HYPOCHOLESTEROLEMIC EFFECT OF FLAXSEED AND

ITS OIL IN THE OVARIECTOMIZED GOLDEN SYRIAN

HAMSTERS

By

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NOMENCLATURE

ACAT   Acyl CoA:cholesterol acyltransferase

AHA   American Heart Association

ALA   Alpha-linolenic acid

Apo A   Apolipoprotein A

Apo B   Apolipoprotein B

BMI   Body Mass Index

CRP   C-reactive protein

CVD   Cardiovascular disease

DHA   Docosahexaenoic acid

EPA   Eicosapentanoic acid

FO   Flaxseed oil

GI   Glycemic index

HDL   High density lipoprotein

HERS   Heart and Estrogen/Progestin replacement

HMG-CoA   3-hydroxy-3-methyl-glutaryl-coenzyme A

HMGR   3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HRT   Hormone replacement therapy

IDL   Intermediate density lipoproteins

LCAT   Lecithin cholesteryl-acyl transferase

LDL	Low density lipoprotein
Lp(a)	Lipoprotein a
n-3	Omega-3
OVX	Ovariectomy
PUFA	Polyunsaturated fatty acid
SDG	Secoisolariciresinol diglycoside
Sham	Sham operated
SRB-1	Scavenger receptor B1
TC	Total cholesterol
TG	Triglyceride
VLDL	Very low density lipoprotein
WF	Whole flaxseed
WHI	Women's Health Initiative

CHAPTER I

INTRODUCTION

According to the most recent survey by the American Heart Association (1), cardiovascular disease (CVD) accounts for 37.3 percent of all deaths in the United States. CVD is the leading cause of mortality and a major contributing factor of women's disability all over the world (1); (2); (3). Ovarian hormone deficiency puts postmenopausal women at an increased risk of CVD (4). Hormone replacement therapy (HRT) is used to relieve menopausal symptoms and is thought to reduce the risk of CVD in postmenopausal women. However recent findings from the Women's Health Initiative indicate that the risks for HRT outweigh its health benefits. HRT is associated with increased risk of endometrial cancer, breast cancer, hypertension, hyperlipidemia, and gallbladder disease (5); (6). In order to avoid the risks associated with HRT postmenopausal women and their practitioners are in search for alternative regimens.

Many functional foods with potential health benefits have been recently investigated for their role in reducing CVD. Flaxseed is one functional food which has the potential for reducing CVD risks. Flaxseed is a very rich source of lignans, α-linolenic acid (ALA) and soluble fiber mucilage, all of which individually or collectively may play a significant role in reducing women's CVD risk (7;) (8); (9); (10);

(11); (12); (13); (14); (15; 16); (17). Clinical studies (18); (11; 19) have demonstrated that the consumption of approximately 40 to 50g of flaxseed resulted in 5 to 9% reduction in total cholesterol. Animal findings support the hypocholesterolemic property of flaxseed (7); (8); (9); (10); (11); (12); (13); (14); (15); (16). However, the mode of action by which whole flaxseed modulates cholesterol and lipid profiles is relatively unknown. Moreover, what component(s) of flaxseed are responsible for this cholesterol lowering effect needs further investigation.

Earlier findings have shown the hypocholesterolemic and anti-atherosclerotic properties of flaxseed in ovarian hormone deficiency in hamsters (20). The purpose of this study has to determine whether flaxseed oil exerts the same cholesterol lowering effect as does whole flaxseed and to elucidate the mode of action by which whole flaxseed and its oil modulates serum and liver lipid profiles in ovariectomized hamsters.

Cholesterol metabolism is greatly influenced by levels of LDL receptor, SRB-1, HMGR , and 7-α hydroxylase. Hepatic levels of some key enzymes important in cholesterol metabolism, namely, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), Microsomal cytochrome P-450 cholesterol 7-α hydroxylase (7-α hydroxylase), LDL receptor and scavenger receptor B1 (SRB-1) will be assessed. HMGR is an enzyme catalyzing the rate limiting step of cholesterol biosynthesis, i.e. conversion of HMG-CoA to mevalonic acid. Regulation of HMGR activity is the primary means for controlling the level of cholesterol biosynthesis. 7-α. hydroxylase is an enzyme involved in the rate limiting step in bile acid synthesis from cholesterol. Scavenger receptor B1, a HDL receptor, plays an important role in metabolism of cholesterol by mediating cholesterol uptake in the liver in a process known as reverse cholesterol transport. LDL receptor is

involved in endocytosis of LDL and other lipoproteins by cells and in the release of free cholesterol into liver. By looking at the levels of these key regulatory proteins in liver microsomes, we can gain insight into the mechanism by which flaxseed and its oil is able to exert hypocholesterolemic action.

The **hypothesis** of this study is that flaxseed exerts its hypocholesterolemic action by modulating the levels of one or more of the key enzymes important in cholesterol metabolism, namely, SRB-1, HMGR, LDL receptor, and 7-α hydroxylase.

To test our hypothesis, we have the following **specific aims**;

1) To determine the effect of whole flaxseed and its oil on serum and liver lipid profiles in ovariectomized Golden Syrian hamsters

2) To determine the effect of whole flaxseed and its oil on protein levels of key enzymes involved in cholesterol metabolism, namely, SRB-1, HMGR, LDL-receptor, and 7-α hydroxylase.

CHAPTER II

REVIEW OF LITERATURE

Cardiovascular disease in women

Despite advances in the treatment of CVD, it remains the leading killer of women in the United States and in most developed areas of the world (2); (21). In the United States alone, more than half a million women die due CVD each year, exceeding the number of deaths in men and the next 7 causes of death in women combined. This translates into approximately 1 death every minute (3). Coronary heart disease (CHD) accounts for the majority of CVD deaths in women and is a prime target for prevention (22); (2). Because CHD is often fatal and nearly two thirds of women who die suddenly have no previously recognized symptoms, it is essential to prevent CHD (23). Other forms of atherosclerotic/thrombotic CVD, such as cerebrovascular disease and peripheral arterial disease, are critically important in women. While the incidence of heart disease is considerably lower among premenopausal women in comparison to men of the same age, it increases following menopause placing them at an equal risk for CVD as their male counterparts (4). Therefore, CVD is a major public health concern particularly due to the rapidly increased number of women reaching menopause , expected to exceed 45 million by the year 2020.

Health concerns in postmenopausal women

Obesity

Due to changing ways of life in modern society and sedentary lifestyle, obesity has become a serious growing problem worldwide. Elderly (≥70 yrs) and postmenopausal women are among the most sedentary and obese segments of the U.S. population and there is strong relationship between obesity and risk for CVD (24). Obesity in women has been associated with a variety of factors, including genetic predisposition, social class, early age of menarche, lack of physical activity excessive alcohol consumption and diet low in fruits and vegetables (25). Most women become overweight during and after menopause in the fourth and fifth decade of life. These changes in body composition may be due to reduced levels of circulating estrogen, and an increase in the androgen-estrogen ratio which is a likely factor for shifting fat distribution (26).

In postmenopausal years, women develop a central pattern of fat distribution and an increased risk of developing CVD (27); (28). In a cross-sectional study, Gower and colleagues (1998) recruited 141 healthy pre- and postmenopausal women aged 35-65 years to investigate if menopause-related differences in lipids are associated with greater estimated intra-abdominal adiposity. The results of this study indicated that postmenopausal women had greater total body fat, central skinfolds and intra abdominal fat and also had higher plasma concentrations of total and LDL-cholesterol and triglycerides than premenopausal women (27).

Diabetes

CVD is the major cause of morbidity and mortality in patients with diabetes. According to the recent report of the American Diabetes Association, 18.2 million people in the United States have diabetes. Although an estimated 13 million have been diagnosed, 5.2 million people are unaware that they have the disease (29). Heart disease strikes people with diabetes twice as often as people without diabetes. Deaths from heart disease in women with diabetes have increased 23% over the past 30 years (29). Type 2 or adult onset diabetes has long been known as a risk factor for CVD and is conservatively estimated to increase the risk of a fatal event by twofold. The association between diabetes and CVD has been suggested to be stronger in women than in men, indicating that diabetes eliminates the advantages of being female.

Ovarian hormone deficiency

Estrogen has a protective effect in women but in postmenopausal women ovarian hormone deficiency is the primary cause of problems associated with menopause. Hormone deficiency affects all parts of the body, and so, all tissues dependent on the hormone such as breast, ovaries, uterus, bones and heart are compromised. Hormonally dependent tissues cannot and do not function optimally or maintain their integrity when the required hormone is absent. Hormone deficiency causes an increase in the incidence of coronary artery disease, strokes, osteoporosis and possibly Alzheimer's disease. The primary effects of hormone deficiency are hyperlipidemia and hypercholesterolemia increasing their risk for CVD.

Hyperlipidemia/ hypercholesterolemia

Hyperlipidemia is an elevation of lipids in the bloodstream. These lipids include cholesterol, cholesterol esters, phospholipids and triglycerides which are transported in the blood as part of large molecules called lipoproteins. Hypercholesterolemia is defined as an increase in total cholesterol and low density lipoprotein levels.

Although hyperlipidemia is a risk factor for the development of CVD in both men and women, there are several important ways in which women differ from men. Cholesterol levels change over time differently in men and women. Men reach their peak cholesterol level at about age 50 which women peak a little later, at about age 60, after which the level declines gradually. HDL levels are higher in women than in men at all ages (30). LDL is less predictive and HDL cholesterol is more predictive of CVD risk in women. Postmenopausal women often experience hypercholesterolemia because of ovarian hormone deficiency, which puts them at a high risk of CVD.

<u>Cholesterol</u>

There are close to 100 genes devoted to the synthesis, transport, metabolism, and regulation of cholesterol. Cholesterol, cholesterol metabolites, and its immediate biosynthetic precursors play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, maintenance of nerve cells, stress responses, salt and water balance and calcium metabolism. Cholesterol is a tetracyclic molecule (Figure 1) which has roles in membrane structure as well as being a precursor for the synthesis of the steroid hormones such as estrogens, androgens, sterols and bile acids.

Steroid hormones play a key role in regulation of metabolism. These hormones act primarily by protein- receptor mechanism to control gene expression in the appropriate target tissue. In the nervous system, it is needed for myelin sheath formation in neuronal cells and also as neurotransmitters. Cholesterol is also a very important component of cell membrane where it maintains appropriate membrane fluidity. A large amount of cholesterol is also found in brain tissues.

Bile acids are the primary degradation products of cholesterol. The bile acids are made in liver, stored in the gall bladder, and secreted into the small intestine. They aid in solubilization of lipids, facilitating digestion by intestinal lipases in the small intestine.
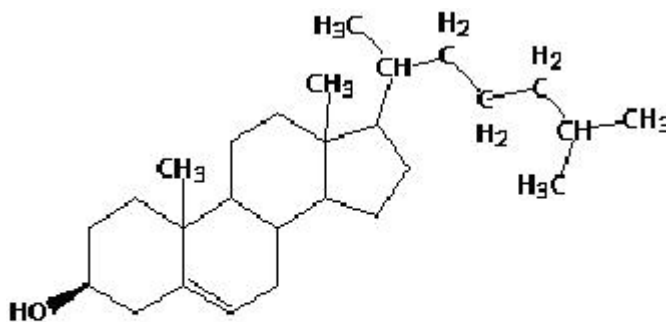
Figure 1. Structure of cholesterol

The general structure of cholesterol consists of two six-membered rings side-by-side, a third six-membered ring off the top corner of the right ring, and a five-membered ring attached to the right side (Figure 1). The central core of this molecule, consisting of four fused rings, is shared by all steroids, including estrogen (estradiol), progesterone, corticosteroids such as cortisol (cortisone), aldosterone, testosterone, and Vitamin D.

Cholesterol metabolism

A relatively constant level of cholesterol in the body (150 - 200 mg/dL) is maintained by various controlling mechanisms. The level of cholesterol synthesis is regulated based on the dietary intake of cholesterol. The cholesterol levels are maintained at a steady level by a number of mechanisms:

1) Regulation of HMGR activity and levels.

2) Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL-mediated reverse transport.

3) Regulation of hepatic rates of sterol biosynthesis.

4) Regulation by conversion to bile acids through 7-α hydroxylase activity.

5) Regulation of excess intracellular free cholesterol through the activity of acyl-CoA:cholesterol acyltransferase, ACAT.

While normal cholesterol level is essential to life, its involvement in atherosclerosis has been associated with CVD and stroke. In a healthy organism, an intricate balance is maintained between the biosynthesis, utilization, transport, degradation and reabsorption of cholesterol.

There are two sources of cholesterol in the body. One is the *de novo* synthesis of cholesterol by the liver and second is from dietary cholesterol. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles. The same is true of cholesteryl esters, the form in which cholesterol is stored in cells.

Cholesterol is transported in the plasma predominantly as cholesteryl esters associated with lipoproteins. All of the plasma lipids are associated with proteins to

form water-miscible lipoprotein complexes. Classification of lipoproteins is generally based on density gradient ultracentrifugation. Each lipoprotein contains different proportions of protein, phospholipids, triglyceride, and cholesterol, thereby giving rise to the different densities of each class (31); (32). Lipoproteins are made up of a neutral lipid core composed of triacylglycerol and/or cholesterol esters. Around this core is a coat of apoprotein, phospholipids and cholesterol (31). Five major classes of lipoproteins have been sequentially separated based on their molecular weights by ultra-centrifugation, and are designated as chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). An accepted view of lipoprotein structure suggests the existence of an outer shell of polar phospholipid and protein groups, with an inner hydrophobic core of primarily triglycerides and cholesteryl esters (31).

Chylomicrons

The chylomicrons, which are derived from intestinal absorption of triglycerides, have densities of less than 0.95 g/ml and diameters ranging from 750 to 6000 Å. Their primary composition is triglyceride (80-90% by weight), free and esterified cholesterol (2-7%), phospholipid (3-6%), and protein (1-2%) (31); (33). Since chylomicrons transport exogenous triglycerides from the intestinal mucosa via the thoracic duct to the circulation, their formation fluctuates with the load of absorbed triglycerides.

Chylomicrons are cleared rapidly from the blood. Therefore, their presence in the post-absorptive state, approximately 12 to 16 hours after the last meal, is indicative of

defective handling of dietary fat. The composition of chylomicrons is altered in the circulation, with the action of endothelial lipoprotein lipase facilitating triglyceride hydrolysis by extra hepatic tissues. The activity of this enzyme in individual tissues varies with nutritional and physiological states, such as fasting, exercise, pregnancy, and lactation, thereby affecting triglyceride uptake and plasma levels (34). Chylomicron remnants, with 80% less triglyceride, are then taken up by the parenchymal cells of the liver via receptor-mediated endocytosis after binding to specific remnant receptors (33; 35; 36).

Very low density lipoproteins (VLDL)

VLDLs are of endogenous origin and are synthesized and secreted primarily by liver and intestine. They are isolated from chylomicron-free plasma at densities less than 1.006 g/ml; and their particles vary in diameter between 300 and 800 Å. They are composed of 55-65% triglyceride, 10-15% free and esterified cholesterol, 15-20% phospholipid, and 5-10% protein. Triglycerides carried in VLDL are transported in plasma to utilization sites such as heart, muscle, mammary gland and storage sites such as adipocytes. Following lipoprotein lipase action, the triglyceride-depleted particles are released into the bloodstream, and are then the primary source of circulating LDL cholesterol. As VLDLs undergo de-lipidation, the particles change their composition, structure, and biological properties. As VLDL become smaller and denser, percent contribution from the proteins, phospholipids, and cholesterol increases the proportion of triglyceride decreases. Thus, the heterogeneity of VLDL represents a dynamic equilibrium of particles from several sources (37; 38).

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After VLDLs (and possibly chylomicrons) have interacted with lipoprotein lipase at multiple sites, particles of density greater than 1.006 g/ml are formed. These remnant particles are operationally defined as IDL and has a density range of 1.006-1.019 g/ml and a diameter of about 250 Å. Triglycerides contribute only 20% of the mass of IDL and it is still regarded as triglyceride-rich and may be further reduced to LDL and even HDL (37).

Some of the circulating VLDL remnants are removed directly by the liver in a process mediated by Apo E. The surface of liver cells contains receptors that recognize and bind to the Apo E; the remnant lipoprotein is then internalized and removed from the circulation (39). However, another fraction of VLDL-remnants remains in the circulation and this is the fraction that is converted to LDL. Usually, about two-thirds of IDL is taken up by the liver, and one-third goes to LDL. Thus, the proportion of VLDL fraction converted to IDL and then to LDL greatly influences the concentrations of serum LDL (35).

Low density lipoprotein (LDL)

Low density lipoprotein, a spherical particle, contains mostly cholesteryl ester in its nonpolar core; its surface apolipoprotein consists almost exclusively of one Apo B-100 molecule. Low density lipoproteins are the end product of a chain of de-lipidation steps of VLDL and chylomicron particles involving lipoprotein lipase, hepatic lipase, and lecithin:cholesterol acyltransferase (LCAT). Studies suggest that direct hepatic secretion of LDL is a normal process, its magnitude varies from species to species and may be markedly heightened in disease states, as in familial hypercholesterolemia (31; 35; 40).

Low density lipoproteins normally transport 60 to 75% of the total plasma cholesterol. They are isolated from chylomicron-free plasma in the density range 1.019-1.063 g/ml and have a diameter of about 220 Å. They are composed of about 50% free and esterified cholesterol, 20% phospholipid, 20% protein, and 10% triglyceride (31). The small size and high cholesterol content of LDL-cholesterol may be the reason for its high atherogenic property. Apolipoprotein-B serves as a marker for LDL-cholesterol turnover as it is the only protein found on the surface of LDL.

Serum LDL concentration is affected by the fractional removal rate of LDL-cholesterol from the circulation, and this rate is determined in large part by the availability of cell-surface receptors for LDL-cholesterol (41). In normal cells, the LDL is internalized via endocytosis. The endocytotic vesicle containing LDL then fuses with a primary lysosome, where the protein moiety is degraded completely to amino acids and the cholesteryl esters are hydrolyzed by cholesterol esterase. Re-esterification of excess cholesterol for storage is catalyzed by acyl CoA:cholesterol acyltransferase (ACAT). Endogenous cholesterol synthesis within the cells is suppressed by HMGR when exogenous cholesterol is delivered via LDL to the tissues. The need for cellular cholesterol for membrane and steroid hormone synthesis regulates the number of LDL membrane receptors (35; 38).

High density lipoprotein (HDL)

High density lipoproteins are isolated in the density range 1.063-1.21 g/ml and are the smallest of the lipoproteins, with a diameter of 70-120 Å (42; 43)They normally account for 20-25% of total plasma cholesterol and are composed of about 50% protein,

25% phospholipid, 20% free and esterified cholesterol, and 5% triglyceride. The greater density of HDL compared with other lipoproteins reflects its greater protein and lesser lipid content. Their cholesteryl ester to free cholesterol ratio is approximately 3:1 (43). HDLs have been divided into two classes: HDL_2 (density range 1.063-1.125 g/ml) is composed of about 60% lipid and 40% protein and HDL_3 (density range 1.125-1.21 g/ml) contains about 55% proteins (43).

The liver is the primary site for HDL biosynthesis and secretion while the intestine is considered secondary site of secretion. Nascent HDL (HDL precursor complexes) formed in vivo may be transformed in the plasma to mature spherical particles. The nascent HDL particles are converted to mature spherical circulating HDL through the action of plasma Lecithin cholesterol acyltransferase (LCAT). This enzyme is responsible for removing phospholipids from HDL and replacing them with cholesteryl esters, as well as facilitating the loss of Apo E from nascent HDL. The end result of these processes appears to be the net movement of cholesterol from peripheral tissues to hepatic tissue for removal and breakdown (31; 42).

LCAT is an extra cellular enzyme that is synthesized and secreted by the liver, circulates in the plasma, and acts on plasma HDL. This enzyme also promotes non-enzymatic transfer of cholesteryl esters from HDL to VLDL and LDL. The cholesteryl esters formed by the LCAT reaction are distributed among HDL, VLDL, and LDL, which increases their total cholesterol content. This process may serve to increase the rate of cholesterol removal by a receptor-mediated process, and may in addition provide a mechanism for directing cholesterol transport toward specific tissues. Patients with

familial LCAT deficiency show an accumulation of plasma unesterified cholesterol, with all lipoproteins having an abnormal lipid and apolipoprotein composition.

Removal of cholesterol

The liver and intestine are the only organs with the capacity to effectively remove cholesterol from the circulation. The transfer of HDL cholesteryl esters to the liver for elimination could theoretically be achieved by: 1) the uptake and catabolism of whole HDL particles; 2) selective removal of cholesteryl esters from mature HDL, with its subsequent recycling; and/or 3) initial transfer of cholesteryl ester to lower density lipoprotiens, with subsequent hepatic removal (44). The transfer of cholesteryl esters from HDL to LDL is facilitated by the cholesteryl ester transfer protein. The increased net transfer of cholesteryl esters by the cholesteryl ester transfer protein results in decreased HDL concentration (44).

Cholesterol is excreted in the bile as free cholesterol or as bile salts. Most of the cholesterol in the liver is converted to bile salts and excreted via bile duct. The end products of cholesterol utilization are the bile acids which are synthesized in the liver. Synthesis of bile salts is one of the predominant mechanisms for the excretion of excess cholesterol. Bile salts are synthesized from cholesterol by the liver with the help of enzyme 7α-hydroxylase which hydroxylates cholesterol to 7-hydroxycholesterol (45). This substrate undergoes several transformations to form bile salts, cholyl Co-A and chenodeoxycholyl Co-A. The most abundant bile acids in human bile are chenodeoxycholic acid (45%) and cholic acid (31%). These are referred to as the primary bile acids. Within the intestines, the primary bile acids are acted upon by bacteria and

converted to the secondary bile acids, deoxycholate (from cholate) and lithocholate (from chenodeoxycholate). Both primary and secondary bile acids are reabsorbed by the intestines and delivered back to the liver via the portal circulation. Within the liver, the carboxyl group of primary and secondary bile acids is conjugated via an amide bond to either glycine or taurine before being resecreted into the bile canaliculi. These conjugation reactions yield glycoconjugates and tauroconjugates, respectively (45). Bile acids are carried from the liver to the gallbladder where they are stored for future use. The ultimate fate of bile acids is secretion into the intestine, where they aid in the emulsification of dietary lipids. In the gut the glycine and taurine residues are removed and the bile acids are either excreted (only a small percentage) or reabsorbed by the gut and returned to the liver. This process of secretion from the liver to the gallbladder, to the intestines and finally reabsorbtion is termed the enterohepatic circulation.

Bile acids have physiologically significant functions such as 1) their synthesis and excretion in the feces is the significant mechanism for the elimination of excess cholesterol, 2) the bile acids deoxycholate and lithocholate and phospholipids solubilize cholesterol in the bile, thereby preventing the precipitation of cholesterol in the gallbladder, 3) bile acids facilitate digestion of triacylglycerols by acting as emulsifying agents which makes fats accessible to pancreatic lipases, and 4) also bile acids facilitate absorption of fat soluble vitamins due to their emulsifying property.

HMG CoA reductase (HMGR)

HMGR is an enzyme involved in cholesterol biosynthesis. The reaction catalyzed by HMGR is the rate limiting step of cholesterol biosynthesis, and this enzyme

is subject to complex regulatory controls. Regulation of HMGR activity is the primary means for controlling the level of cholesterol biosynthesis. The enzyme is controlled by four distinct mechanisms: feed-back inhibition, control of gene expression, rate of enzyme degradation and phosphorylation- dephosphorylation. The first three control mechanisms are exerted by cholesterol itself. Cholesterol acts as a feed-back inhibitor of pre-existing HMGR as well as inducing rapid degradation of the enzyme. The latter is the result of cholesterol-induced polyubiquitination of HMGR and its degradation in the proteosome. Regulation of HMGR through covalent modification occurs as a result of phosphorylation and dephosphorylation. The enzyme is most active in its unmodified form. Phosphorylation of the enzyme decreases its activity. Hormones such as glucagon and epinephrine negatively affect cholesterol biosynthesis by increasing the activity of the inhibitor of phosphoprotein phosphatase inhibitor-1. Conversely, insulin stimulates the removal of phosphates and, thereby, activates HMGR activity. Additional regulation of HMGR occurs through an inhibition of its activity as well as of its synthesis by elevation in intracellular cholesterol levels.

