ECF group generated the greatest contractile force (5.34 ± 0.47 g). This force was greater than that developed by the CF group (5.04 ± 0.38 g) and significantly ($P < 0.05$) greater than the force (4.01 ± 0.35 g) developed by the CC group.

Arterial rings were then treated with ethanol (200 mg/dl) and the contractile responses evaluated. The sample recording from an ECF rabbit is presented in Figure 2a shows a slowly developed and maintained contractile curve. The CC, CF and ECF groups generated maximal contractions of 51.6 ± 7.5 , 71.2 ± 12.6 and 104.9 ± 31.5 milligrams, respectively (Figure 2b). The F value for intergroup differences resulting from administration of 200 mg/dl ethanol was 2.12

Figure 1. Maximal contractile force generated by arterial rings in response to 80 mM potassium chloride. Abbreviations: CC, cholesterol consuming; CF, cholesterol-free, and ECF, Ethanol-consuming and cholesterol-free. Data are represented as mean \pm SEM. *Significantly different from CC group ($P < 0.05$).

Grams Tension Developed

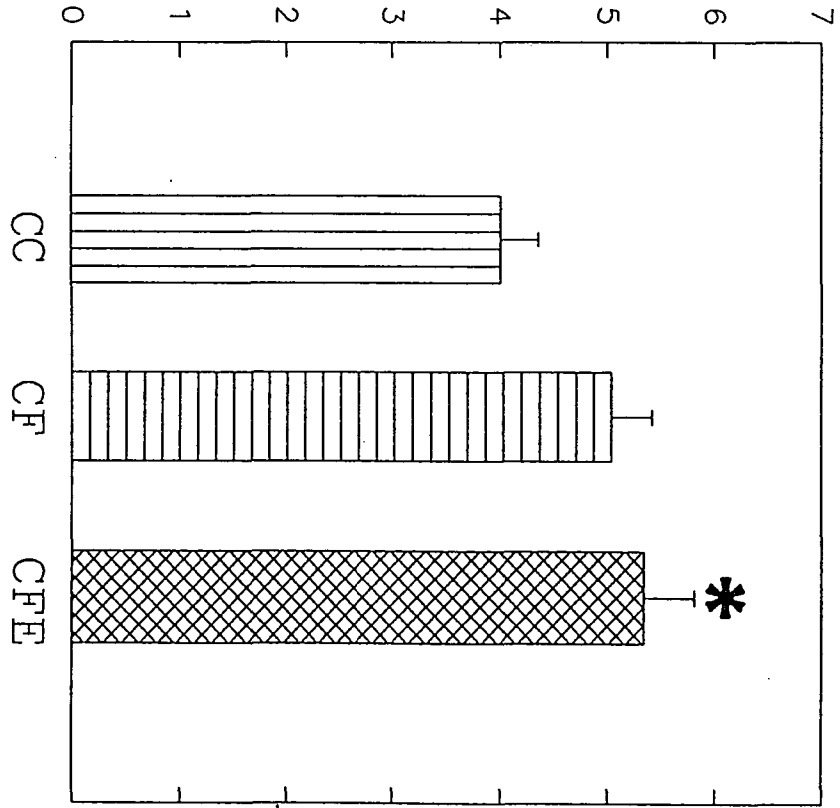
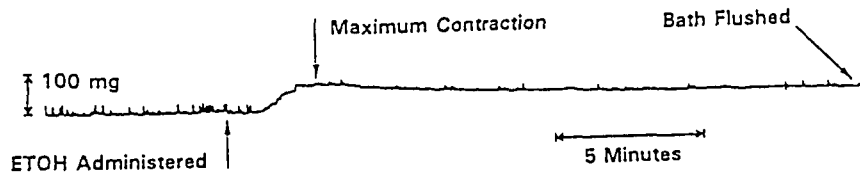
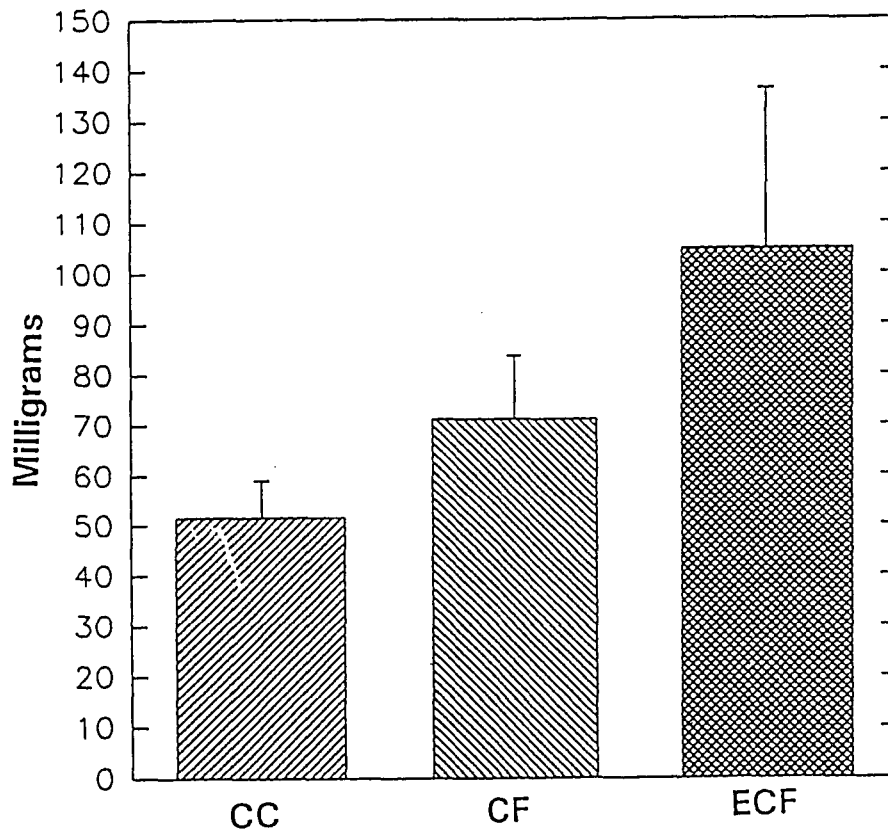


