FLAXSEED CONSUMPTION POSITIVELY INFLUENCES LIPID PROFILES IN POSTMENOPAUSAL WOMEN

By

LISA JEAN HAMMOND

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Oklahoma State University

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Thesis Advisor

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NOMENCLATURE

AHA	American Heart Association
ALA	alpha-linolenic acid
Apo A	apolipoprotein A-1
Apo B	apolipoprotein B
CRP	c-reactive protein
CHD	coronary heart disease
CVD	cardiovascular disease
DHA	docosahexaenoic acid
EPA	eicospentaenoic acid
ERT	estrogen replacement therapy
FDA	Food and Drug Administration
HDL	high-density lipoprotein
HRT	hormone replacement therapy
LDL	low-density lipoprotein
MUFA	monounsaturated fatty acid
NCEP	The National Cholesterol Education Program
NHANES	National Health and Nutrition Examination Survey

PUFA	polyunsaturated fatty acid
SERM	selective estrogen replacement therapy
TC	total cholesterol
TG	triglyceride
VLDL	very low-density lipoprotein

CHAPTER I

INTRODUCTION

Coronary heart disease (CHD) is the number one cause of death in men and women in all developed countries (Bales, 2000). Premenopausal women have a lower incidence of heart disease than their male counterparts, however, following menopause the CHD rate for women is virtually the same as men (AHA, 2002).

Ovarian hormone deficiency brings about physiological changes that are concurrent with an increased risk for heart disease; most predominant is the undesirable alteration in serum lipids (Nathan & Chaudhuri, 1997). Elevated lipoproteins in the blood have been strongly correlated with an increased risk for CHD (Kannel et al., 1971).

Observational studies have shown that the use of exogenous estrogen in the form of estrogen replacement therapy (ERT) can reduce and sometimes prevent these unfavorable cardiovascular changes brought about by menopause. Clinical studies have also supported the role of ERT in correcting dyslipidemias, but the long-term benefits of ERT still need to be investigated. In fact, recent clinical studies have failed to show long-term cardioprotective benefits of ERT (Hulley et al., 1998). Also, unopposed estrogen use is associated with an increased risk for uterine and breast cancer, and for thrombosis (Tsang et al., 2000). The addition of progesterone to the hormone replacement therapy (HRT) lowers the risk of hormone-induced endometrial hyperplasia but it may also reduce the cardiovascular benefits of the exogenous estrogen (Tsang et al., 2000).

Along with the undesirable side effects and risks of ERT, many women are unwilling to take medications on a long-term basis or unable to afford it (Keating et al.,

1999; Rabin et al., 1999). Only a maximum of 30% of postmenopausal women use ERT (Lobo, 1995) and women often discontinue its use after only one year (Barrett-Connor et al., 2000).

Researchers are attempting to develop safer alternatives to ERT. Selective estrogen receptor modulators (SERMs) may be a viable option. SERMs are a group of medications that possess tissue specific estrogenic or antiestrogenic effects (Grese & Dodge, 1998; Weryha et al., 1999; Cosman & Lindsay, 1999). These compounds were first developed for the treatment of estrogen receptor positive breast cancer and were found to not only function as estrogen antagonists in breast but, at the same time, as estrogen agonists in bone and cardiovascular tissue (Cosman & Lindsay, 1999). These first generation SERMs, like tamoxifen, were found to increase the risk of some uterine diseases but second generation SERMs, like raloxifen, do not cause uterine stimulation (Cosman & Lindsay, 1999; Grese & Dodge, 1998). Pharmaceutical research is being conducted to develop SERMs that could replace traditional HRT by relieving menopausal symptoms such as hot flashes, heart disease, and osteoporosis, without the side effects of estrogen (Grese & Dodge, 1998; Weryha et al., 1999; Cosman & Lindsay, 1999).

Recent reports indicate that phytoestrogens exert their effects in a similar manner as SERMs (Brzezinski & Debi, 1999). Phytoestrogens are non-steroidal compounds found in a variety of plants. Food sources rich in phytoestrogens may provide women with a practical and safe alternative for the alleviation of postmenopausal increases in cholesterol levels.

Diet has been shown to influence serum cholesterol levels and heart disease outcomes. Phytoestrogens are found in many foods including legumes, grains, sprouts,

fruits, and vegetables (Setchell & Cassidy, 1999). The three major classes of phytoestrogens are isoflavones, lignans, and coumestans (Thompson, 1998). Flaxseed is the richest source of lignans (Thompson, 1998), a group of polyphenolic compounds with antioxidant activity. Flaxseed is believed to have many health benefits including: protection against certain forms of cancer (Thompson, 1998); reduction in cardiovascular disease risk (Arjmandi et al., 1998; Cunnane et al., 1995; Prasad et al., 1998); and, partly due to its anti-inflammatory properties, impedance in the progression of kidney disease (Clark et al., 1995; Ogborn et al., 1999, Velasquez & Bhathena, 2001).

The **hypothesis** of this study is that the daily consumption of flaxseed by postmenopausal women, not on HRT, improves their lipid profiles as well as reduces serum levels of c-reactive protein (CRP), a general marker of inflammation. To test this hypothesis we have three specific aims:

Specific aim 1: To determine whether daily consumption of 40 grams ground flaxseed, by postmenopausal women, reduces total cholesterol (TC), low-density lipoprotein (LDL) -cholesterol, and triglyceride (TG) levels.

Specific aim 2: To evaluate whether daily consumption of 40 grams ground flaxseed, by postmenopausal women, maintains/increases high-density lipoprotein (HDL) cholesterol and apolipoprotein A (apo A) levels, both of which have favorable effects on lipid profiles.

Specific aim 3: To examine whether daily consumption of 40 grams ground flaxseed, by postmenopausal women, decreases serum levels of CRP.

CHAPTER II

REVIEW OF LITERATURE

PREVALENCE OF HEART DISEASE IN THE UNITED STATES

Coronary heart disease (CHD) is the leading cause of mortality in the United States for men and women as well as the leading cause of death in all other industrialized countries (AHA, 2002). About 12.4 million Americans have some form of CHD (AHA, 2002).

Economically, CHD has a severe impact. In the United States alone the direct costs of health care as well as indirect costs of loss in productivity as a result of CHD are currently estimated to be around $100.8 billion annually (AHA, 2002).