7-α hydroxylase

Microsomal cytochrome P-450 cholesterol 7-α hydroxylase (7-α hydroxylase) is an enzyme involved in bile acid synthesis from cholesterol. The rate-limiting step in bile acid biosynthesis is catalyzed by 7-α hydroxylase. The expression of this enzyme is subject to feedback regulation by sterols. Bile acids play two opposing roles in the maintenance of cholesterol homeostasis. In one role, they are the end products of cholesterol catabolism, and their biosynthesis and excretion serve to decrease the levels

of cholesterol in the liver. In the second role, their presence in the intestine facilitates the solubilization of dietary fats and cholesterol and the subsequent uptake of these essential nutrients. In this manner, bile acids increase whole body cholesterol levels (46).

Scavenger receptor B 1 (SRB1)

HDL can pick up the cholesterol deposited on arteries by LDL and deliver it to the liver for disposal in bile (42). More cholesterol can be disposed when HDL levels are high. One of the ways in which HDL acts is through the action of Scavenger Receptor B1. SRB1 is an HDL receptor in the liver and plays an important role in the metabolism of cholesterol by mediating cholesterol uptake in the liver in a process known as reverse cholesterol transport.

SRB-I may facilitate both uptake and efflux of cholesterol down the cholesterol-concentration gradient over the plasma membrane, in the hepatocytes, cholesterol is used for VLDL-synthesis, converted to bile acids, or secreted directly into the bile. Hepatic SRB-I is negatively regulated by 17 β-estradiol (47); (48), vitamin E (49), and cholesterol. Polyunsaturated fatty acids increases hepatic SRB-I levels in hamster, whereas the saturated fatty acid myristic acid suppresses hepatic SRB-I expression (50); (51).

Hepatic LDL receptor

LDL and HDL are serum cholesterol transporting molecules. When cells require cholesterol, cells increase cholesterol synthesis and often up-regulate LDL receptors to obtain lipoprotein cholesterol. LDL receptor facilitates cellular uptake of

cholesterol rich low density lipoprotein. LDL receptor is involved in endocytosis of LDL and other lipoproteins. It is also engaged in the release of free cholesterol into the liver. LDL receptor functions by mechanisms such as incorporation into plasma membrane, inhibition of new LDL receptors and inhibition of cholesterol synthesis.

The concentrations of these lipoprotein particles are influenced by nutritional and hormonal stimuli. For example, dietary cholesterol and saturated fat can separately and together elevate LDL-cholesterol in humans, and alteration of hepatic LDL receptor activities is one way that dietary lipids can change plasma LDL concentrations. Dietary cholesterol decreases hepatic LDL-receptor protein and activity by transcriptional mechanisms *in vivo*.

Despite of all these complex regulatory processes to control cholesterol levels, hypercholesterolemia is a major determinant of CVD risks and is very frequently seen in both men and women especially postmenopausal women. The reasons for elevated cholesterol levels could vary greatly among individuals depending on diet, lifestyle, smoking, disease condition, genetics, and other factors.

Hormone Replacement Therapy (HRT)

Postmenopausal women often experience hypercholesterolemia due to ovarian hormone deficiency putting them at increased risk for CVD. Hormone replacement therapy is being used to reduce the increased risk of CVD. A number of studies have confirmed that women who use HRT are more likely to have healthier lifestyle and health behaviors that may reduce their susceptibility to some diseases.

Hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) may prevent cardiovascular disease in postmenopausal women by reducing serum cholesterol. Estrogen therapy has been shown to improve lipid profiles, bone quality due to action of estrogen receptor-linked calcium transport in the intestine (52), blood pressure through direct action on vascular endothelium and also slowing the buildup of atherosclerotic plaque (53).

Nanda et al. (53) reported that estrogen reduces serum LDL-C levels and increase serum HDL-C levels. Estrogen replacement therapy have also been shown to increase triglyceride levels through increase in VLDL particles (54); (55). VLDL particles are known to be atherogenic but those produced due to use of exogenous estrogen are low in cholesterol concentration and are believed to be less likely to enter the arterial walls because of their buoyancy (55). Estrogen also increases all major cholesterol transport pathways and up-regulate LDL receptors thus allowing for more LDL uptake by the liver for production of steroids or bile salts. Estrogen also increases Apo-A and HDL-C levels possibly due to decrease in hepatic lipase activity. Though ERT was shown to have promising results in alleviating postmenopausal symptoms, the results from recent follow up studies have been surprising and all together unexpected. The Women's Health Initiative (WHI) study started in 1993 had two components, the estrogen only treatment arms (0.625 mg/day of conjugated equine estrogen) which enrolled 10,739 postmenopausal women, aged 50-79 years, with prior hysterectomy, and the estrogen plus progestin component (0.625 mg/day conjugated equine estrogens plus 2.5 mg/day medroxyprogesterone acetate) in which 16,608 postmenopausal women aged 50-79 years with an intact uterus at baseline were recruited by 40 US clinical centers in 1993-1998.

Both treatments were stopped in 2002 after only a 5-6 yr follow-up as neither of the treatments reduced the incidence of CVD in postmenopausal women but were increasing the risk of stroke in the estrogen only group and invasive breast cancer in the estrogen plus progestin group (5); (6).

The Heart and Estrogen/Progestin replacement (HERS) therapy which gave the same dose of hormones as WHI study to 2763 postmenopausal women with CHD and followed them for 4 yrs found no advantage of the therapy for these women. The HERS trial found that taking estrogen plus progestin for up to 4 years did not prevent further heart attacks or death from previous heart disease in postmenopausal women who already had a previous heart attack or known heart disease.

Less than 30% of postmenopausal women in the United States receive HRT and of these women most discontinue use after one year (56). Most women stop taking HRT after severe symptoms of menopause such as hot flashes are relieved and some stop due to financial reasons. The main reasons for stopping estrogen therapies by most women is due to side-effects such as irregular and break out bleedings, and the increased incidence of cancers and thrombosis. Hormone replacement therapy is now recommended only for women who have osteoporosis and severe menopausal symptoms such as hot flushes and mood swings (56). Postmenopausal women all over the world are searching for alterative treatments to reduce the risk of CHD without the increased risk and side effects. Dietary modifications are an important option to reduce lipid profile without the associated side effects.

Dietary Modifications Beneficial in Improving Lipid Profile

The increased awareness of the role that diet plays in human health has led to the recognition of the importance of food components in preventing chronic diseases. The rates of death due to heart disease, cancers especially colon cancers, have been shown to be reduced due to incorporation of fiber in the diet (57). The American Heart Association endorses Step I and Step II diets created by the National Heart, Lung, and Blood Association's National Cholesterol Education Program (NCEP) to reduce risk of CVD by reducing high blood cholesterol levels.

The Step I diet restricts total fat to no more than 30 percent of total calories, saturated fat to no more than 10 percent of total calories, and cholesterol to less than 300 mg/day. This diet is intended as the starting point for patients who have high cholesterol levels. The Step II diet goals are lower for saturated fat (less than 7 percent) and cholesterol (less than 200 mg/day). They are intended for people who had attained Step I diet goals and still has a high cholesterol level ($\geq$240 mg/dL) or who had a heart attack (57).

For people at high risk or who have known CVD the Therapeutic Lifestyle Changes (TLC) diet is preferred. This is the "next generation" of the Step II diet recommended in May 2001. The dietary goals for TLC similar to Step II diet but it provides therapeutic options for LDL-lowering such as intake of plant sterols at least 2 g/day and increased intake of soluble fiber to at least 10-25 g/day. Along with the caloric restriction it also includes moderate exercise to expend at least 200 kcal/day (57). All these dietary recommendations emphasize the importance of diet in reducing or preventing CVD.

Fiber

Dietary fiber refers to the fraction of the edible part of plants or their extracts, or synthetic analogues that (i) are resistant to digestion and absorption in the small intestine, usually with complete or partial fermentation in the large intestine; and (ii) promote one or more of the following beneficial physiological effects, namely, laxation, reduction in blood cholesterol, and modulation of blood glucose. Person who consume foods high in dietary fiber (whole grain cereals, fruits and vegetables) have a lower prevalence of risk factors for CVD including hypertension, obesity, and type 2 diabetes mellitus (58; 59); (60). Recent large, prospective studies also show a direct inverse association between high fiber food intake and the development of coronary heart disease and stroke (61). While several etiologies have been considered, the biologic mechanisms whereby a high-fiber diet exerts beneficial cardiovascular effects are not entirely known.

Fiber has been widely accepted to play a significant role in reducing total blood cholesterol particularly LDL (62); (11) thereby decreasing the risk of coronary heart disease. Studies done in both humans (9); (10); (11); (12) and animals (13); (14); (15; 16) suggest that dietary fiber lowers cholesterol by enhancing bile acid synthesis and their fecal excretion which results in increased hepatic cholesterol synthesis. Also, gut bacteria ferment soluble fiber to form short chain fatty acids (SCFA) which are readily absorbed and at concentrations of 15-30 mmol/L inhibit *in vivo* hepatic cholesterol and fatty acid synthesis (16).

Researchers (63); (9) have reported moderate, but significant, reductions in plasma cholesterol when subjects consumed increased amounts of mixed vegetables and fruits for long periods of time. Perhaps the beneficial effects of soluble fiber can be seen

most clearly when it is consumed as part of a balanced diet. A recent comprehensive meta-analysis suggests that 3 g soluble fiber from oats (three servings of oatmeal, 28 g each) can decrease total cholesterol and LDL by 5 mg/dL (64). A clinical study in 59 subjects with elevated LDL (131–191 mg/ dL) already on a step I cholesterol-lowering diet (1) showed that daily intake of 20 g of fiber, predominantly as water-soluble guar gum and pectin, lowered LDL by 9% over 1 year of treatment (65); (66). These studies demonstrate the well-known effects of fiber consumption on lipids, particularly LDL.

A study by Jenkins and colleagues (67) compared the cholesterol-lowering potential of food with that of a statin. Thirty-four hyperlipidemic participants underwent three 1-month treatments in random order, a very-low-saturated fat diet, the same diet plus 20 mg lovastatin (statin diet), and a diet high in plant sterols (1.0 g/1000 kcal), soy-protein foods (21.4 g/1000 kcal), almonds (14 g/1000 kcal), and viscous fibers from oats, barley, psyllium, and the vegetables okra and eggplant (10 g/1000 kcal). LDL-C concentrations decreased by 8.5%, 33.3%, and 29.6% after 4 wk of the control, statin, and vegetable diets, respectively. The statin and the vegetable diets did not differ significantly (P=0.288) in their ability to reduce LDL-C. Hence, the authors concluded that dietary combinations are similar in potency to statin drugs in improving the lipid profile (67).

Thus increased fiber consumption also lowers the risk of developing heart disease and help fight obesity. High-fiber foods help reduce starving and move waste through the digestive tract faster and easier reducing the contact of harmful substances with the gastrointestinal tract. A diet adequate in fiber-containing foods is also usually rich in micronutrients and nonnutritive ingredients that have additional health benefits. Dietary

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intervention programs are necessary to educate people about dietary modifications to reduce the risk of CVD and other nutrition-related disorders.