Figure 2. Maximal contractile force generation by arterial rings in response to 200 mg/dl ethanol. a) Sample recording of the contractile response by a ring from an ECF rabbit. b) Comparison of the maximal contractile force generated by all groups. Abbreviations: CC, Cholesterol consuming; CF, Cholesterol-free, and ECF, Ethanol-consuming and cholesterol-free. Data represented as mean \pm SEM; *Significantly different from CC group ($P < 0.05$). Data are represented as mean \pm SEM; no statistically significant ($P < 0.05$) differences were detected between groups.

a)



b)



($P > F = 0.15$), indicating only a trend toward significant differences between the groups.

Although statistically significant intergroup differences were not detected, arteries from the ECF rabbits had the greatest mean contraction in response to 200 mg/dl ethanol and the largest SEM. In contrast, the arterial rings from the CC rabbits had the smallest mean and a relatively narrow SEM. The range of the maximal contractile force generated by the individual rings within the CC group were 0 to 110 milligrams, within the CF group the range was 0 to 180 milligrams and within the ECF group the range was 0 to 310 milligrams. The range of the mean contractile force generated by the four rings from each rabbit in the three groups were 35 to 86, 25 to 116 and 11 to 205 milligrams for the CC, CF and ECF groups, respectively.

Norepinephrine (10^{-7} M) applied to ring segments #1 and #3, generated maximal contractile forces of 3.69 ± 0.32 , 5.09 ± 0.39 and 5.31 ± 0.30 grams for the CC, CF and ECF groups, respectively (Figure 3). The responses of the CF and ECF groups were both significantly greater ($P < 0.05$) than that of the CC group.

When 5-HT (10^{-7} M) was applied to arterial ring segment #2 and #4 (Figure 4), maximal contractile forces of 2.35 ± 0.24 , 2.13 ± 0.21 and 2.89 ± 0.59 grams were generated by the CC, CF and ECF groups, respectively. No statistically

Figure 3. Maximal contractile force generated by arterial rings in response to 10^{-7} M norepinephrine. Abbreviations: CC, cholesterol consuming; CF, cholesterol-free, and ECF, Ethanol-consuming and cholesterol-free. Data are represented as mean \pm SEM; *Significantly different from CC group ($P < 0.05$).