CHD mortality rates are 50.8% and 49.2% for men and women respectively but it is a common misconception that women have a much lower risk for developing heart disease than men, due to the fact that heart disease manifests in women 10 years later than in men. In the Framingham study, 2873 women were monitored for 24 years and during that time no premenopausal women experienced a myocardial infarction or died from coronary heart disease (Gordon et al., 1978). Researchers found that the incidence of CHD in postmenopausal women, between the ages of 45 to 54 years, was twice that of premenopausal women of the same age group (Gordon et al., 1978). Subsequent human trials have resulted in consistent findings (Nathan & Chaudhuri, 1997). These gender differences have been seen in laboratory animals as well (Nathan & Chaudhuri, 1997). It was this increase in CHD in women corresponding to the cessation of ovarian hormone production that led researchers to investigate the cardioprotective effects of estrogen.

Risk Factors for Coronary Heart Disease Following Menopause

There are many risk factors for heart disease: age, family history of premature CHD, elevated TC, elevated LDL-cholesterol, low levels of HDL-cholesterol, high serum TG, elevated CRP, hypertension, current smoking, and obesity are just a few examples (Davidson & Maki, 1998). Women potentially have the additional risk factor of postmenopausal status without ERT.

This classification is due to the epidemiological evidence showing an increase in the incidence of CHD following menopause. Menopause brings about physiological changes that can negatively affect the cardiovascular system. Studies have shown that following menopause, there is a dramatic increase in blood pressure (Staessen et al., 2001). The increase in blood pressure is believed to be due to changes in vascular tone and/or changes in endothelium-derived chemicals that influence dilation and constriction of blood vessels (Koh et al., 2001; Brooks et al., 1997). One study conducted by Noto et al. (2000) involving 24-hour blood pressure monitoring revealed periodic increases in blood pressure of postmenopausal women throughout the 24-hour period. These increases were more pronounced during sleep and in those with surgical menopause. An increase in intra-abdominal fat is also seen which can put women at an increased risk for insulin resistance and CHD (Tchernof & Poehlman, 2000; Toth et al., 2000).

More extensively studied is the change in serum lipid levels that coincide with cessation of ovarian function. The Framingham Study revealed that following surgical or natural menopause, women experienced a significant rise in total cholesterol (Hjortland et al., 1976). Cholesterol levels for this report were from examinations performed two years apart, one prior to menopause and one after, therefore this increase was considered to be

seen within a short period of time. Since the Framingham results, studies have consistently shown an increase in TC and LDL-cholesterol concentrations following menopause (Pansini et al., 1993; Kim et al., 2000; Pasquali et al., 1997; Fukami et al., 1995; Stevenson et al., 1993; de Aloysoio et al., 1999). However, the effect of menopause on HDL-cholesterol levels remains contentious. Studies have shown that, with the onset of menopause, HDL-cholesterol concentrations decrease (Tsang et al., 2000) while other studies have shown an increase in HDL-cholesterol concentrations (Kim et al., 2000; Pansini et al., 1993). HDL-cholesterol levels have also been shown to remain constant during the transition from premenopausal to postmenopausal (Gardner et al., 2000; Fukami et al., 1995; Pasquali et al., 1997; de Aloysio et al., 1999). Study findings on TG levels following menopause are also varied. While some studies have found no change in TG levels (Gardner et al., 2000; Fukami et al., 1995; Pasquali et al., 1997; de Aloysio et al., 1999), others have reported increases (Kim et al., 2000; Tsang et al., 2000).

Another risk factor for heart disease is elevated CRP concentrations. CRP is produced in the liver, along with other acute phase proteins, following cytokine stimulation (Lagrand et al., 1999). CRP is a sensitive marker of inflammation due to bacterial infection, trauma, or tissue damage (Koenig et al., 1999). Serum levels of CRP respond rapidly to inflammation as well as its resolution (Du Clos et al., 2000). CRP is believed to function as either a surveillance molecule (Du Clos, 2000) or a host defense (Volanakis, 2001) to protect against pathogens and to remove necrotic cells. Normally, CRP levels are less than 8 mg/dL. Elevated CRP levels in apparently healthy individuals are associated with increased risk of cardiovascular events and increases following a myocardial infarction correlate with severity and outcome (Lagrand et al., 1999; Ridker

et al., 2000). Therefore, an elevated CRP levels with or without other CHD risk factors is cause for alarm and modifications of lifestyle.

PREVENTION AND TREATMENT OF CORONARY HEART DISEASE IN WOMEN

LIFESTYLE MODIFICATION

The first step in treating hypercholesterolemia, depending on the degree of severity and the existence of concomitant CHD risks, is lifestyle changes. Changes may include smoking cessation, weight reduction, increased physical activity and dietary modification.

The National Cholesterol Education Program (NCEP) and the American Heart Association (AHA) recommendations include; a diet high in plant foods, choosing fat-free or low fat dairy and meats, and consuming two servings of fish per week (AHA Scientific Position). These guidelines are specified in the AHA's Step I and Step II diets. The AHA no longer uses the designations "Step I" and "Step II" but the dietary guidelines remain the same. The revised guidelines focus on an overall healthy diet and not on specific numbers and percentages. On the Step I diet, 30% or less of the total calorie intake comes from fat. Of that 30%, less than 7% is to come from saturated fat, up to 10% from polyunsaturated fat and 20% or less from monounsaturated fat. The Step I diet also limits the intake of cholesterol to less than 300 mg/day. The Step II diet is used if CHD is already present or if lipid goals are not met after following the Step I diet. It further restricts intakes of cholesterol and saturated fat.

The Step I and Step II diets can reduce LDL-cholesterol levels as much as 9% and 20%, respectively (Stone et al., 1996; Bunyard et al., 2002). It has been shown repeatedly

that reduction of dietary cholesterol and saturated fat will reduce the risk of CHD (Gylling & Miettinen, 2001). Other components of a cholesterol lowering diet are soluble fibers, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

It has been well documented that diets high in fiber reduce the risk of heart disease. Clinical studies have shown that fiber reduces LDL-cholesterol by an average of 9% (Fernandez, 2001). Fiber has also been shown to increase HDL-cholesterol in rats (Bagger et al., 1996). A prospective cohort study of participants from the first National Health and Nutrition Examination Survey (NHANES I) data showed that a diet high in soluble fiber, in the form of legumes, is inversely related to CHD risk (Bazzano et al., 2001). One study revealed a significantly greater decrease in TC and LDL-cholesterol in a hypocaloric diet that included oats compared to a hypocaloric control diet (Saltzman et al., 2001).