Omega-3 fatty acids and omega-6 fatty acids

Omega 6-polyunsaturated fatty acids and omega 3-polyunsaturated fatty acids (PUFA) are necessary for proper growth and development and are therefore of nutritional importance. Omega-3 fatty acids are long-chain polyunsaturated fatty acids (18-22 carbon atoms in length) with the first of many double bonds beginning with the third carbon atom from the methyl end of the fatty acid molecule. The fish-based and fish-oil-based omega-3 polyunsaturated fatty acids (also referred to as n-3 PUFA) consist of eicosapentanoic acid (EPA) (20 carbon atoms, 5 double bonds) and docosahexanoic acid (DHA) (22 carbon atoms, 6 double bonds). Whereas plant foods and vegetable oils lack EPA and DHA, some do contain varying amounts of the n-3 PUFA alpha-linolenic acid (ALA), which has 18 carbon atoms and 3 double bonds. Many vegetable oils are greatly enriched in omega-6 fatty acids (mainly as linoleic acid in corn, safflower, sunflower and soybean oils), but canola oil (nonhydrogenated), ground flaxseed and walnuts are rich sources of ALA. The typical North American diet consists of very high intake of n-6 PUFA, mostly as linoleic acid (LA) (12-15 g/day) and 1-3 g of ALA per day but only 0.10-0.15 g of EPA plus DHA per day.

Greenland Eskimos have a low mortality rate from CHD (68); (69); (70) despite a high intake of fat (about 40% of their total caloric intake). This so-called 'Eskimos paradox' led to a series of epidemiological studies in the late 1970s by Danish investigators Bang and Dyerberg, which suggested a close correlation between the

observed low incidence of CHD amongst the Inuit and their high consumption of fish and fish-eating mammals (including seal, walrus, whale), resulting in a diet rich in the long chain n-3 PUFAs (71); (72). These PUFAs include EPA and DHA which are scarce or absent in land animals and plants. The Greenland Inuits' intake of saturated fat was low (9% of total calories), whereas their dietary intake of n-3 PUFAs was high (4.2% of total calories). These observations are in marked contrast to the much higher rates of CVD among Danish, whose diet had a comparable amount of total fat (42% of total calories) but a much lower intake of n-3 PUFAs (< 1% of total calories) and a much higher intake of saturated fat (approximately 22% of total calories) (71); (72). Since then, several cross-cultural epidemiological studies among the coastal population of Japan and Alaskan Natives have produced broadly similar data, showing an inverse relationship between n-3 PUFAs from fish intake and CHD death rates, thus suggesting a potential role of marine n-3 PUFAs in the prevention of CHD (72); (73); (74).

This increased interest has resulted in a large number of epidemiological, animal and clinical studies investigating the potential role of the intake of fish, fish oils or the specific n-3 fatty acids EPA and DHA in prevention and therapy. The combined results from these studies suggest that n-3 fatty acids may be a beneficial factor in the development of CVD some forms of cancer and diseases with an immunoinflammatory component. Today, there is little doubt that n-3 fatty acids are important and play a role in the modulation and prevention of human diseases, in particular CVD.

Walnut, almonds, olives and flaxseed are also good plant sources of n-3 fatty acids (75). Good health requires a ratio of 2:1 for omega-3 to omega-6 fatty acid where as the average American diet has an average ratio of 1:20 to 1:50. Flaxseed has a ratio of 4:1

for omega-3 to omega-6 fatty acid and studies have shown this ratio to be ideal for cholesterol lowering and in turn reducing the risk factors for CVD (76). Since flaxseed is a rich source of n-3 fatty acids, it should increase the ratio between n-3 versus n-6 fatty acid intakes which would be considered a positive influence on maintaining CVD health.

Flaxseed

Flax (Lignum usitatissimum) is an economically important oilseed crop, especially for Canada, which produces about 40 % of the world's flaxseed. Flaxseed consumption in various forms as a food ingredient and for its medicinal properties dates from 5000 BC since its cultivation. Flaxseed is the most prominent oilseed studied to date as a functional food, since it is a leading source of the omega-3 fatty acid ALA (52 % of total fatty acids) and the phenolic compounds known as lignans (>500 µg/g). It is also a very rich source of soluble fiber known as mucilage.

Flaxseed is also a rich source of dietary fibers, which are known to reduce in diabetes, coronary heart disease risk, preventing colon and rectal cancer and reduce the incidence of obesity. Flaxseed gum behaves like typical viscous fibers with ability to reduce blood glucose response and improve blood glucose profile (18).

Whole flaxseed consumed either raw or defatted reduces total and LDL cholesterol in humans, confirming the multicomponent cardioprotective effect of flaxseed (18); (19). Studies in women show the vital role of flaxseed in mediating bone health (11) and its strong phytoestrogenic and therapeutic effect in reducing the risk of hormone related cancers (77); (78); (79); (80).

The most researched biologic activities of flaxseed have been relegated to ALA, lignans, and soluble polysaccharides (gum) since flaxseed is the most abundant source of these components. A case in point is the reduced risk of cancer that has been attributed to the biological effects of both ALA and the lignan secoisolariciresinol diglycoside (SDG), and protection against cardiovascular disease attributable to ALA, flaxseed gum and proteins. Accurate documentation of the therapeutic effects of flaxseed and its components that contribute uniquely to disease prevention, health protection and as a deterrent to degenerative diseases will increase its potential for use as a functional food. It could be highly beneficial to postmenopausal women instead of undergoing HRT and having to face risks associated with it. Flaxseed can provide postmenopausal women with the source of phytoestrogens to combat menopausal symptoms at the same time reduce risks of CVD, cancer and a number of other diseases.

Alpha linolenic acid

A number of studies involving humans (11), rabbits (81) and hamsters (82); (17); (20) have shown the hypocholesterolemic effects of ALA from flaxseed. Lucas et al (20) have shown that flaxseed reduces plasma cholesterol and atherosclerotic plaque formation in ovariectomized Golden syrian hamsters (20). A number of studies confirm the hypocholesterolemic effects of flaxseed in various experimental models (17); (83); (84); (85); (86).

ALA can be easily converted to cardioprotective n-3 fatty acids namely EPA and DHA in the body. Harper and colleagues (87) studied the effects of daily supplementation with 3 g of ALA on plasma concentration of long-chain (n-3) fatty acids in a

predominantly African-American population with chronic illness. They found that ALA increases the EPA concentration by 60% and DPA levels by 25%. Thus, flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans (87); (88).

Another study assessed the effects of ALA and LA on inflammatory markers and lipids and lipoproteins in hypercholesterolemic subjects (89). Participants (n = 23) were fed 2 diets low in saturated fat and cholesterol, and high in PUFA varying in ALA (ALA Diet) and linoleic acid (LA Diet) compared to the average American diet (AAD). The two high-PUFA diets decreased serum total cholesterol, LDL cholesterol and triglycerides similarly (P < 0.05); the ALA Diet decreased HDL cholesterol and apolipoprotein AI compared with the AAD (P < 0.05). ALA reduced the inflammatory markers vascular cell adhesion molecule-1 (VCAM-1), E- selectin and C-reactive protein (CRP) significantly. ALA appears to decrease CVD risk by inhibiting vascular inflammation and endothelial activation beyond its lipid-lowering effects (89) which confirms its role as an anti inflammatory agent.

Lignans

Phytoestrogens are naturally occurring plant compounds which have estrogenic and/or anti-estrogenic activity. They are heterocyclic phenols with structural similarities to estrogenic steroids and are constituents of many foods including beans, sprouts, cabbage, spinach, soyabeans, grains and hops. There are three main groups of phytoestrogens, the isoflavones, coumestans and lignans. Phytoestrogens share several features in common with estradiol, including a pair of hydroxyl groups separated by a similar distance and the

presence of a phenolic ring which is a prerequisite for binding to the estrogen receptor. Based on structure considerations alone, it is not surprising that these compounds bind to estrogen receptors (ER); however, their apparent action as both partial estrogen agonists and antagonists makes it hard to predict how interaction of phytoestrogens with ER will modify the activity of endogenous estrogens.

Lignans are naturally occurring plant components that are structurally similar to the mammalian estrogen and exhibit estrogenicity. Enterodiol (Figure 2) and enterolactone are formed in the gastrointestinal tract from the plant lignan precursors namely matairesinol and seco-isolariciresinol. Few studies have been initiated to thoroughly address factors affecting the absorption, metabolism and excretion of these compounds. Evidence from an in vitro fermentation model suggests that, in a way similar to the isoflavones, the conversion of lignan precursors to their metabolites is enhanced in the presence of high-carbohydrate substrate (90); (91). This suggests that a high-fiber diet may well speed up the conversion of the lignan precursors to the metabolites enterodiol and enterolactone.

Flaxseed is by far the richest dietary source of plant lignans (92). Lignans are not associated with the oil fraction so flaxseed oils do not typically provide lignans. A variety of factors may affect the lignan contents of plants, including geographic location, climate, maturity, and storage conditions (92). The main lignan in flaxseed is secoisolariciresinol diglycoside (SDG) (Figure 2). When flaxseed is eaten, SDG is converted by bacteria in the colon to mammalian lignans: enterolactone and enterodiol. The amount of enterolactone and enterodiol found in the blood and urine of humans and animals is related to the amount of plant lignans eaten (92).

Lignans have both estrogenic and anti estrogenic effects depending on the doses. Supplementation with flaxseed alters estrogen metabolism in postmenopausal women to a greater extent than does supplementation with an equal amount of soy (93). A number of studies support the anti carcinogenic and antioxidant effects of flaxseed lignan i.e. SDG or enterodiol and enterolactone (94); (92); (95); (96).

Prasad et al (7) have shown that flax lignan complex is able to reduce the atherosclerosis plaque formation by 40% , LDL by 14 % and increase HDL by 30 % in hypocholesterolemic rabbits. Lignan complex isolated from flaxseed may, therefore, be beneficial in preventing hypercholesterolemic, atherosclerosis and reducing risk factors for coronary artery disease. It has also been shown to reduce oxidative stress, delay the development of type 2 diabetes in rats and possess potent antioxidant activity, (97); (8). Lignans have also been shown to inhibit established human breast cancer growth and metastasis in a nude mice model (98). Hence, along with its cardiovascular benefits lignans also show anticancer and antioxidant properties which make them component of the diet.

Secoisolariciresinol diglycoside (SDG)

Enterolactone   Enterodiol
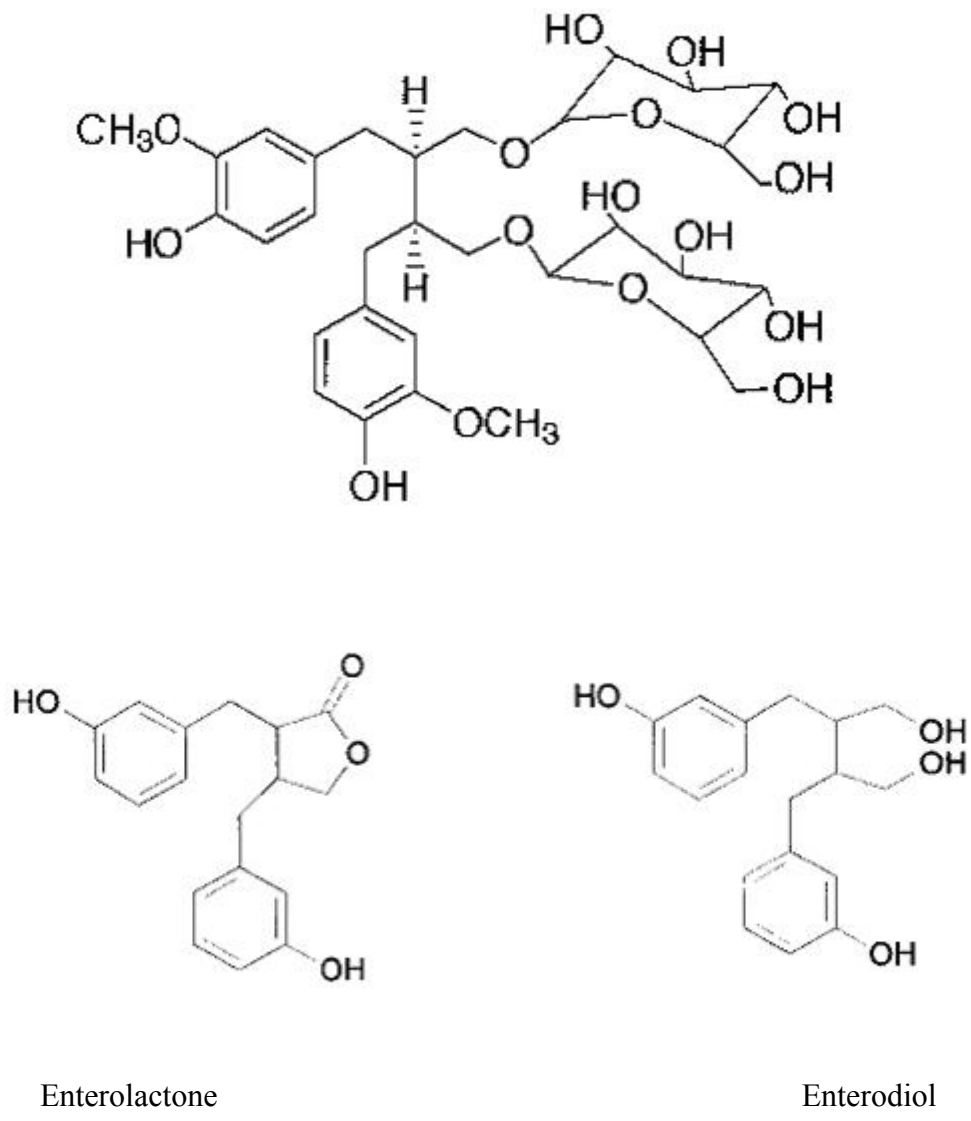
Figure 2. Structure of enterolactone, entrodiol, and Secoisolariciresinol diglycoside