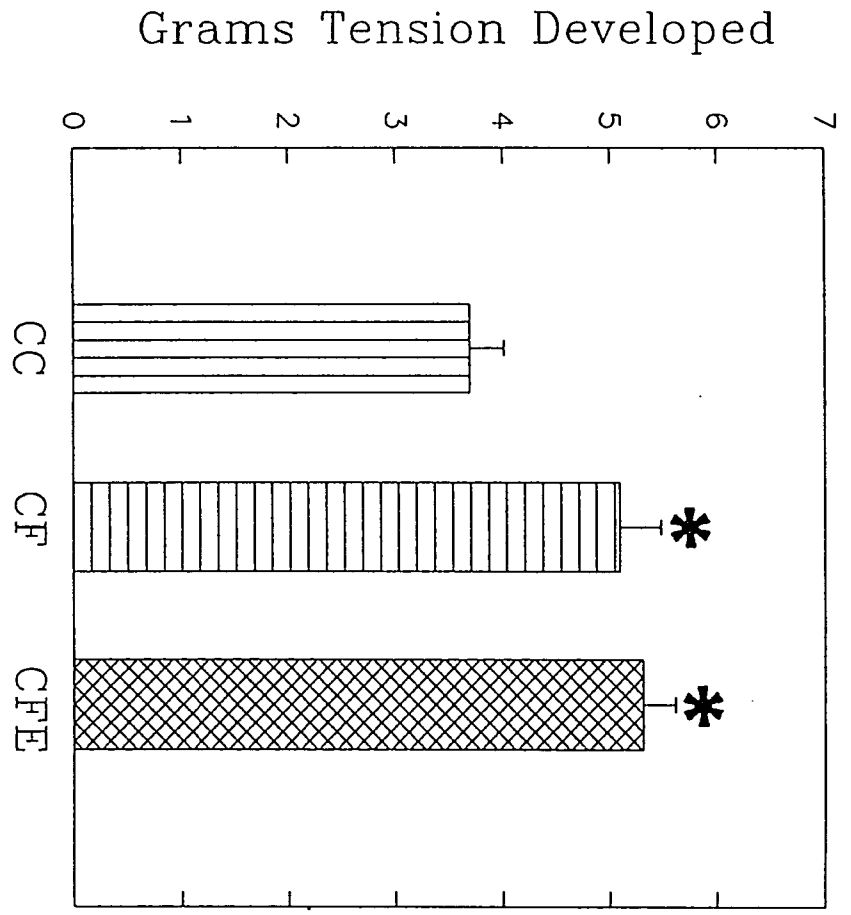
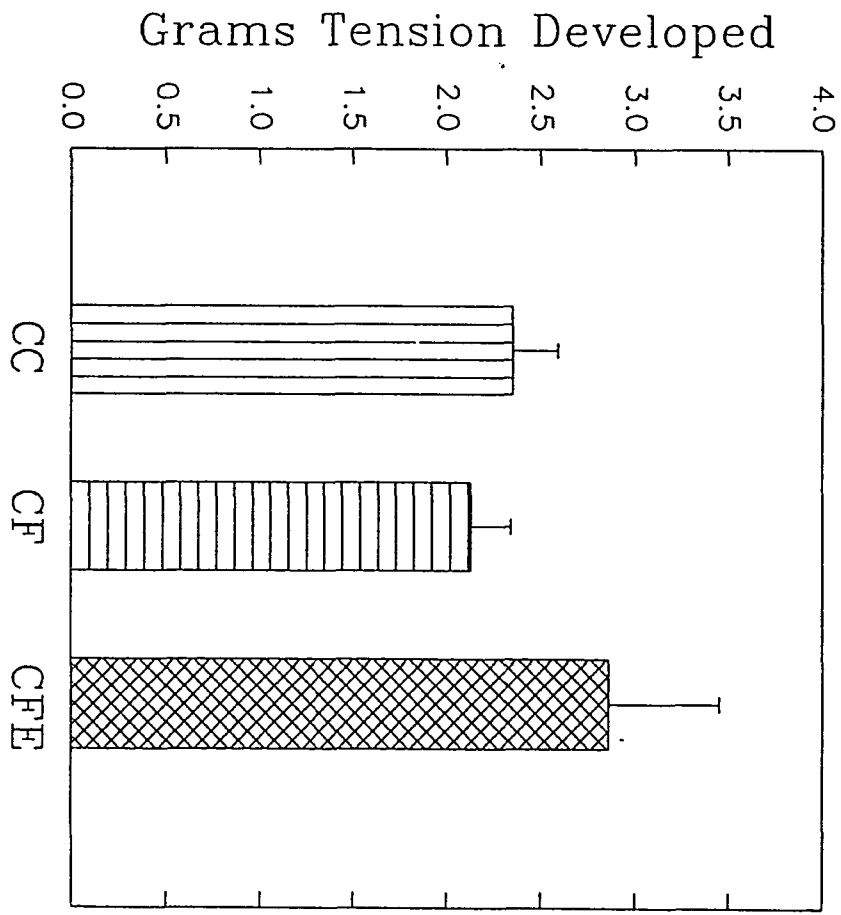


Figure 4. Maximum contractile force generated by arterial rings in response to 10^{-7} M serotonin. Abbreviations: CC, cholesterol consuming; CF, cholesterol-free, and ECF, Ethanol-consuming and cholesterol-free. Data are represented as mean \pm SEM; no statistically significant ($P < 0.05$) differences were detected between groups.



significant differences in the forces generated by the groups were observed.

Discussion

This investigation of atherosclerotic rabbits demonstrates that an ECF diet promotes a significant increase in vasoconstrictive force generation in response to KCl and NE when compared to rabbits consuming a CC diet. Furthermore, a trend toward ethanol-induced contractile potentiation was also observed in regards to the ECF diet. However, dietary ethanol did not potentiate increases in the contractile force generated in response to 5-HT. When the CF group was compared to the CC group, only the NE response was significantly different. Because no differences between the CC, CF and ECF groups were noted in regards to rabbit body weight or arterial ring weight, the increased contractile forces associated with ethanol consumption were apparently not associated with differences in either the weight of the rabbits or the weight of the arterial rings.

Aortic strips from atherosclerotic rabbits treated with KCl have been observed to have a greater maximal contractile force when compared to non-cholesterol-fed controls (Merkel et al., 1990; Kishi and Numano, 1984). In addition, a

reduction in the ED_{50} of KCl for atherosclerotic rabbit aortas has also been demonstrated (Ibengwa and Suzuki, 1986). These studies of KCl-mediated vasoconstriction suggest that receptor-independent changes which modify the vasoconstrictive properties of arteries are associated with atherosclerotic development.

The present investigation carries the trend a step further by suggesting that switching atherosclerotic rabbits from a cholesterol-rich to a cholesterol-free diet promotes increased contractile force generation, when compared to rabbits maintained on a cholesterol-supplemented diet. The fact that KCl induced contractions in the ECF group were significantly greater indicates that at least part of the increased contractile ability is due to non-receptor mediated contractile effects. This surprising result suggests that, in terms of sensitivity to vasospasm, animal health might be deleteriously affected by the switch to a cholesterol-free diet, as occurs when humans consume a lipid lowering diet to treat hypercholesterolemia.

Ethanol-induced contractions have been observed in arterial strip preparations from the canine coronary artery and arterial rings from the human umbilical artery (Altura, Altura and Carella, 1983; Savoy-Moore et al., 1989). It has been demonstrated that clinical levels of circulating ethanol inhibit acetylcholine stimulated release of endothelium-

derived relaxing factor (Hatake, Wakabayashi and Hishida, 1993). Acute ethanol administration has also been shown to inhibit the basal production of endothelium-derived relaxing factor (Persson and Gustafsson, 1992). The administration of an endothelium-dependent relaxation inhibitor (nitro-L-arginine) has been shown to promote small contractile responses that are similar to those generated by ethanol in this investigation (Minor et al., 1990).