The AHA's recommendation to substitute a portion of dietary saturated fat with MUFAs such as canola oil and olive oil, and PUFAs such as soybean oil, corn oil, fish oil, and flaxseed oil are based on studies showing improved lipid profiles as a result of these dietary changes. A high-fat (34% of calories from fat) MUFA diet reduced TC by 10%, LDL-cholesterol by 14%, and TG by 13% (Kris-Etherton et al., 1999). Gustafsson et al. compared the lipid lowering effect of MUFAs in the form of canola oil with PUFAs in the form of sunflower oil and found similar reductions in total-, LDL-, and HDL-cholesterol (Gustafsson et al., 1994). Triglyceride levels increased significantly in both groups with a much greater increase in the PUFA group.

The AHA's recommendation to consume fish twice a week is partly the result of studies associating high fish consumption with a low risk of CHD. Marine oils contain α-

linolenic acid (ALA), an essential omega-3 fatty acid, and its metabolites eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Mantzioris et al., 2000). At high doses omega-3 fatty acids from fish oils lower cholesterol and triglycerides and have anti-thrombotic and anti-inflammatory activity (Simopoulos et al., 1991). Consumption of flaxseed oil, another rich source of ALA, results in increases in tissue EPA but not DHA while consumption of fish oil results in elevated tissue concentrations of both EPA and DHA (Mantzioris et al., 2000). However, clinical studies using ALA in the form of flaxseed oil have shown that flaxseed oil may also protect against cardiovascular disease. Study results have also indicated increased arterial compliance or elasticity (Nestel et al., 1997) and reduced platelet aggregation (Allman et al., 1995).

Many different food items labeled functional foods are under investigation for their lipid lowering effects; soy foods, flaxseed, oatmeal, garlic, and grapes are just a few examples. In 1999 the Food and Drug Administration (FDA) approved the health claim that the daily consumption of 25 grams of soy protein reduces the risk of heart disease (DHHS, 1999). The group petitioning the FDA presented more than 40 studies verifying the health benefit of soy foods. The FDA agreed that soy protein reduces TC and LDL-cholesterol but the component that is behind this lipid lowering effect has yet to be determined; it could be isoflavones, saponins, phytic acid, trypsin inhibitors, fiber, or globulins present in soy protein (Hasler et al., 2000).

Cholesterol Lowering Drugs

Diet modification and weight loss are quite effective in lowering LDL-cholesterol and TC, and exercise can increase HDL-cholesterol levels but if these lifestyle changes

are not possible or are not sufficient, medications will often be prescribed. There are a variety of drugs with different mechanisms of action. The type of drug chosen depends on the nature of the dyslipidemia. Statin or other 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor drugs inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Statin is the most potent of the cholesterol lowering drugs but its expense does limit its use. These drugs lower LDL-cholesterol and TG concentrations and can raise HDL-cholesterol concentrations in some individuals. Niacin increases HDL-cholesterol and decreases LDL-cholesterol and TG levels but can cause itching and flushed skin due to the release of histamine and prostaglandins. However, timed-release niacin reduces these symptoms. Fibric acid derivatives such as gemfibrozil and fenofibrate have little effect on LDL-cholesterol levels but they do reduce TG and increase HDL-cholesterol concentrations. Bile acid sequestrants are effective in reducing LDL-cholesterol levels but may raise TG levels (Kuncl & Nelson, 2000).

Hormone Replacement Therapy

ERT may prevent cardiovascular changes, such as increased serum cholesterol levels, that lead to CHD. Much of our current knowledge of the cardioprotective effects of estrogens has been derived from studies involving ERT or HRT. Estrogen therapy has been shown to improve lipid profiles and through direct action on vascular endothelium improve blood pressure and slow the buildup of atherosclerotic plaque (Nathan & Chaudhuri, 1997).

Atherosclerosis is responsible for the majority of CHD related deaths (AHA, 2002). It is an inflammatory condition characterized by the development of plaque on the

walls of blood vessels. Myocardial infarction or stroke can result when plaque becomes dislodged forming a thrombus. The mechanisms behind estrogens ability to slow the progression of plaque development are not well understood. Although the benefits of estrogen may affect any stage in the progression, it is the early stages, such as cholesterol reduction, that have been most extensively studied. It has been estimated that the improvement of serum lipid levels is responsible for 20% of estrogens benefit (Rosano & Panina, 1999).

Animal and human studies have repeatedly shown estrogens ability to reduce serum LDL-cholesterol levels and increase HDL-cholesterol levels. ERT also increases circulating TG concentrations in the form of very low-density lipoprotein (VLDL) particles. Although VLDL particles are generally considered to be atherosclerotic, those produced following the use of exogenous estrogens are believed to be less likely to enter the arterial wall because they are buoyant and cholesterol-poor (Knopp et al., 1996).

Estrogen increases all major pathways for cholesterol transport (Knopp et al., 1996). Upregulation of LDL receptors allows more LDL particles to be taken up by the liver and used for the production of steroids or bile salts. HDL-cholesterol production is increased, as is the production of apo A. There is also a decrease in hepatic lipase activity, this causes LDL-cholesterol particles to accumulate a higher percentage of triglycerides resulting in a larger and more buoyant molecule that is less likely to become trapped in the arterial wall. Hepatic lipase also increases the uptake of HDL-cholesterol; therefore, by decreasing the activity of hepatic lipase with estrogen more HDL-cholesterol remains in circulation (Knopp et al., 1996).