In a prospective cohort study of 1,889 Finnish men followed for an average of 12 years, those with the highest serum enterolactone levels (a marker of plant lignan intake) were significantly less likely to die from coronary heart disease or cardiovascular disease than those with the lowest levels (99). Flaxseed lignans thus have a number of health

benefits including antioxidant, anticarcinogenic, hypocholesterolemic and antiinflammatory properties (94); (92); (95); (96); (89).

Hamsters as a suitable model for postmenopausal hypercholesterolemia

A suitable and economical animal model of ovarian hormone deficiency can greatly enhance the understanding of postmenopausal-elevated risk of coronary heart disease. The male golden Syrian hamster is a small animal model that is frequently used for studying cholesterol metabolism and atherogenesis (100); (101); (102). The rate of cholesterol synthesis in male golden Syrian hamsters can be easily altered in response to changes in cholesterol intake (103). Low doses of dietary cholesterol inhibit synthesis of cholesterol in the hamster, allowing a considerable influx and efflux of cholesterol and bile acids independently from cholesterol synthesis (103). The hamster liver reacts more sensitively than that of the rat in response to a negative sterol balance, with an increase in LDL receptor activity and a less robust alteration in hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase activity. Overall, the plasma lipoprotein profile, bile acid pool composition, and metabolic responses to dietary changes make the hamster more comparable to humans than other frequently used animal models such as the rat or rabbit (103).

However, diet-induced hypercholesterolemia does not model the postmenopausal-associated rise in cholesterol in humans. Arjmandi et al (103) used twenty-two 90-day-old female Golden Syrian hamsters and divided them into two groups. The hamsters were either ovariectomized or sham-operated and given free access to a standard cholesterol-free laboratory diet for 65 days. Ovariectomized hamsters had significantly ($P < 0.05$)

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elevated serum total cholesterol concentrations (16.6%) as well as abdominal fat mass (56%; *P*< 0.01) despite equal food intake compared with the sham-operated group. The observed increase in serum total cholesterol concentrations of ovariectomized hamsters resembles those reported in postmenopausal women (104). These findings, coupled with the known similarities in lipid metabolism between humans and hamsters qualifies the hamster as a model for studying cholesterol metabolism in ovarian hormone deficiency. Ovariectomized hamsters undergo changes in serum cholesterol and fat distribution similar to those experienced by postmenopausal women, and thus may serve as an appropriate model for postmenopausal hypercholesterolemia.

CHAPTER III

MATERIAL AND METHODS

Animals and treatment groups

Forty eight, 6 month old female Golden Syrian hamsters (Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed three per cage and kept in an environmentally controlled laboratory. Hamsters were acclimatized for three days and were given a semi-purified casein based and cholesterol free powdered diet. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee at Oklahoma State University were strictly followed. After acclimation, hamsters (n=12/group) were either ovariectomized (OVX; three groups) or sham- operated (sham; one group). After surgery, hamsters were given one of three dietary treatments for 90 days: control diet (SHAM and one OVX group), diet supplemented with 15% of whole flaxseed (OVX + WF); or diet supplemented with flaxseed oil (OVX + FO). Experimental groups are shown in Table 1. Hamsters were pair fed to the mean food intake of the SHAM operated group and had free access to deionized water. Food intake and body weights were monitored routinely and a twelve hour light: twelve hour dark cycle was followed.

Composition of experimental diets

Compositions of the various dietary treatments are shown in Table 2. Diets had similar carbohydrate, fiber, protein, and fat content and were a modification of the formulation by Terpstra et al. (105). The flaxseed oil group (OVX + FO) had equivalent amount of flaxseed oil to that of 15 % WF. This will determine the contribution of flaxseed oil to the hypocholesterolemic effect of whole flaxseed.

Animal necropsy and processing of tissue samples

After 90 days of dietary treatment, hamsters were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg body weight) and xylazine (5 mg/kg body weight) and bled from the abdominal aorta. The collected blood samples and serum were separated by centrifugation at 1500 x g for 20 min at 4 $^{\circ}$C. Aliquots of serum were frozen and kept at -80 $^{\circ}$C for later analyses. The liver was immediately removed, rinsed with ice-cold saline. The total weight of the liver was recorded. The liver was kept in a sealed container and stored at -80 $^{\circ}$C until analyzed. Small intestine was flushed of its contents using saline, blotted and weighed. Uterus was removed and weighed to confirm the success of ovariectomy. Spleen was weighed and discarded.

Serum and liver lipid parameters

Serum triglycerides (TG) and total- and HDL-cholesterol concentrations were determined enzymatically using commercially available kits from Alfa Wassermann Inc. (West Caldwell, NJ). These tests were performed using the Alfa Wassermann Clinical Analyzer (West Cadwell, NJ) following the manufacturer's instructions. The clinical

analyzer was calibrated using Gemcal reference serum (Alfa Wassermann, Inc; West Caldwell, NJ) before each test. Alfa Wassermann quality control (QC-1 and QC-2) were used as control in all tests. Non-HDL cholesterol concentrations were calculated by subtracting HDL-cholesterol from the total cholesterol.

The reagent for measuring total cholesterol concentrations contains cholesterol esterase, which releases cholesterol from its esters. This released cholesterol and endogenous free cholesterol are both oxidized by cholesterol esterase to produce hydrogen peroxide which forms a red colored quinoneimine complex when combined with 4-aminoantipyrine (AAP) and p-hydroxybenzoic acid. The intensity of the red complex was measured photometrically at 505nm and is directly proportional to cholesterol concentration.

The triglyceride reagent works similarly to the cholesterol assay. The triglycerides are converted to produce H_2O_2 through a series of enzymatic reactions. Hydrogen peroxide produced reacts with p-chlorophenol and AAP in a reaction catalyzed by peroxidase to produce a red colored quinoneimine complex which absorbs strongly at 505 nm and is directly proportional to the triglyceride concentration.

The HDL-C assay uses a unique detergent which solubilizes only the HDL lipoprotein particles, thus releasing HDL cholesterol to react with cholesterol esterase and cholesterol oxidase, in the presence of a chromogen to produce color. The detergent also inhibits the reaction of the cholesterol enzymes with LDL, VLDL and chylomicron lipoproteins by adsorbing to their surfaces. The amount of chromogen formed, determined by measuring the increase in absorbance bichromatically at 592/692nm, is directly proportional to the HDL cholesterol concentration.

Total liver lipids were determined using the Folch gravimetric method (106). Approximately 2 g liver sample was homogenized with 25 ml chloroform: methanol (2:1) mixture, mixed thoroughly and the solution was filtered into 50 ml centrifuge tube. Sodium chloride solution (0.73 %) was added to the chloroform: methanol mixture and shaken well. After the two phases are distinctly separated, the top aqueous layer was completely aspirated. The organic layer was transferred to 25 ml volumetric flask and made upto the mark with chloroform. An aliquot of the organic layer was used for cholesterol analysis and remainder of the solution was poured into a pre-dried and pre-weighed aluminium pan. The solvent was evaporated off under fume hood overnight then dried in an oven at 100 ^{0}C for 1 hour. The aluminium pan was cooled in a dessicator for 30 minutes and weighed. The amount of liver lipid was expressed as mg lipid/ g liver.

Liver total cholesterol was determined using a color reagent of glacial acetic acid– $FeSO_4$–H_2SO_4 (107). The solvent of the aliquot of the liver extract was evaporated to dryness using nitrogen gas under fume hood and then 15 ml saponification solution (30% KOH and 6% pyrogallol in ethanol) was added to all tubes and heated in shaking water bath for 10 minutes at 88 °C. After cooling 5 ml of distilled water and 10 ml hexane were added and shaken vigorously. After the phases were separated, 5 ml of supernatant was evaporated to dryness in culture tube under nitrogen gas. Four hundred μl of acetone: ethanol (1:1), 6 ml saturated $FeSO_4.7H_2O$ in glacial acetic acid, and 2 ml concentrated H_2SO_4 was added into each tube. The solution was vortexed and allowed to stand for 10 minutes and absorbance was measured at 490 nm. The cholesterol concentrations were determined based on the standard curve.

Western blotting analyses

To get SRB1, HMGR, LDL receptor, and 7 α hydroxylase levels in liver, microsomes were prepared for western blot analysis. To prepare liver microsomes, liver was cut in small pieces with scissors in ice cold TEDK-S buffer and then homogenized in cold Teflon glass homogenizer. Homogenate was centrifuged at 10000 rpm for 10 minutes and then the supernatant was ultracentrifuged at 38000 rpm for 60 minutes using a Beckman 50.2 Ti ultracentrifuge (Beckman Coulter, CA). The pellet containing microsomes were resuspended in 1.0 mL TEDK buffer and aliquots were stored at -80 °C.

Eighty micrograms of liver protein from microsomes was separated on an 8% SDS polyarcylamide gel using electrophoresis and transferred onto a PVDF membrane (Immobilon, Millipore) using semi-dry transfer. The immunoblot was blocked for one hour in a TBS solution containing 5% non-fat milk at room temperature. This was followed by over night incubation at room temperature with primary antibodies.

Antibodies for scavenger receptor B1 (Novus Biologicals, Littleton, CO), LDL receptor (Cortex Biochemi, Inc., Davis, CA, USA), 7-α hydroxylase (generously provided by Southwestern Medical Center, Dallas, TX), and HMG CoA reductase (Upstate Cell Signalling solutions, Lake Placid, NY) were diluted in TBS solution with 5 % milk at a dilution of 1:2000, 1:5000, 1:1000 and 1:600, respectively.

Blots were washed with 10 ml TBS and TBS/Tween for 10 minutes three times and then incubated for another two hours at room temperature in a TBS solution containing 5% non-fat milk with secondary antibody anti-rabbit HRP conjugate (Upstate Cell Signalling Solutions, Lake Placid, NY) at a dilution of 1:2000. Membranes were washed with TBS and TBS/Tween as earlier and developed by adding 1 ml each of

chemilumenesent immunostar HRP peroxide buffer and enhancer (Bio-Rad, Hercules, CA) , shaked for 1 min and visualised using the Versadoc system (Bio-Rad,Hercules, CA).

Statistical Analyses

Statistical analyses involved computation of least square means and standard error (SE) of the means for each of the treatment groups using SAS version 9.11 (SAS Institute, Cary, NC). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. Differences were considered significant at $P < 0.05$.