Based on the similarity of the contractile effects produced by inhibition of EDRF and the contractile effects created by ethanol administration in this investigation, one could speculate that the contractions observed in this investigation might be caused by ethanol-dependent inhibition of basal endothelium-dependent relaxation, thereby sensitizing the artery to underlying constrictive tendencies.

Even though the ethanol-induced contractions observed in this investigation were small relative to those generated by KCl, NE or 5-HT, it must be remembered that blood flow through a vessel is proportional to the fourth power of the radius. As a result, very small changes in the radius of a vessel can have very large effects on blood flow. In addition, because the lumen of an atherosclerotic vessel may already be considerably filled in by the atherosclerotic lesion mass, a relatively tiny additional decrease in lumen diameter could promote a deficiency in oxygen supply,

resulting in angina or heart attack. The potential of small changes in diameter to affect blood flow is probably important in understanding why ethanol consumption by some human patients is a stimulant for variant angina (Takizawa et al., 1984).

For the ethanol-mediated contractions observed in this investigation, only a trend toward differences between groups could be demonstrated. The arteries of the CC groups showed a tight cluster of relatively lower contractile responses (51.6 ± 7.5). In contrast, ECF treated rabbits had a mean and SEM (104.9 ± 31.5) approximately twice that observed for the CC group. These observations support a trend toward differences between the CC and EFC groups and suggest that ethanol consumption may potentiate sensitivity to ethanol-induced contraction.

Previous studies have demonstrated that contractile properties mediated by 5-HT are potentiated by atherosclerosis (Kishi and Numano, 1984; Henry and Yokoyama, 1980; Kalkman, Neumann and Branner, 1989) and an increase in the number of 5-HT receptors has also been observed in atherosclerotic rabbits (Nanda and Henry, 1980). Constrictive hyper-reactivity in atherosclerotic rabbits has been associated with the 5-HT₂ receptor (Kalkman, Neuman and Braunel, 1989). Because atherosclerotic lesions were observed in the arterial lumens of 73 or the 76 arterial

segments used in this investigation, the arteries of the CC, CF and ECF groups were presumed to be 5-HT hyper-reactive at the beginning of the second 45 day period. The present investigation suggests that neither the CF nor the ECF diet have an effect on 5-HT mediated contraction.

In humans, hyper-reactivity to adrenergic stimulation has been clearly linked with atherosclerotic development (Chester et al., 1980; Mudge et al., 1976). It has been demonstrated by in vivo experiments with rabbits and dogs, that high levels of plasma low-density lipoprotein cholesterol potentiate α_1 -adrenergic vasoconstriction (Bloom et al., 1975; Hof and Hof, 1988). An increase in the number of α_1 -adrenergic receptors has also been observed in atherosclerotic rabbit aortas (Nanda and Henry, 1980). Furthermore, Du and Woodman (1992) observed a transient increase in pressor responses to NE and phenylephrine after a high cholesterol diet had been administered for 4 weeks. In contrast to the observations of Du and Woodman (1992), several studies of rabbits fed atherosclerotic diets have determined that, when aortic sensitivity is evaluated in vitro, vascular sensitivity to NE and phenylephrine can remain unchanged or become slightly depressed (Ibengwa and Suzuki, 1986; Kishi and Numano, 1980; Verbeuren et al., 1983; Verbeuren et al., 1986).

The contractile properties of the thoracic aorta and the effects of administration of a cholesterol-free diet to atherosclerotic rabbits was evaluated by Kappagoda et al. (1990). They did not observe significant changes in NE induced contractile forces after feeding a 2.5% cholesterol diet to rabbits for 3 weeks followed by administration of a cholesterol-free diet for the next 12 weeks. In a similar study (Kappogoda et al., 1989) fed 2.0% cholesterol diet for 6 weeks, followed by a CF diet for an additional 36 weeks, after which again differences in NE sensitivity did not develop.

The results observed by Kappogoda et al. (1989) and Kappagoda, Thomson and Senaratne (1990) are in contrast to the results observed in our investigation, where significant changes in the vascular responsiveness to NE were linked to consumption of a CF diet. The lower amount of cholesterol administered to the rabbits in our study, 0.8% (w/w) and the relatively short period of administration of a cholesterol-free diet may have been a cause for these differences.

The present investigation suggests that changes in diet, from a cholesterol-rich to a CF status, promote potentiation of NE-induced contractions. Because excessive α_1 -adrenergic stimulation in humans has been implicated in causing essential hypertension and angina pectoris, our results suggest that if a human is switched from a high cholesterol

diet to a low cholesterol-diet, especially one including ethanol, their vasculature would become more sensitive to NE mediated vasoconstriction, but not to 5-HT mediated vasoconstriction.

While several studies have suggested that consumption of dietary ethanol inhibits the development of atherosclerosis, the effect of ethanol on the development or regression of previously existing atherosclerosis has remained undetermined. This investigation evaluated the effect of a CF diet on aortic contractile properties and indicates that when ethanol is administered as part of a CF diet to atherosclerotic rabbits, ethanol induces changes in the artery that potentiate constriction. Clinically, it is an assumption that a CF diet by atherosclerotic patients will promote an improvement of vascular health (Patsch, Patsch and Gotto, 1989). However, based on the increased tendency toward vasospasm, this investigation suggests that circulatory health of humans might be deleteriously affected by changing from a CC diet to a cholesterol-free diet and especially by including dietary ethanol in a CF diet administered for the treatment of atherosclerosis.