Several observational studies have shown that ERT is associated with a decrease in all cause mortality rates by 30-50% (Greendale et al., 1999). Previously, the majority of the research conducted on ERT and CHD has been observational and animal studies. In the last five years however, large clinical studies have been conducted to investigate the effects of ERT on CHD risk factors and CHD events in postmenopausal women with and without heart disease. Some of the findings have been surprising and all together inconclusive. The Heart and Estrogen/Progestin Replacement Study (HERS) found no advantage to HRT in women with heart disease. The study included 2763 postmenopausal women less than 80 years of age with CHD. The intervention consisted of either 0.625mg conjugated equine estrogen plus 2.5mg medroxyprogesterone acetate or placebo. After 4 years, no differences were seen between the two groups and during the first year of the study there was actually a greater incidence of CHD-related events in the treatment group (Hulley et al., 1998). This increased incidence of CHD may have been due to the prothrombotic or proinflammatory effect of exogenous estrogen (Herrington & Klein, 2001). In the Estrogen Replacement and Atherosclerosis (ERA) study, 309 postmenopausal women with coronary artery atherosclerosis were randomly assigned to one of three treatment groups; unopposed conjugated equine estrogen, estrogen plus medroxyprogesterone, or placebo. After three years of treatment, no differences were seen between the three groups in the progression of atherosclerosis (Herrington & Klein, 2001). The only study to look at the effect of estrogen in primary prevention of CHD was the Estrogen in the Prevention of Atherosclerosis Trial (EPAT). This study was a two-year placebo controlled study of 199 postmenopausal women. The subjects had elevated LDL-cholesterol levels but no evidence of CHD. Women with

diabetes mellitus or a history of smoking were excluded from the study. Those with LDL-cholesterol levels greater than 160 mg/dL were given lipid lowering medications. In this study estrogen was beneficial, the subjects that received unopposed estrogen had a lower rate of progression of atherosclerosis compared to those that received the placebo as measured by intima-media thickness of the right common carotid artery (Hodis et al., 2001; Herrington & Klein, 2001).

It has been reported that less than 30% of postmenopausal women in the United States receive HRT (Lobo, 1995) and out of these women most discontinue use after one year (Barrett-Connor et al., 2000). Most women begin taking ERT for relief of hot flashes and stop taking them when the potential for hot flashes has passed. Women may also quit taking ERT for financial reasons (Barrett-Connor et al., 2000). Resistance to HRT use is, in part, due to the risk of side effects such as irregular bleeding and the increased risk for the development of certain cancers and thrombosis (Lobo, 1995). These undesirable effects of estrogen therapy are a result of its non-specific estrogenic effect.

Selective Estrogen Receptor Modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are nonsteroidal compounds that produce tissue-specific estrogenic or antiestrogenic response (Weryha et al., 1999). SERMs were initially designed as antiestrogens for the treatment of estrogen receptor positive breast cancer. Following the widespread use of tamoxifene, the first antiestrogen, it was discovered that it had an estrogen-like affect on bone metabolism (Weryha et al., 1999). Tamoxifene was also found to cause endometrial hyperplasia, as does estrogen replacement therapy (Weryha et al., 1999). The second generation of SERMs, including

raloxifene, have antiestrogenic affects on breast tissue and estrogenic affects on bone and the cardiovascular system but do not cause endometrial hyperplasia as tamoxifene or first generation SERMs have (Grese & Dodge, 1998). The effects of SERMs on the risk factors of cardiovascular disease vary from the effects seen with HRT. Both reduce TC and LDL-cholesterol concentrations but SERMs do not cause the rise in TG or HDL-cholesterol levels that HRT does (Cosman & Lindsay, 1999; Grese & Dodge, 1998). SERMs also have been shown to reduce fibrinogen levels, another possible risk factor for heart disease, while HRT does not cause this reduction (Cosman & Lindsay, 1999; Grese & Dodge, 1998). SERMs may replace HRT for treatment of postmenopausal symptoms such as hot flashes as well as preventing the chronic diseases associated with estrogen deficiency such as heart disease and osteoporosis (Brzezinski & Debi, 1999).

PHYTOESTROGENS

Phytoestrogens exert tissue specific estrogenic/antiestrogenic effects as seen with SERMs (Brzezinski & Debi, 1999). Phytoestrogens are non-steroidal plant compounds that are structurally and functionally similar to estrogen and SERMs (Brzezinski & Debi, 1999). There are three main classes of phytoestrogens: isoflavones, coumestans, and lignans (Humfrey, 1998). Isoflavones are most abundant in soybeans, coumestans are derived from alfalfa sprouts and clover, and lignans are found in a variety of grains and oilseeds with flaxseed having the highest concentration (Lissin & Cooke, 2000).

Isoflavones were first discovered in 1931 but the hormonal effects were not realized until the mid-1940's (Setchell, 2000). Urinary metabolites were unintentionally discovered in human urine in the early 1980's, this sparked the interest in the use of

phytoestrogens in human nutrition (Setchell, 2000). So far, most of the phytoestrogen research has been done on the isoflavones found in soybeans (Lissin & Cooke, 2000).

Phytoestrogens are widespread in our food supply, they are found in cereal, grains, beans, fruits and vegetables. Epidemiological data demonstrates a lower incidence of chronic diseases such as heart disease and osteoporosis in people consuming diets rich in phytoestrogens (Lissin & Cooke, 2000). Asian countries that consume a plant-based diet high in soy foods have lower rates of female CHD mortality, breast cancer mortality, and lower rates of endometrial cancer (Burke et al., 2000). There are also fewer reported menopause-related vasomotor symptoms (Burke et al., 2000). To further emphasize the health benefits of the traditional Asian diet, a recent survey of Japanese citizens found a relationship between the deterioration of the health of the nation and the increasingly Westernized dietary patterns (Setchell, 1998).

Phytoestrogens exist in plants as glucose conjugates, or glycones, and before the phytoestrogens can be absorbed the glucose molecule must be removed (Anderson & Garner, 1997). Hydrolysis takes place in the jejunum by glucosidases to produce aglycones (Setchell & Cassidy, 1999). Aglycones are further metabolized by intestinal bacteria to more estrogenically potent compounds (Setchell & Cassidy, 1999). The production of bacterial metabolites varies greatly between individuals depending on their gastrointestinal flora, antibiotic use, and other constituents of the diet (Anderson & Garner, 1997; Setchell & Cassidy, 1999).

The aglycones and its bacterial metabolites are absorbed and distributed throughout the body in the same manner as other fat-soluble molecules and undergo

enterohepatic circulation (Anderson & Garner, 1997). Phytoestrogen metabolites are stored in the liver and adipocytes and excreted in the urine (Anderson & Garner, 1997).

Phytoestrogens compete with estradiol for binding to estrogen receptors (Grese & Dodge, 1998) but with higher affinity for estrogen receptorβ (ERβ) than for estrogen receptorα (ERα) (Setchell & Cassidy, 1999). ERβ are found in tissues such as brain, thymus, lung, blood vessels, breast, uterus, prostate, bladder, and bone (Setchell & Cassidy, 1999).