CHAPTER IV

RESULTS

Food intake, body and tissue weights

There were no significant differences in initial body weight as hamsters were randomly assigned to the various treatment groups based on their initial body weights. After 90 days of treatment, body weights among the group were still similar. Hamsters from each group consumed approximately 7 grams of food per day. This food intake was similar among animals as they were pair fed to the mean food intake of the sham group. Ovariectomy caused atrophy of uterine tissue, indicating the success of the surgical procedure. There were no significant differences in liver and small intestine weights among any of the treatment groups (Table 3).

Serum and liver lipid parameters

Figure 1 shows the effects of ovariectomy and diet on serum total cholesterol concentrations. As we have previously observed, ovariectomy significantly increased serum total cholesterol concentrations. Whole flaxseed brought down the OVX- induced increase in serum total cholesterol to the sham level. Flaxseed oil has an intermediate effect on serum total cholesterol concentration. Serum cholesterol was reduced by approximately 12 and 4 % by WF and FO, respectively.

The effects of ovariectomy and diet on other lipid parameters are shown in Table 4. There were no significant differences in serum triglycerides and non-HDL cholesterol concentrations among the groups. Similar to total cholesterol, HDL cholesterol was increased due to ovariectomy. Similar to serum total cholesterol concentrations, HDL-cholesterol was increased (P=0.0212) due to ovariectomy. This increase in HDL concentration was neutralized by FO while WF had an intermediate effect. There were no significant differences in liver total lipids and cholesterol among the groups.

Protein levels of some key enzymes involved in cholesterol metabolism

Protein levels of SRB1, a receptor associated with HDL cholesterol, were slightly (P=0.0591) reduced in ovariectomized hamsters compared to the sham-operated hamsters (Figure 4). Whole flaxseed caused a significant (P=0.0093) decrease in the SRB1 protein levels in comparison to the sham animals but flaxseed oil had an intermediate effect.

Ovariectomy caused an increase in the protein level of LDL receptor compared to sham. WF and FO maintained the OVX-induced increase in protein levels of LDL receptor (Figure 5).

Protein level of 7-α hydroxylase, an enzyme involved in synthesis of bile acids and HMGR, the rate limiting enzyme in cholesterol synthesis was not affected by ovariectomy. However, OVX hamsters fed either WF or FO had higher protein levels of both 7-α hydroxylase and HMGR compared to the sham and OVX animals fed the control diet (Figures 5 and 6).

CHAPTER V

DISCUSSION

This study investigated whether flaxseed oil was as effective as whole flaxseed in lowering serum cholesterol in hamster model of postmenopausal hypercholesterolemia. Our findings show that whole flaxseed was more effective in reducing serum cholesterol levels than flaxseed oil. This may be due to the other components in whole flaxseed that may contribute to its cholesterol-lowering properties. Whole flaxseed contains other components aside from the oil content, which have been shown to have hypocholesterolemic effect, e.g. lignans (7); (8), and fiber (9); (10; 11); (12); (13); (14); (15; 16).

Flaxseed is one of the richest sources of alpha linolenic acids which have also been shown to reduce cholesterol in hamsters (17). Apart from ALA, flaxseed is one of the richest edible source of the lignan precursor's, secoisolariceresinol diglycoside (SDG) and materesinol, which are converted to enterolactone and enterodiol, respectively. Evidence suggests that SDG isolated from flaxseed directly lowers serum cholesterol (7). Flax lignan complex isolated from flaxseed exerts hypocholesterolemic action in hypercholesterolemic rabbits (108). Fiber has been widely accepted as playing a significant role in reducing total blood cholesterol particularly LDL-cholesterol (62); (11). Studies done in both humans (9); (11); (12) and animals (13); (14); (15) suggest that dietary fiber lowers cholesterol by enhancing bile acid synthesis and their fecal excretion which results in increased hepatic cholesterol synthesis.

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Effects of whole flaxseed or its powder on lipid parameters in hypercholesterolemic and non-hypercholesterolemic patients have not been consistent although majority of the results indicate it can modestly reduce total cholesterol and LDL by 1.6% to 18%. Flaxseed reduces plasma total and HDL concentrations to levels similar to the sham groups and it increases the plasma triglyceride levels compared to those treated with control diet (20). Neither liver total lipid cholesterol nor total lipid levels are significantly affected by WF (20). FO has been shown to have inconsistent results with a few studies finding a modest reduction in TG with large doses, but most with no effects (11). In the present study, we observe that WF reduced the ovariectomy induced rises in total serum cholesterol similar to the sham levels while FO has intermediate effect. This indicates that alpha linolenic acid is not the only component involved in hypocholesterolemic properties of WF but other components such as lignans and soluble fiber mucilage may also contribute to its cholesterol lowering properties. Ovariectomy significantly increased HDL levels. WF brought HDL- concentration down although not to the sham levels and FO has no effect on HDL cholesterol. HDL is an important lipoprotein involved in picking up cholesterol deposited by the arteries and delivering it to the liver, thereby reducing the risk of atherosclerosis. Lower HDL levels may be due to the lower levels of total cholesterol in WF treated hamsters compared to sham operated. Levels of non HDL cholesterol and TG are unaffected by WF and FO although triglyceride levels in WF and FO tended to be higher than that of sham which is consistent with our earlier findings (20) .

In this study, we also investigated the effect of whole flaxseed and its oil on some key regulators of cholesterol metabolism: 7-alpha hydroxylase, HMG CoA reductase,

LDL receptor, and SRB1. In the present study, we observe that SRB1 protein levels tended (P=0.0591, P=0.2969) to reduce in ovariectomized hamsters fed control and FO diet. Whole flaxseed further reduced SRB1 protein levels. SRB1 helps in the uptake of cholesterol in the liver which can be removed through the formation of bile acids (50). Reduction of SRB1 levels may be one of the mechanisms by which OVX causes hypercholesterolemia. WF or FO does not increase the levels of SRB1 indicating that flaxseed doesn't exert its hypocholesterolemic effect through modulation of SRB1.

The protein levels of LDL receptors were found to be significantly higher in ovariectomized hamsters and it was not affected by diet. Hepatic LDL receptors bind LDL and release cholesterol in the liver. Higher levels of LDL receptor levels in ovariectomized hamsters may be a response to the increased circulating levels of cholesterol, which may increase the uptake of cholesterol in the liver at least on the tansitory basis. LDL-receptor levels remained unaffected by the incorporation of flaxseed in the diet, indicating that this is not the mechanism by which the hypercholesterolemic action is exerted.

7-α hydroxylase levels were similar between the sham operated and ovariectomized hamsters fed the control diet. OVX hamsters fed WF or FO showed significantly higher 7-α hydroxylase protein levels. 7-α hydroxylase is the key enzyme involved in bile acid synthesis (31; 35), thus it has a very important role in getting rid of excess cholesterol. In ovariectomized hamsters fed control diet, lower levels of 7-α hydroxylase may indicate less clearance through the liver which can lead to elevated circulating cholesterol levels. WF and FO showed significantly higher levels of 7-α hydroxylase than Sham and OVX groups. Significantly higher levels of 7-α hydroxylase in both WF and FO indicate that it

may be the major reason behind the cholesterol lowering effect of WF and FO. Up regulation of 7-α hydroxylase may be one of the key mechanism by which flaxseed and its oil can bring about reduction in serum cholesterol.

In the results, we observed that HMGR levels were significantly higher in WF and FO groups than in Sham and OVX group. HMGR is the rate controlling enzyme in cholesterol biosynthesis. It has been shown earlier (35) that HMGR levels are modulated by the cholesterol levels in the body in a negative manner. That explains the lower levels of HMGR in ovariectomized hamsters since they have significantly higher levels of cholesterol. In case of WF and FO they had lower levels of cholesterol; hence in order to meet the cholesterol demand HMGR was up regulated. This finding points towards the possibility that flaxseed per se has no effect on HMGR levels rather this enzyme levels are modulated based on the cholesterol levels in the body.

In summary, the findings after present study show that WF as a whole is more potent in reducing circulating levels of cholesterol than FO. The cholesterol lowering effect of whole flaxseed cannot be totally attributed to its oil content but there are other components that may be responsible such as lignans and fiber. Our findings indicate that the main pathway in cholesterol metabolism involved in cholesterol lowering property of flaxseed is by increasing the protein levels of 7-α hydroxylase which is involved in the conversion of cholesterol to bile.

Table 1

Treatment groups and experimental diets

Treatment Groups (n=12/group)	Experimental Diets
Sham	Semi purified casein based and cholesterol free powdered diet (control)
OVX	Control
OVX + WF	Control supplemented with 15 % (w/w) whole flaxseed (WF)
OVX + FO	Control supplemented with flaxseed oil (FO) equivalent to the oil content provided by 15 % WF

WF = Whole flaxseed
FO = Flaxseed oil

Table 2

Composition of experimental diets

Ingredient	Control Diet	15% Whole Flaxseed (WF) g/kg diet	Flaxseed Oil equivalent to 15% (WF)
Carbohydrates (total)	395.6	395.6	395.6
Rice Flour[a]	395.6	344.4	395.6
Flaxseed[b]	0.0	51.2	0.0
Fiber (total)	144.4	144.4	144.4
Wheat bran[c]	72.2	72.2	72.2
Cellulose[d]	72.2	59.3	72.2
Flaxseed[b]	0.0	12.9	0.0
Protein (total)	240.6	240.6	240.6
Casein[c]	240.6	205.6	240.6
Flaxseed[b]	0.0	35.1	0.0
Fats (total) [e]	154.0	154.0	154.0
Hydrogenated coconut oil[d]	96.3	96.3	96.3
Safflower oil [d]	19.3	2.3	2.3
Soybean oil [d]	38.5	4.6	4.6
Flaxseed [b]	0.0	50.8	50.8
Choline chloride [d]	2.9	2.9	2.9
Potassium bicarbonate [g]	19.3	19.3	19.3
Vitamin mix [h]	9.6	9.6	9.6
Mineral mixture [i]	33.7	33.7	33.7

a California Natural Products (Lathrop, CA, USA).

b Flaxseed is of the variety NORBI and from Agriculture and
  Agri-Food Canada, Saskatoon, Sask., Canada.

c Natural Ovens of Manitowoc (Manitowoc, WI, USA).

d Harlan-Teklad (Madison, WI, USA).

e Of the total dietary fat, approximately 57.2% is saturated fat, 11.4%
  is monounsaturated fat, and 26.8% is polyunsaturated fat for all dietary
  treatments.

f Flaxseed oil from Barleans (WA, USA)

g Sigma Chemicals (St. Louis, MO, USA).

h Vitamin mixture, (TD #40060, Harlan Teklad, Madison, WI, USA,
  g/kg): *p*-aminobenzoic acid, 11.0132; ascorbic acid, coated (97.5%), 101.664; biotin, 0.0441;   folic
  acid, 0.1982; Vitamin B12 (0.1% in mannitol), 2.9736; calcium panthothenate, 6.6079; choline
  dihydrogen citrate, 349.6916; inositol, 11.0132; menadione, 4.9559; niacin, 9.9119; pyridoxine HCl,
  2.2026; riboflavin, 2.2026; thiamine HCl, 2.2026; dry Vitamin A palmitate (500,000 U/g), 3.9648; dry
  Vitamin D3 (500,000 U/g), 0.4405; dry Vitamin E acetate (500 U/g), 24.2291; corn starch, 466.6878.

i Mineral mixture (TD #170911, Harlan Teklad, Madison, WI, USA,
  g/kg): Ca, 6.232; P, 3.993; K, 3.829; Na, 2.109; Cl, 3.472; S, 0.644; Mg, 0.465; I, 0.0006; Fe,   0.0252;
  Cu, 0.0052, Mn, 0.0501; Zn, 0.0118.