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CHAPTER 6. GENERAL CONCLUSIONS

The data in this dissertation suggest that dietary ethanol is not beneficial to human health. It demonstrates how ethanol causes constriction of umbilical arteries and the constriction of ovine umbilical arteries could be important for modeling how dietary ethanol causes fetal alcohol syndrome in human infants. This dissertation demonstrates that constriction of arteries in atherosclerotic rabbits fed a cholesterol-free diet actually increases constrictive force generation in response to norepinephrine. In terms of vasoconstriction, the data suggest that ethanol consumption as part of a cholesterol-free diet is associated with increased contractile force generation in response to potassium chloride and ethanol, as well as norepinephrine. Finally, the research demonstrates that in rabbits atherosclerotic regression is not promoted by the short-term removal of cholesterol from the diet and that inclusion of ethanol in the diet does not induce atherosclerotic regression. Furthermore, because the ethanol diet was also associated with the maintenance of high plasma triglyceride and cholesterol concentrations, both of which are known risk

factors for atherosclerotic development, ethanol should be contraindicated during administration of cholesterol-free diets.

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**APPENDIX 1. STATISTICAL ANALYSIS OF TIME-DEPENDENT
CHANGES IN PLASMA LIPID CONCENTRATIONS.**

Introduction

The investigation of the effects of a cholesterol-lowering diet administered with or without dietary ethanol, described in Chapter 3, included both time and treatment dependent changes in the plasma lipid profile. Treatment-dependent changes and the their statistical significance have been addressed previously in Chapter 3. However, the complicated nature of the experimental subjects made statistical evaluation of time-dependent changes rather complicated and difficult to interpret. The experiment is complicated by the fact that all animals were part of a single population of rabbits between days 0 and 45, and that only after blood sampling on day 45 were these animals randomized to three different treatment groups.

Personal communications with Dr. D.F. Cox in the (Department of Statistics, Iowa State University) revealed that a perfect statistical test to evaluate the statistical significance of these time-dependent changes is not available. Because of this fact, time-dependent changes in Chapter 3 were described in qualitative terms. Dr. Cox

suggested that one of the most easily interpretable statistical tests for time-dependent changes would be Student's t-test. However, there are some problems associated with using a Student's t-test for this purpose which need to be addressed.

One of the requirements for the proper use of a Student's t-test is that there be two different populations. While different populations were available at day 90, data from day 0 and 45 were collected from a single population. As a result, one of the primary requirements for the proper use of a Student's t-test was broken. In regards to publication and potential confusion associated with the misuse of Student's t-test, the statistical analysis of time-dependent changes in the lipid profile is addressed in this appendix and not in Chapter 3.

Statistical Methods

A Student's t-test was used to evaluate the statistical significance of the time-dependent changes in plasma lipid concentrations. To use the Student's t-test for this purpose, the populations at days 0 and 45 were, from the standpoint of statistics, considered as populations that were separate from the populations receiving the cholesterol

consuming (CC), cholesterol-free chow (CF) and cholesterol-free chow with ethanol supplement (ECF) diets at day 90, when in fact they were not separate. The software used for this test was provided on the B-stat statistical program (Mc Graw Hill Inc., New York). This program utilized the means and standard errors of the mean to determine a T-value and probability of significance.

A detailed example of how the evaluations were carried out is as follows for the plasma triglycerides of the CF group at the end of the experiment (day 90, 80.0 ± 11.8 mg/dl) verses the plasma triglycerides of the entire population of rabbits treated alike at day 0 (54.9 ± 7.7 mg/dl). The t-value was calculated to be -1.661 and the $P < 0.111$.

Results and Discussion

The mean and standard error of the mean for plasma triglycerides, total cholesterol, VLDL-cholesterol, LDL-cholesterol and HDL-cholesterol of the entire population is presented in Table 1, the day 90 values were described in Chapter 4. Statistical analysis of the differences between the day 45 and day 90 values, and between the day 0 and day 90 values are summarized in Table 2.

Table 1. Plasma lipid data for the entire population of animals treated alike on days 0 and 45, expressed as the representative mean \pm SEM.*

mg/dl	Day 0	Day 45
Triglycerides	54.9 \pm 7.75	437.9 \pm 49.17
Total Cholesterol	77.0 \pm 15.51	2281.7 \pm 133.96
VLDL-Cholesterol	23.0 \pm 5.21	1316.4 \pm 56.1
LDL-Cholesterol	32.2 \pm 10.67	731.3 \pm 63.4
HDL-Cholesterol	21.7 \pm 2.31	238.6 \pm 42.8

* Abbreviations: SEM, Standard error of the mean, VLDL, Very low-density lipoprotein; LDL, Low-density lipoprotein; HDL, high density lipoprotein.

Table 2. Results of Student T-tests comparing day 45 population means obtained on day 90 for the CC, CF and ECF groups.

Day 90 Group *	<u>Day 45 vs Day 90</u> P-value	<u>Day 0 vs Day 90</u> P-value
<u>Plasma Triglycerides</u>		
CC	< 0.048	< 0.000
CF	< 0.000	< 0.111
ECF	< 0.967	< 0.000
<u>Total Cholesterol</u>		
CC	< 0.010	< 0.000
CF	< 0.000	< 0.000
ECF	< 0.000	< 0.000
<u>VLDL-Cholesterol</u>		
CC	< 0.009	< 0.000
CF	< 0.000	< 0.000
ECF	< 0.000	< 0.000
<u>LDL-Cholesterol</u>		
CC	< 0.000	< 0.000
CF	< 0.000	< 0.000
ECF	< 0.044	< 0.000
<u>HDL-Cholesterol</u>		
CC	< 0.030	< 0.407
CF	< 0.008	< 0.020
ECF	< 0.012	< 0.000

* Abbreviations: CC, Cholesterol consuming; CF, Cholesterol-free; ECF, Ethanol-consuming and cholesterol-free.