In premenopausal women, consumption of soy foods has caused lengthening of the menstrual cycle and changes in certain hormone levels (Setchell & Cassidy, 1999). In postmenopausal women, epidemiological data have suggested that phytoestrogens reduce the incidence of hot flushes, breast cancer, and osteoporosis (Setchell & Cassidy, 1999). Clinical trials have had variable results of the effect of phytoestrogens on hot flushes and maturation index (Setchell & Cassidy, 1999). It has been suggested that the differences in the outcomes of these clinical studies is due to differences in study design and the variability in phytoestrogen metabolism in study participants (Setchell & Cassidy, 1999). Variation in isoflavone content of crops may also play a role in these study outcomes (Lissin & Cooke, 2000).

LIGNANS

Lignans are a class of phytoestrogens that are found in a variety of plants but most abundantly in grains and oily seeds (Thompson, 1998). The most prominent lignans, enterodiol and enterolactone, are derived from secoisolariciresinol diglycoside (SDG) and matairesinol, respectively, through the action of colonic bacteria (Thompson, 1998).

After formation in the colon, lignans are absorbed, undergo enterohepatic circulation, and are eventually excreted in the urine.

Structural similarities between lignans, as well as other phytoestrogens, and estrogen led researchers to investigate the possible estrogenic effects of lignans. Many in vitro studies have demonstrated the hormonal effect of lignans (Thompson, 1998) and it is believed that these hormonal effects contribute to the cancer preventive effects of lignans.

The cancer preventive effects could also be due to the antioxidant properties of lignans (Prasad, 1997b; Kitts et al., 1999). Free radicals cause DNA damage, which can potentially allow the proliferation of cancerous cells (Thompson, 1994). Free radical damage has also been associated with atherosclerosis, diabetes, cataracts, inflammatory disorders, and aging (Thompson, 1994).

FLAXSEED

Flaxseed is the richest source of the lignan precursor SDG (Thompson, 1998). It is an edible grain that is of interest due to the health promoting properties of its various components. Along with a high lignan concentration, flaxseed is a rich source of ALA, an omega-3 fatty acid. Flaxseed contains an average of 35% of its mass as oil, of which about 15% is ALA (Prasad, 1999). Flaxseed is also high in soluble fiber, which has repeatedly been shown to reduce blood cholesterol levels.

The majority of the research into the health benefits of flaxseed has been in the area of cancer treatment, but other areas of research include polycystic kidney disease (Ogborn et al., 1999), lupus nephritis (Ogborn et al., 1999), arthritis (Nordstrom et al.,

1995), hypercholesterolemia (Arjmandi et al., 1998; Cunnane et al., 1995), atherosclerosis (Prasad et al., 1998), and arterial function (Talom et al, 1999). Flaxseed's ability to protect against cancer, particularly of the colon, skin and breast, may be due to the weak estrogenic/antiestrogenic effects as well as the antioxidant properties of lignans (Thompson, 1998).

Clinical studies of the estrogenic effect of flaxseed have not been conclusive. For example, one study conducted by Hutchins et al. (2001) found that serum concentrations of 17β-estradiol and estrone sulfate were significantly reduced in postmenopausal women after seven weeks of consuming 5 or 10 grams of ground flaxseed per day. Another study of premenopausal women found that 10 grams of flaxseed per day did not affect circulating estrone or estradiol levels (Phipps et al., 1993).

The effects of flaxseed on cardiovascular health have been more apparent. Animal studies have shown that flaxseed reduced TG, TC, and LDL-cholesterol in rats receiving 20% of their diet from flaxseed for 90 days (Ratneyake et al., 1992). A high flaxseed diet (20%) also improved endothelial vasorelaxation in spontaneously hypertensive rats (Talom et al., 1999). Talom et al. (1999) believe that these findings were due to the ALA content of the flaxseed since similar results have been seen with fish oils.

At least three studies have shown the positive effects of flaxseed on atherosclerosis (Prasad, 1997a; Prasad et al., 1998; Prasad, 1999). All three studies showed that flaxseed reduced hypercholesterolemic atherosclerosis in rabbits. The first study (Prasad, 1997a) reported a reduction in atherosclerotic plaque without a reduction in cholesterol levels. Prasad et al. (1998) used low ALA flaxseed and found that it reduced atherosclerotic plaque along with TC and LDL-cholesterol. Finally, in 1999,

Prasad demonstrated rabbits fed SDG isolated from flaxseed had reduced atherosclerotic plaque in addition to lower levels of TC and LDL-cholesterol.

Flaxseed has also been shown to reduce TC and LDL-cholesterol concentrations in human subjects. Jenkins et al. (1999) reported that hyperlipidemic subjects (n=29) consuming partially defatted flaxseed (50 g/day) incorporated into muffins along with a self-selected AHA Step II diet for 3 weeks had lower serum levels of TC (4.6%), LDL-cholesterol (7.6%), apo B (5.4%), and apo A (5.8%). Bierenbaum et al. (1993) showed that the incorporation of flaxseed along with 800 IU of vitamin E per day for three months reduced TC and LDL-cholesterol levels of 15 hyperlipidemic subjects. A double-blind cross-over study comparing the effects of sunflower seed and flaxseed on serum cholesterol levels of hyperlipidemic postmenopausal women showed that 38 grams of flaxseed per day in the form of bread products significantly reduced LDL-cholesterol by 14.7% (Arjmandi et al., 1998). Findings by Cunnane et al. (1993; 1995) further corroborate the hypocholesterolemic effect of flaxseed. Both studies found that 50 grams of flaxseed (traditional and high ALA varieties) significantly lowered TC and LDL-cholesterol in normocholesterolemic young women (mean age-24 years).

CHAPTER III

MATERIALS AND METHODS

SUBJECT RECRUITMENT

Following approval of the study protocol by the Institutional Review Boards at Oklahoma State University and the University of Oklahoma Health Sciences Center, fifty-eight postmenopausal women were recruited from the Oklahoma City area though radio and newspaper advertisement. The inclusion criteria for the subjects were: postmenopausal for 1 to 12 years (determined by 1 year of amenorrhea), less than sixty-five years of age, and not on HRT for at least three months prior to starting the study. Women were excluded from the study if they were taking any prescribed medications that would affect lipid or bone metabolism. Women were also excluded if they had any of the following conditions: cancer, liver disease, thyroid disorder, gastrointestinal problems, insulin-dependent diabetes mellitus, pelvic inflammatory disease, or endometrial polyps. After receiving a written and oral explanation of the study protocol, subjects signed a consent form. A complete medical history was obtained prior to initiating the treatments. All subjects were given a routine physical and gynecological examination including a vaginal smear, performed by a gynecologist and evaluated by a pathologist. The subjects were asked to maintain their usual dietary and physical activity routines.