Table 3

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on food

intake, body and tissue weights

Measures	SHAM	OVX	OVX + WF	OVX + FO
Food intake, *g/day*	7.3 ± 0.2	6.7 ± 0.2	6. 7 ± 0.2	6.4 ± 0.2
Body weight, *g*				
Initial	161 ± 4	162 ± 4	162 ± 4	163 ± 4
Final	162 ± 4	169 ± 4	166 ± 4	160 ± 4
Tissue weights, *g*				
Uterus	0.505 ± 0.027[a]	0.153 ± 0.026[b]	0.173 ± 0.026[b]	0.167± 0.028 [b]
Liver	4.36 ± 0.18	4.64 ± 0.17	4.48 ± 0.17	4.32 ± 0.19
Small Intestine	2.20 ± 0.15	2.08 ± 0.14	2.19 ± 0.14	2.45 ± 0.15

Values are mean ± SE, n=12;means that don't share the same letters are different at P<0.05.

Table 4

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on serum

and liver lipid parameters

Measures	SHAM	OVX	OVX+WF	OVX+FO
Serum lipid, *mg/dL*				
HDL cholesterol	82.1 ±4.2^b	97.0 ±4.1^a	86.9±3.9^{ab}	94.2±4.1^a
Non-HDL cholesterol	49.2±2.8	53.1±2.7	45.3±2.6	49.6±2.7
Triglycerides	53.2±9.8	74.2±9.4	82.3±9.0	74.3±9.4
Liver lipids, *mg/g liver*				
Total lipids	48.8±2.6	49.9±2.5	47.2±2.5	46.3±2.7
Cholesterol	4.72±0.32	4.75±0.31	4.50±0.31	4.90±0.34

Values are mean ± SE, n=12;means that don't share the same letters are different at P<0.05.
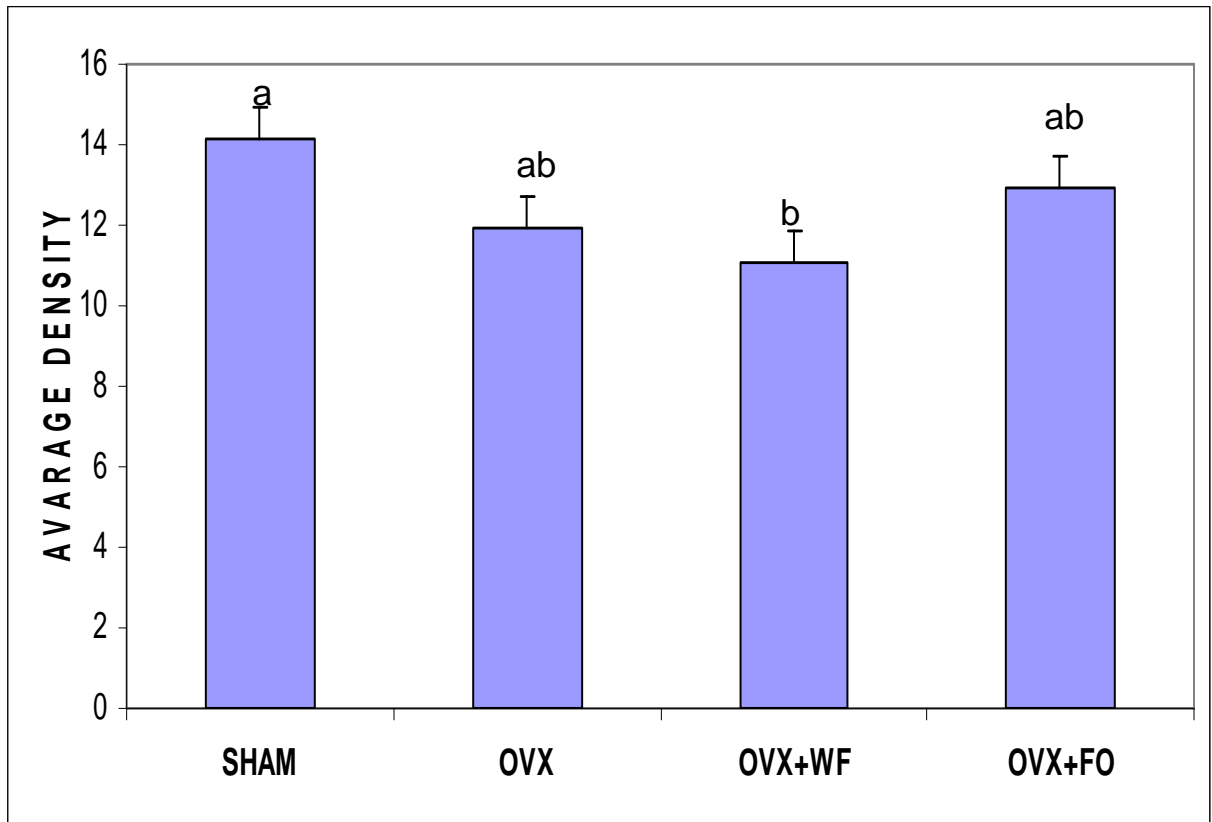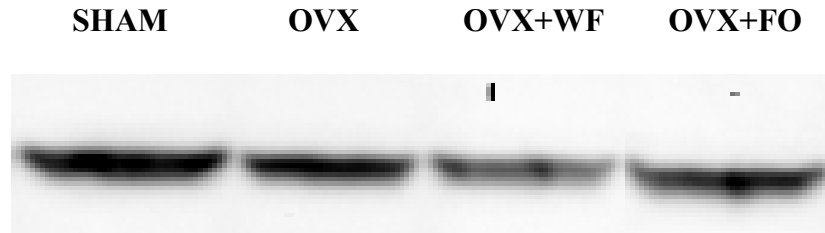
Figure 3

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on serum

total cholesterol concentrations of Golden Syrian hamsters
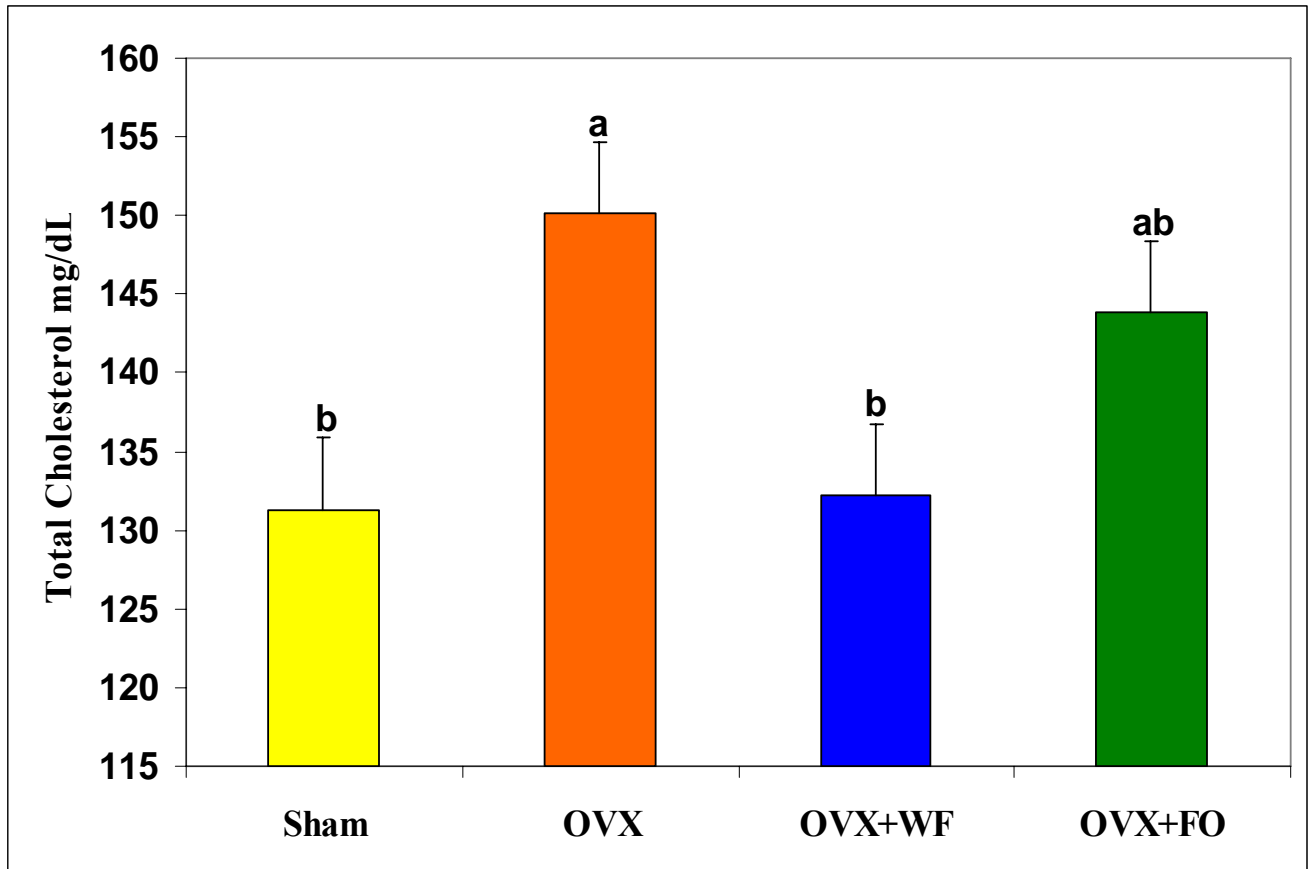
Values are mean ± SE, n=12;bars that don't share the same letters are different at P<0.05.
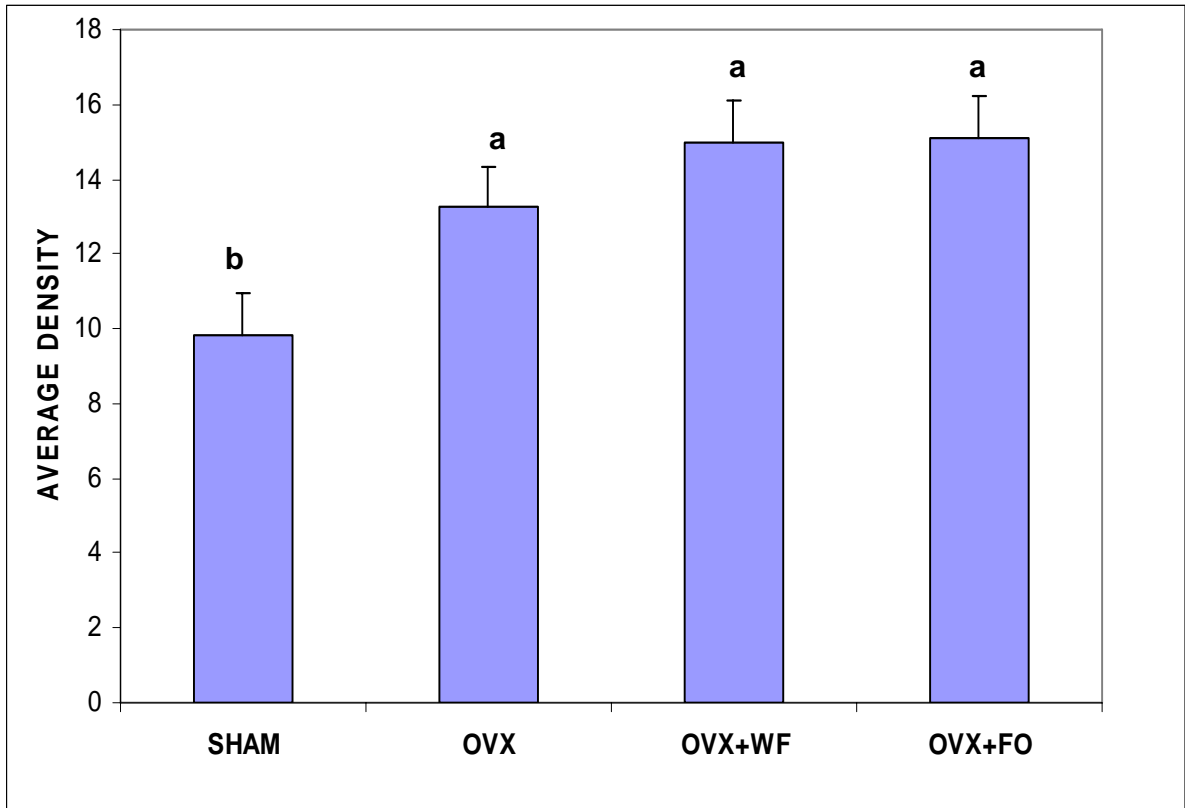
Figure 4

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on protein levels of Scavenger receptor B1 (SRB1) levels in liver microsomes of Golden Syrian hamsters

Values are mean ± SE, n=3;bars that don't share the same letters are different at P<0.05.