The data shown in Table 2 support the idea that inclusion of ethanol in the lipid lowering diet of hypercholesterolemic rabbits inhibits the resolution of the developed hypercholesterolemia in association with a cholesterol-supplemented diet and that consumption of a cholesterol-free diet without ethanol will be the best for promoting a return of high plasma triglyceride and cholesterol concentrations to normal values. However, when considering these conclusions it is again important to remember that the use of a Student's t-test to make these comparisons requires that one neglect one of the primary rules for the use of a Student's t-test, that of separate populations.

**APPENDIX 2. SHEEP UMBILICAL ARTERIES AND THE VASOCONSTRICTOR
DOSE-RESPONSE RELATIONSHIP FOR ETHANOL: PRELIMINARY DATA.**

Introduction

Dietary ethanol is the cause of fetal alcohol syndrome in human infants; therefore, ethanol consumption can have a profound and permanent effect on the health of the unborn fetus (Clarren and Smith 1978). The pathological mechanism of fetal alcohol syndrome has not been determined, but a reduction in fetal oxygen supply has been implicated. Because ethanol is known to have a vasoconstrictive effect on arterial smooth muscle, ethanol could promote a reduction in fetal blood flow by reducing arterial diameter and increasing blood flow resistance (Mukherjee and Hodgen 1982; Altura et al. 1983; Savoy-Moore et al. 1989). Changes in prostaglandin E have also been associated with the promotion of teratogenic changes associated with FAS (Randall et al. 1991).

Sheep umbilical arteries can be used as a human model (Fiscus and Dyer 1981) because the ovine fetus is similar to the human fetus in respect to its weight, blood flow requirements and umbilical artery dimensions. The effect of ethanol has been previously characterized for constriction of human umbilical arteries (Altura et al. 1983; Mukherjee and

Hodgen 1982; Savoy-Moore et al. 1989; Fiscus and Dyer 1981), but the effects of ethanol in the ovine model have not been determined.

This preliminary investigation sought to generate a dose-response relationship for ethanol promoted ovine umbilical artery vasoconstriction. However, more importantly this short investigation served to familiarize the investigator with the techniques used in handling isolated tissues and administration of drugs to isolated tissues. The data presented in this study represents a very small number of experiments because the supply of animals was terminated prematurely due to a shortage of animals from the Principal Investigator Dr. D. C. Dyer (Department of Veterinary Physiology and Pharmacology, Iowa State University).

Materials and Methods

At 10 to 20 days pre-term, three ewes were euthanized with a overdose of sodium pentobarbital/magnesium chloride solution (Sleep-Away, Fort Dodge Laboratories, Fort Dodge, Iowa). Fetuses were removed after abdominal laparotomy, and the umbilical cord was cut 1.5 to 2.0 cm from its origin at the fetal abdomen. The distal end of the umbilical cord was cut at the first cotyledonous branch point. Umbilical cord

removal was completed within 10 minutes of anaesthetic induction, and the cord was immediately placed in a modified Krebs solution (115.21 mM NaCl, 4.70 mM KCl, 1.80 mM CaCl₂, 1.16 mM MgSO₄, 1.18 mM KH₂PO₄, 22.14 mM NaHCO₃, 7.88 mM glucose and 0.0267 mM EDTA) and oxygenated with a gas mixture consisting of 95% O₂ and 5% CO₂. The umbilical arteries were removed from the umbilical cord within 60 minutes of removal from the fetus. The adventitial tissue of the arteries was removed, and the arteries were stored at 4°C and oxygenated with the 95% O₂ and 5% CO₂ gas mixture for 1 to 24 hours before final sectioning and physiological evaluation.

Just prior to constrictor evaluation, the umbilical arteries were returned to room temperature and cut into 4 mm long cross-sectional rings. Care was taken to avoid disruption of the endothelium at all times. After 8 rings had been cut from each umbilical artery, the rings were attached to fine wire triangles and mounted in 10 ml tissue baths. The tissue baths were maintained at 37°C, oxygenated with the 95% O₂ and 5% O₂ gas mixture and perfused with modified Krebs.

The arterial ring triangles were attached to Grass FT03 force-displacement transducers (Grass Instruments, Quincy, Illinois) linked to a Beckman R611 recorder (Beckman Instruments, Schiller Park, Illinois) or monitoring and recording changes in the tension maintained by the arterial

rings. After being placed in the tissue baths, 12 grams of tension was applied over a 20 second period. The rings were then allowed to relax over the next 40 minutes, after which the tension was gradually adjusted until a 2.0 gram resting tension could be maintained.

Two slightly different protocols were used to evaluate the vasoconstrictive effects of ethanol, and, for this experiment, their results were considered equivalent. In one protocol, KCl at a bath concentration of 140 mM was used to develop a maximal contraction in all arterial rings. The KCl was then flushed, the 2 gram resting tension re-established and ethanol (95% ethanol, Chemistry Stores, Iowa State University, Ames, Iowa) administered at 0, 50, 100 and 200 mg/dl bath concentrations, with two rings of the eight receiving each dose. The second protocol applied an ethanol dose of 150 mg/dl to three rings, an ethanol dose of 300 mg/dl to three rings and a 140 mM dose of KCl was applied to the remaining two rings. The magnitude of the ethanol induced contractions were expressed as a percentage of the respective KCl-induced contractile maximum.