EXPERIMENTAL DESIGN

Upon entry into the study, subjects were randomly assigned to one of two dietary treatments (n = 29 per treatment) in a double blind comparative control study. In order to randomly assign subjects, as they entered the study they drew their subject number from a collection of all possible numbers. The same procedure was followed to assign subjects to either group A (flaxseed) or group B (wheat). The dietary treatments consisted of 40 grams of either ground whole flaxseed or wheat-based comparative control regimen to be consumed daily for a period of three months. Both regimens were similar in macronutrient compositions (Table 1). Study participants were given suggestions for incorporating the products into their diets. Subjects also received 1,000 mg elemental calcium plus 400 IU vitamin D supplements to provide some protection against rapid bone loss. Unused flaxseed or wheat-based regimens, supplements, and a self-recorded treatment intake log were returned in order for investigators to monitor intake. Treatment regimens and supplements were provided monthly.

DIETARY ASSESSMENT

Seven-day food frequency questionnaires were administered at baseline and at the end of the study. The subjects used measuring cups and food portion models while filling out the food frequency questionnaire in order to estimate serving sizes. Nutrient analysis was performed using food analysis software (Food Processor version 7.4, ESHA Research, Salem, OR).

Anthropometric Measurements

At baseline standing height, knee height, and weight were obtained as well as circumferences of the waist, hip, wrist, and upper arm. To estimate body fat percentage three skin fold measurements were taken at the abdomen, suprailiac crest, and tricep and those values were used in the following equation:

$$\text{Percent body fat} = 0.41563\ (X_1) - 0.00112\ (X_1)^2 + 0.03661\ (X_2) + 4.03653$$

where X_1 = sum of triceps, abdomen, and suprailiac skinfolds and X_2 = age in years (Lee, 1996).

Measurements were repeated at the end of the study excluding standing height, knee height and wrist circumference. Weight was monitored throughout the study. The procedures for obtaining these anthropometric measurements were derived from the NHANES III survey (Kuczmarski, 1994).

All body weights were measured using a medical scale (Health-O-Meter, Continental Scale Corp., Chicago, IL) and subjects were dressed in street clothes without shoes. Height was measured using the same medical scale. Subjects stood with heels together, heels and back of head touching the metal bar of the stadiometer.

Blood Collection and Processing

Fasting blood samples were collected prior to initiation of treatment and after three months. All blood samples were kept in ice until processing. After centrifugation at 1500x *g* for 15 minutes, serum and plasma were aliquoted into smaller volumes and

stored at -80°C until analysis. At the end of the study, all samples were tested simultaneously, in duplicate.

SERUM ANALYSES

All lipid parameters, excluding LDL-cholesterol, were measured using a Cobas-Fara II Clinical Analyzer (Monclair, NJ). The machine was calibrated using Roche Calibrator or HDL Direct Calibrator (Roche Diagnostic Systems; Somerville, NJ) before each test. BioRad QCS Level 1 and Level 2 (Irvine, CA) were used as controls in all test except for apo A and apo B when Roche Apolipoprotein Standard was used.

The reagent used for measuring total cholesterol concentrations in serum contains cholesterol esterase, which releases cholesterol from its esters, and cholesterol oxidase, which oxidizes the free cholesterol to produce hydrogen peroxide. A quinoneimine complex is produced when hydrogen peroxide is combined with 4-aminoantipyrine and phenol, which is measured photometrically at 500 nm.

Serum triglyceride levels were measured using a Roche reagent also. The triglyceride reagent works similarly to the cholesterol reagent in that triglycerides are converted to produce hydrogen peroxide through a series of enzymatic reactions and the hydrogen peroxide reacts with 4-chlorophenol and 4-quinoneimine to form a quinoneimine complex that can be read at 490-550 nm.

High-density lipoprotein content of the serum samples was determined by a direct method (Ultimate HDL Direct; Roche Diagnostic Systems). Compounds in the reagents solubilize cholesterol from lipid fractions other than HDL. The cholesterol in HDL is then determined enzymatically.

The Roche reagent for apo A-1 contains antiserum which forms a precipitate with apo A-1 in serum samples and the turbidity is measured at 340 nm. The apo B reagent follows the same principle. Roche Apolipoprotein standard was used for both tests.

Antibody Reagent Set II for CRP (DiaSorin; Stillwater, MN) was used to determine serum levels of CRP. Again the Cobas-Fara was utilized. Antiserum is added to the serum sample and antigen-antibody complexes form. After an incubation period, absorbance is measured and CRP concentration is interpolated from a standard curve.

Low-density lipoprotein cholesterol was calculated using the Friedewald equation: (Friedewald et al., 1972)

$$LDL = (TC) - (HDL) - (TG/5)$$

Data Management and Statistical Analysis

Upon completion of the study, a trained graduate student entered data obtained from the questionnaires into spreadsheets. As serum analyses were completed, results were entered into a spreadsheet. All data obtained on subjects were held in a lock cabinet with limited accessibility.

The experiment was a completely randomized design with repeated measures. Treatment is the main plot factor and was applied to the subject, while baseline and final were the repeated measures factor. Data were analyzed with PC SAS Version 8.1 (SAS Inst., Cary, NC) using PROC MIXED. The simple effects of each factor with the other factor held constant were examined with a SLICE option in an LSMEANS statement. Significant differences were determined using alpha level = 0.05.

CHAPTER IV

RESULTS

SUBJECT PARTICIPATION

Out of the 58 participants recruited, there was a 37% attrition. There were 20 women who completed the flaxseed regimen and 16 who completed the wheat regimen. Nine women dropped out complaining of gastrointestinal discomfort. Another nine participants dropped out because they felt that the study food was unpalatable. One subject had time constraints and was unable to meet with study personnel. One subject resumed menstruation while on the study and had to be excluded and another dropped out for personal reasons. Subjects complaining of gastrointestinal problems and unpalatability of study foods were given suggestions for incorporating the study food into their diet to reduce these problems but they decided to end their participation in the study. Of those in the flaxseed group completing the study, there was an 80% compliance with the flaxseed supplement. There was an 87% compliance with the wheat supplement for the wheat group.