Figure 5

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on protein levels of LDL receptor in liver microsomes of Golden Syrian hamsters

Values are mean ± SE, n=3;bars that don't share the same letters are different at P<0.05.

Figure 6

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on protein levels of 7-α hydroxylase in liver microsomes of Golden Syrian hamsters

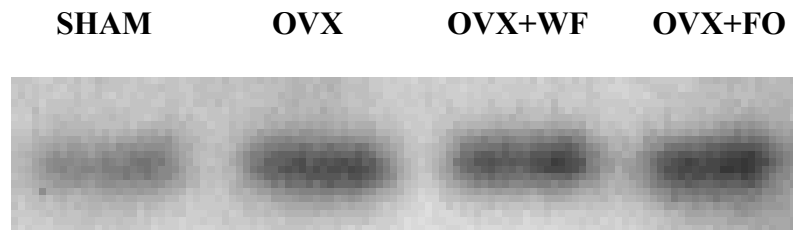*Values are mean ± SE, n=3;bars that don't share the same letters are different at P<0.05.*

Figure 7

Effects of ovariectomy (OVX), whole flaxseed (WF), and flaxseed oil (FO) on protein levels of HMG CoA reductase in liver microsomes of Golden Syrian hamsters

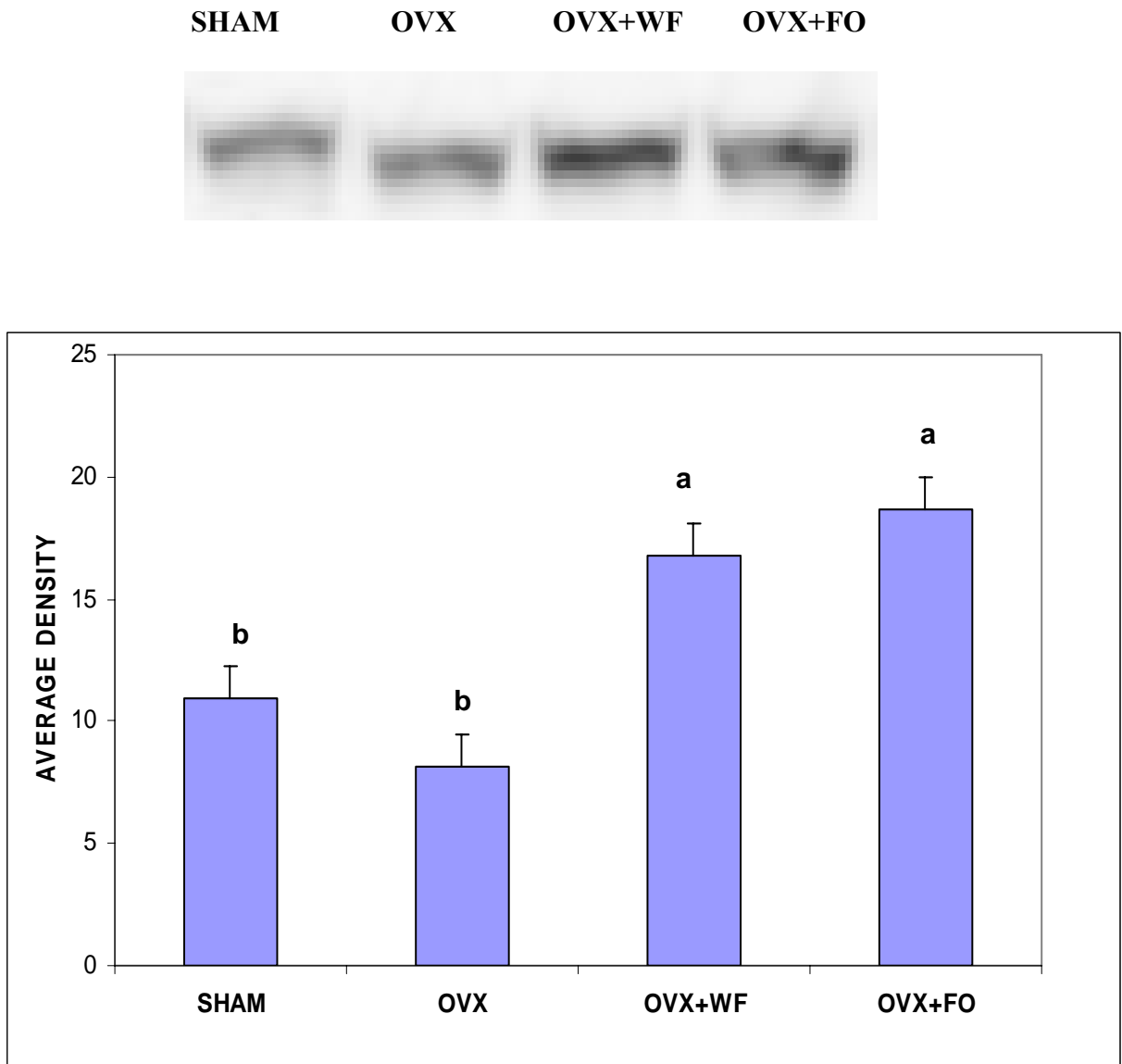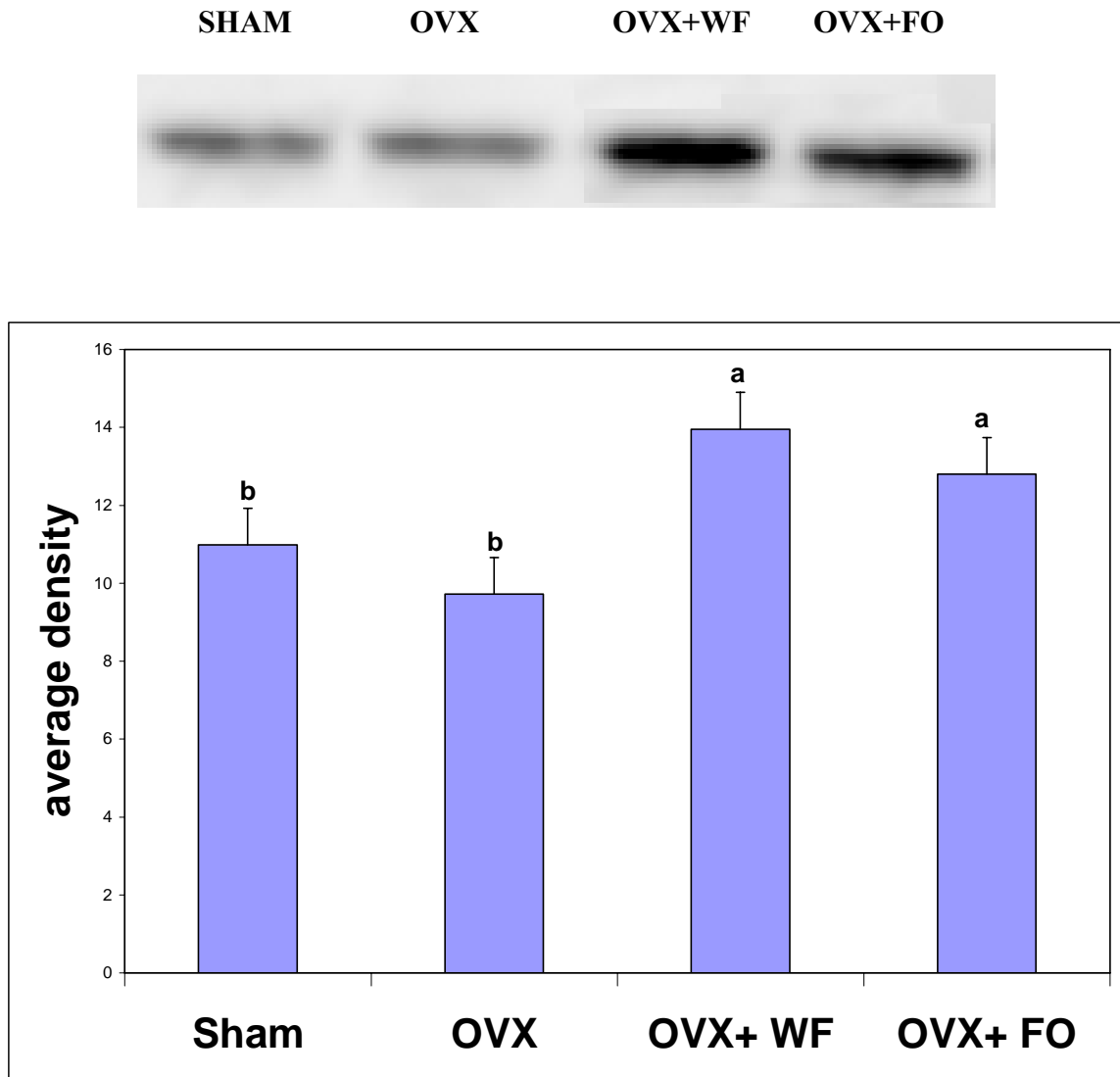*Values are mean ± SE, n=3, bars that don't share the same letters are different at P<0.05.*

APPENDIX

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol Expires: 2/6/2006

Date : Friday, February 07, 2003   Animal Care and Use Protocol (ACUP) No : HE033

Proposal Title: Hypocholesterolemic Mode of Action of Whole Flaxseed

Principal
Investigator:

Edralin Lucas   Bahram Arjmandi
Nutritional Sciences   Nutritional Sciences
425 HES   416 HES
Campus   Campus

Reviewed and   Full Committee
Processed as:

Approval Status Recommended by Reviewer(s) :   Approved

Protocol approved for 3 years and for a total of 96 hamsters.

Signatures :

Kent Olson, IACUC Chairperson   Friday, February 07, 2003
                                         Date

cc: Department Head, Nutritional Sciences
    LAR

Approvals are valid for three calendar years, after which time a request for renewal must be submitted. Any modifications
to the research project, course, or testing procedure must be submitted for review and approval by the IACUC, prior to
initiating any changes. Modifications do not affect the original approval period. Approved projects are subject to
monitoring by the IACUC. OSU is a USDA registered research facility and maintains an Animal Welfare Assurance
document with the Public Health Service Office of Laboratory Animal Welfare, Assurance number AA3722-01.

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VITA

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Thesis: HYPOCHOLESTEROLEMIC EFFECT OF FLAXSEED
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Scope and Method of Study: The health benefits of functional foods, such as flaxseed in lowering cholesterol and lowering the risks of atherosclerosis have already been discovered. Our earlier findings indicate that flaxseed reduced plasma cholesterol induced by ovarian hormone deficiency in Golden Syrian hamsters. This study was designed to investigate whether flaxseed oil (FO) exerts the same hypocholesterolemic effect as whole flaxseed (WF) and to gain insights into the hypocholesterolemic mechanism of action. Forty eight, 6-month-old female, Golden Syrian hamsters were either sham-operated (sham) or ovariectomized (OVX) and randomly assigned to four groups: sham, ovx, ovx plus either WF [15% diet] or FO [equivalent oil content to that of WF diet] and fed for 90 days.

Findings and Conclusions: OVX elevated plasma total cholesterol concentrations in Golden Syrian Hamsters and WF was more effective than FO in reducing the increase in circulating cholesterol. To elucidate the hypocholesterolemic action, we quantified some key regulators of cholesterol metabolism in the liver microsomes using western blot analysis. Results indicate that flaxseed does not exert its hypocholesterolemic action by modulating either of scavenger receptor BI (SRBI), HMG CoA reductase or LDL receptor instead flaxseed and its oil upregulated 7-α hydroxylase, an enzyme involved in bile acid synthesis, leading to cholesterol lowering effect.

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