In one animal, the endothelium was destroyed in three rings by gently turning a metal bar around inside the arterial lumen three times prior to administration of KCl and ethanol; the endothelium was left intact in the remaining four rings (the eighth ring was removed from the study due to

accidental damage during handling). Potassium chloride (140 mM) was then administered followed by ethanol (200 mg/dl) and the effects of endothelial cell removal were evaluated by a paired t-test. All data presented in this experiment were expressed as the mean \pm standard deviation.

Results

The preliminary observations from the three separate trials are reviewed in Figure 1. Isolated tissue bath ethanol concentrations of 50 to 300 mg/dl produced responses ranging from no observed contraction to a contractile force that was 3.81% of the KCl response generated by the artery, respectively. A sham administration of deionized water resulted in no contraction. A regression line described by the equation $y = (0.0113)(x) + (-0.216)$ was obtained from the data points collected in the three trials (correlation coefficient = 0.929). The average KCl response for the arterial rings used to generate the data in Figure 1 was 7.07 ± 0.46 grams.

Finally, the umbilical artery from a single lamb was removed and the endothelial rings with (4 rings) and without an intact endothelium (3 rings) were compared (Figure 2). In rings with an intact endothelium, ethanol (200 mg/dl)

Figure 1. Contractile force generation of sheep umbilical arteries induced by ethanol. Data were collected from three different animals denoted by closed circles, open circles and open triangles; the heavy line indicates the regression line for all data points.

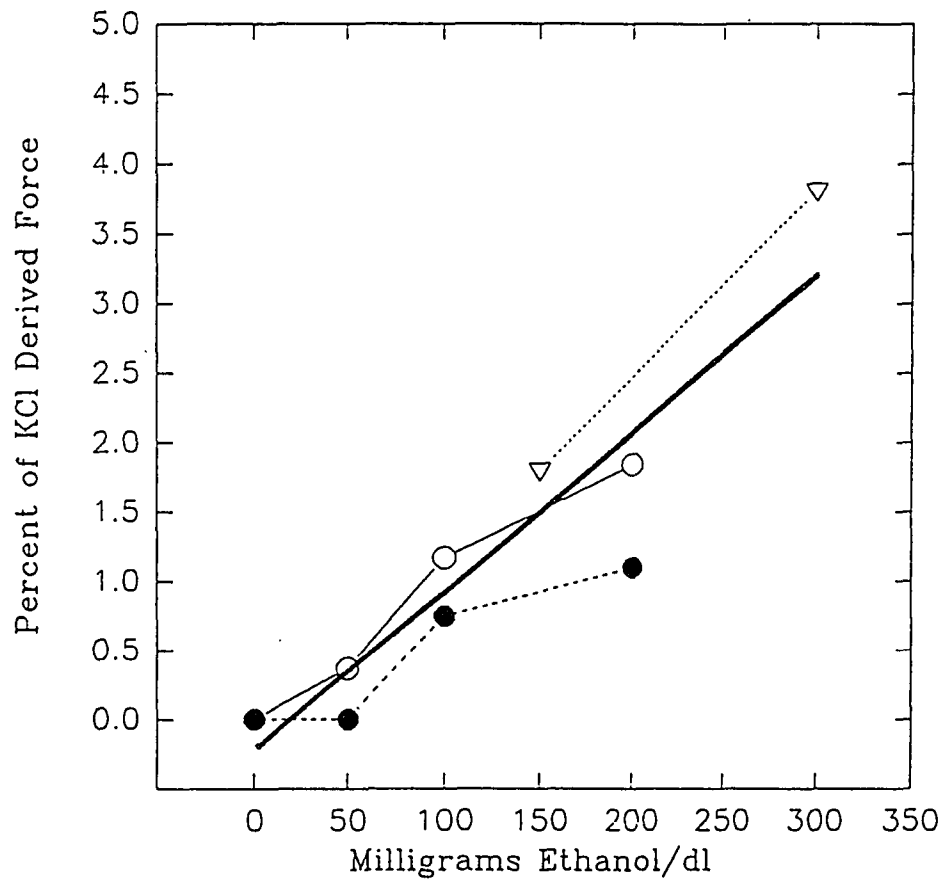
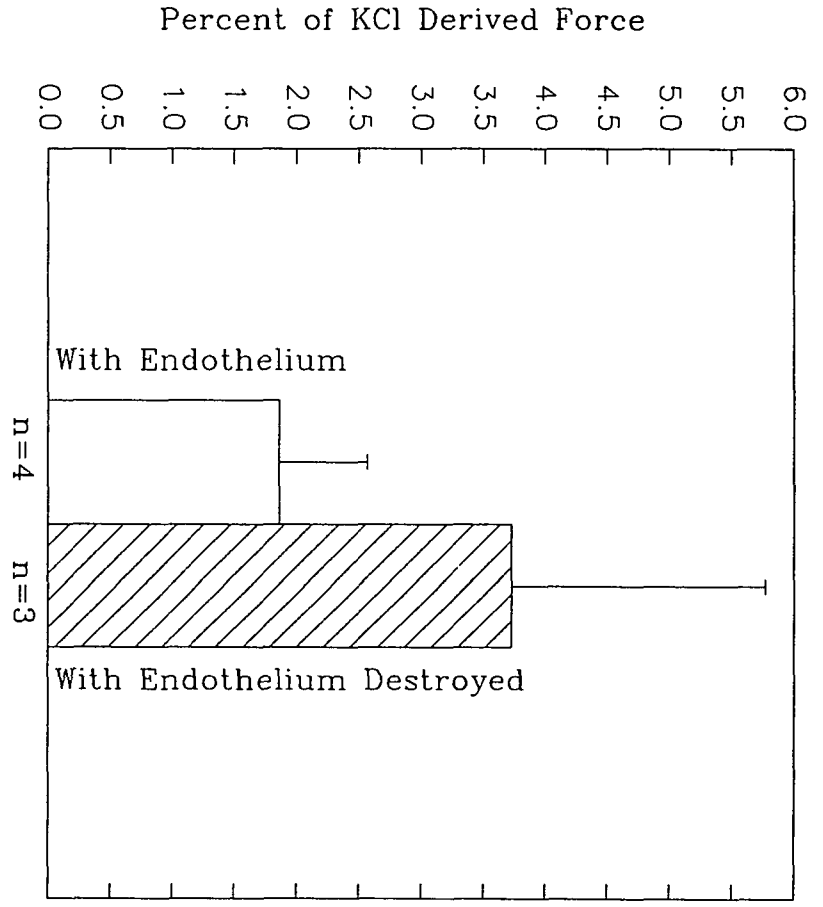