NUTRIENT INTAKE

The average daily nutrient intake, as determined using seven-day food frequency questionnaires, was similar in both groups (Table 2). This report does not include the flaxseed or wheat supplement.

ANTHROPOMETRIC MEASUREMENTS

The results of the anthropometric measurements are recorded in Table 3. There were no significant differences in the baseline and final values of body weight and body mass index (BMI) among the subjects in either treatment group. There was a significant increase in the mean waist circumference measurement in the women in the wheat treatment group (P=0.009). Hip circumference tended to increase in the wheat group (P=0.072), while there was no change in the flaxseed group. There was a significant increase in the waist to hip ratio in the flaxseed group (P=0.025) following treatment. The ratio also increased in the wheat group but not significantly (P=0.079). The calculated percent body fat decreased in both treatment groups but significance was only reached in the flaxseed group.

SERUM ANALYSES

There was a significant decrease in the mean TC level following the flaxseed treatment (P=0.007), as seen in Table 4, while there was no change following the wheat treatment. After three months of the flaxseed regimen, the mean LDL-cholesterol concentration decreased by 4.7% and the mean TG level decreased by 12.8%, however, neither of these values was statistically significant. The mean HDL-cholesterol concentration was also reduced slightly by the flaxseed regimen (P=0.091). Apo A and apo B levels were reduced significantly by the flaxseed regimen but were not affected by the wheat regimen. The mean CRP levels did not change after either treatment regimen (Table 5).

Flaxseed had no estrogenic effect as assessed by serum levels of estradiol (P=0.90) (Table 5).

CHAPTER V

DISCUSSION

We have shown that consumption of whole ground flaxseed can lower total cholesterol in postmenopausal women. These findings are consistent with other clinical studies, using similar amounts of flaxseed, conducted by Jenkins et al. (1999), Bierenbaum et al. (1993), Cunnane et al. (1993;1995), and Arjmandi et al. (1998). In our current study, LDL-cholesterol concentrations decreased by 4.7 % (P=0.218), which is less than the 7.6% reduction seen by Jenkins et al. (1999) and the 14.7% reduction reported by Arjmandi et al. (1998). Both Jenkins (1999) and Arjmandi (1998) used flaxseed incorporated into bread and/or muffins, while whole ground raw flaxseed was used in the current study. This may have contributed to this difference. It is unknown whether the cholesterol-lowering compounds, such as the lignans and α-linolenic acid, in flaxseed are equally as effective in raw and processed forms.

As with Jenkins et al. (1999), we observed that flaxseed reduced serum levels of both apo B and apo A-1. Apo B is positively associated with heart disease. Elevated levels of apo B are seen in CHD patients with or without elevated LDL-cholesterol levels (Douste-Blazy & Kloer, 1989). It is a structural component in atherogenic particles such as VLDL and LDL. Due to its function as a LDL receptor ligand, apo B aids in the delivery of cholesterol to the tissues (Brown & Goldstein, 1986). Apo B particles have been identified, along with LDL particles, in arterial plaque (Hoff et al. 1979). In the current study, apo B was reduced by 10% (P=0.002), a change that could reduce the risk of developing atherosclerosis and CHD.

Apo A is the major apolipoprotein associated with HDL particles and its serum level correlates with HDL-cholesterol levels (Shepherd & Packard, 1989). Apo A is also found, to a lesser extent, in VLDL and chylomicrons. One source of HDL particles is the remnants of VLDL and chylomicrons (Shepherd et al., 1989). On HDL particles, apo A functions as a catalyst for lecithin:cholesterol acytransferase (LCAT), an enzyme responsible for the reverse transport of cholesterol from peripheral tissues to the HDL particles (Glomset, 1968). Flaxseed consumption reduced serum apo A concentration by 6% (P=0.003) however, it remained within normal limits for the study group (1.15-2.20 g/L; Roche Apo A product insert). This decrease in apo A was to be expected with the slight (P=0.09) decrease in HDL-cholesterol.

It is unclear what component in flaxseed is responsible for its hypolipidemic effect or whether it is a combination of components. Prasad (1999) demonstrated that SDG, isolated from flaxseed, reduced atherosclerotic plaque as well as lowering serum TC and LDL-cholesterol concentrations in rabbits. Also, omega-3 fatty acids have been shown to reduce TC and TG (Simopoulos et al., 1991). An additional possible hypolipidemic component of flaxseed is fiber. Fiber has repeatedly been shown to lower serum cholesterol levels (Fernandez, 2001; Saltzman et al., 2001). Because these separate components of flaxseed have hypolipidemic properties, it would be interesting to determine which of these compounds is exerting the cholesterol-lowering effect seen in this study or if these components work synergistically in flaxseed to produce this benefit. An effective dose still remains to be determined as well as a comparison of the effectiveness of raw and processed flaxseed.

Flaxseed supplementation had no effect on serum levels of c-reactive protein. Two possible explanations for this lack of effect are; 1) the serum levels of c-reactive protein were not elevated at baseline in these subjects and 2) that flaxseed may not have a measurable anti-inflammatory effect in humans.

In summary, the results of the present study reveal that reasonable dietary changes, such as incorporating 40 g of flaxseed into the diet, are effective in lowering TC and apo B levels in postmenopausal women. These effects were seen without altering serum estradiol levels.

Table 1

Composition of flaxseed and wheat-based control regimen

Measures	**Flaxseed** *Per 40g*	**Wheat** *Per 40g*
Energy, *kcal*	237.0	202.3
Fat, *g*	12.6	8.9
Fiber, *g*	2.1	2.0
Protein, *g*	8.6	10.6
Mineral, *g*	1.5	1.3
Calcium, *mg*	133	14
Phosphorous, *mg*	0.9	1.1

Gross energy analyzed by bomb calorimetry (Parr 1261 Calorimeter, Parr Inst. Co., Moline, IL), crude protein by the AOAC Kjeldahl method (AOAC, 1984), fat by ether extraction (AOAC,1984), calcium content by atomic absorption spectrophotometer (Perkin Elmer Atomic Absorption Spectrophotometer, model 5100PC, Perkin Elmer, Norwalk, CT) (Hill et al., 1986) and phosphorus was measured using a kit from Roche Diagnostic (Branchburg, NJ).

Table 2

Daily nutrient intake as determined from 7-day food frequency questionnaires.