Figure 2. Comparison of contractile force generation by sheep umbilical arteries with or without an intact endothelium (mean \pm SEM).



promoted a contraction that was $1.86 \pm 0.71\%$ of the KCl response. When the endothelium was destroyed the response was $3.73 \pm 2.04\%$. The significance for this difference was $P < 0.14$. The average KCl response of the rings with intact endothelium was 11.56 ± 1.56 grams, and the average KCl response for rings with a destroyed endothelium was 10.10 ± 1.50 grams.

Discussion

Ovine umbilical arteries, like their human counterparts, are sensitive to the constrictive effects of ethanol. Comparisons between the constrictor forces generated in this and previous investigations are difficult because previous studies have used arterial strip preparations (Savoy-Moore et al. 1989; Altura et al. 1983; Fiscus and Dyer 1981) or morphological changes in artery appearance (Mukherjee and Hodgen 1982). In contrast, this investigation is the first to examine ethanol-induced contractions in a ring preparation.

In our investigation a 50 mg/dl dose of ethanol was found to represent the approximate threshold for detection of ethanol induced constriction in ovine umbilical arteries. The threshold suggested by our study is supported by Altura

et al. (1983) who determined that 52 ± 5.8 mg/dl was the threshold for a human strip preparation. However, a lower human strip-threshold has been observed by Savoy-Moore et al. (1989) who detected contractions at an ethanol dose of 1 mg /dl.

The difficulty with measuring ethanol-induced contractile effects at lower concentrations stems from a loss in the ability to maintain a stable baseline tissue tension needed to observe such small vasoconstrictive forces. When transducers are set to detect changes of as little as 10 to 30 mg of tension, they also tend to record changes in tension produced by aeration bubbles bouncing off of the strings attaching the rings to their force transducers, as well as electrical static in the Beckman recorder itself. As a result, it is very difficult to record changes of tension that are much smaller than about 30 mg.

The physiological mechanisms responsible for ethanol-induced vasoconstriction have not been determined, but have been suggested to include inhibition of endothelium-dependent relaxation (Hatake et al. 1993; Persson and Gustafsson 1992) or potentiation of the constrictor properties of angiotensin II (Savoy-Moore et al. 1989). Altura et al. (1983) observed that the maximal ethanol induced contractions of human umbilical artery were observed to be 68.8% of the maximal serotonin derived contraction. L-N^G-monomethyl-arginine is a

compound that completely abolishes the effects of endothelium-dependent relaxation in rabbit aortas and promotes a 20% increase in tension (Minor et al. 1990). Because the maximal ethanol-derived effects are much greater than that observed when EDRF is pharmacologically abolished, it must be assumed that ethanol derives its constrictor effects by acting on several mechanisms. This assumption is supported by the observations made in this investigation, where a trend suggestive of potentiated ethanol-induced constriction was observed when the endothelium, and endothelium-dependent relaxant effects were mechanically destroyed.

Ethanol may also influence FAS by mediating changes in PGE metabolism. Prostaglandin E is known to induce contractions of the myometrium (Gordeau et al., 1992); but whether ethanol-induced changes in PGE are linked to changes in arterial contractile force during pregnancy is unknown. Bocking et al. (1993) determined that in sheep, establishment of a 148 to 164 mg/dl plasma ethanol concentration is associated with a significant ($P < 0.05$) increase of 44% in placental secretion of PGE₂ when compared to pre-ethanol levels. Furthermore, Randall et al. (1991) administered aspirin (300 mg/kg), to block PGE synthesis, one hour prior to the administration of a 5.8 g/kg intragastric dose of ethanol. Pre-administration of aspirin prior to ethanol was

associated with an 80% reduction in the plasma PGE concentration, significant at $P < 0.05$, and a 50% reduction in the frequency of malformations observed per litter.

In conclusion, this preliminary investigation suggests that ovine umbilical arterial rings respond to ethanol in a manner similar to that observed in human arterial strips. Furthermore, this investigation suggests that the ovine umbilical artery ring-preparation could be useful for the evaluation of the effects of ethanol on the human umbilical vasculature.

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