(Values do not include treatment regimen and calcium plus vitamin D supplement)

Daily intake	Flaxseed			Wheat		
	Baseline	Final	*P* value	Baseline	Final	*P* value
Total energy, (kcal)	1619±137	1529 ± 140	0.55	1786±140	1531 ± 154	0.13
Nutrients (g)						
Protein	64 ± 6	65 ± 6	1.00	74 ± 6	60 ± 7	0.06
Carbohydrates	223 ± 22	208 ± 22	0.58	235 ± 22	205 ± 25	0.33
Dietary fiber	22 ± 2	18 ± 2	0.13	19 ± 2	17 ± 3	0.31
Total fat	56 ± 6	59 ± 6	0.69	64 ± 6	53 ± 6	0.17
Saturated fat	20 ± 3	19 ± 3	0.73	23 ± 3	20 ± 3	0.37
Polyunsaturated Fat	10 ± 1	12 ± 1	0.12	12 ± 1	9 ± 1	0.07
Minerals (mg)						
Calcium	718 ± 111	745 ± 112	0.77	838 ± 113	740 ± 121	0.34
Magnesium	270 ± 26	255 ± 26	0.66	294 ± 26	260 ± 29	0.34
Phosphorus	1038±115	1009 ± 116	0.77	1199±118	954 ± 126	0.03

Values are mean ± SE; n= 20 for flaxseed regimen and 16 for wheat regimen.

Table 3
Subject characteristic

Measures	Flaxseed (n=20)			Wheat (n=16)		
	Baseline	Final	*P* value	Baseline	Final	*P* value
Age (years)	54 ± 8	---	---	55 ± 5	---	---
Time since menopause (years)	11 ± 1.57	---	---	6.44 ± 1.67	---	---
Weight (kg)	78.2 ± 4.3	77.9 ± 4.3	0.650	74.2 ± 4.8	75.1 ± 4.8	0.092
Body Mass Index, (kg/m^2)	29.1 ± 1.6	29.0 ± 1.6	0.648	28.7 ± 1.8	29.1 ± 1.8	0.072
Waist circumference (cm)	95.9 ± 3.7	97.9 ± 3.7	0.280	96.4 ± 4.1	101.9 ± 4.1	0.009
Hip circumference (cm)	112.1 ± 3.5	110.6 ± 3.5	0.134	109.8 ± 3.9	111.9 ± 3.9	0.072
Waist to Hip Ratio	0.86 ± 0.02	0.90 ± 0.02	0.025	0.88 ± 0.02	0.91 ± 0.02	0.079
Body Fat, %	31.3 ± 2.2	29.2 ± 2.2	0.033	38.2 ± 2.3	36.8 ± 2.3	0.172

Values are mean ± SE.

Table 4

Effects of three-month flaxseed supplementation on serum lipid parameters in postmenopausal women

	Flaxseed				Wheat			
	Baseline	Final	*P* value	% Change from baseline	Baseline	Final	*P* value	% Change from baseline
Lipids								
Total cholesterol (mmol/L)	5.76 ± 0.25	5.44 ± 0.25	0.01	-5.5	5.95 ± 0.28	6.13 ± 0.28	0.18	+3
LDL-cholesterol (mmol/L)	3.21 ± 0.25	3.06 ± 0.25	0.22	-4.7	3.52 ± 0.28	3.64 ± 0.28	0.37	+3.4
HDL-cholesterol (mmol/L)	1.89 ± 0.09	1.80 ± 0.09	0.09	-4.5	1.61 ± 0.10	1.67 ± 0.10	0.34	+3.7
Triglycerides (mmol/L)	1.48 ± 0.16	1.29 ± 0.16	0.14	-12.8	1.56 ± 0.19	1.74 ± 0.19	0.20	+11.5
Apo A-1 (g/L)	1.98 ± 0.05	1.86 ± 0.05	0.003	-6	1.94 ± 0.06	1.95 ± 0.06	0.82	+0.5
Apo B (g/L)	1.34 ± 0.07	1.24 ± 0.07	0.002	-7.4	1.38 ± 0.08	1.44 ± 0.08	0.07	+4.3

Values are mean ± SE; n= 20 for flaxseed regimen and 16 for wheat regimen.

Table 5

Effects of three-month flaxseed supplementation on serum 17β-estradiol (E_2) and C-reactive protein concentrations in postmenopausal women

	Flaxseed			Wheat		
	Baseline	Final	*P* value	Baseline	Final	*P* value
Hormone						
E_2 (pmol/L)	34.1 ± 7.4	33.0 ± 7.4	0.90	28.8 ± 8.4	20.0 ± 8.4	0.36
Inflammation						
C-reactive protein (mg/L)	7.62 ± 2	5.47 ± 2	0.31	11.36 ± 2.56	13.54 ± 2.56	0.42

Values are mean ± SE; n= 20 for flaxseed regimen and 16 for wheat regimen.

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Oklahoma State University
Institutional Review Board

Protocol Expires: 10/3/01

Date : Wednesday, October 04, 2000 IRB Application No HE98104

Proposal Title: FLAXSEED PHYTOESTROGENS MAY POSITIVELY AFFECT BONE

Principal Investigator(s) :

Barbara Stoecker
416 HES
Stillwater, OK 74078

Bahram Arjmandi
416 HES
Stillwater, OK 74078

Reviewed and Expedited **Continuation**

Approval Status Recommended by Reviewer(s) : Approved

Signature :

Carol Olson, Director of University Research Complian

Wednesday, October 04, 2000
Date

Approvals are valid for one calendar year, after which time a request for continuation must be submitted. Any modifications to the research project approved by the IRB must be submitted for approval with the advisor's signature. The IRB office MUST be notified in writing when a project is complete. Approved projects are subject to monitoring by the IRB. Expedited and exempt projects may be reviewed by the full Institutional Review Board.

VITA

Lisa J. Hammond

Candidate for the Degree of

Master of Science

Thesis: FLAXSEED CONSUMPTION POSITIVELY INFLUENCES LIPID PROFILES IN POSTMENOPAUSAL WOMEN

Major Field: Nutritional Sciences

Biographical:

Education: Graduated from Jupiter High School, Jupiter, Florida in 1988; received Bachelor of Science degree in Dietetics from Oklahoma State University in 1999. Completed the requirements for the Masters of Science degree with a major in Nutritional Sciences at Oklahoma State University in May, 2002.

Professional Memberships: American Dietetics Association, Oklahoma Dietetics Association.