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Effects of phytoestrogens on lipids in hypercholestrolemic postmenopausal women

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EFFECTS OF PHYTOESTROGENS ON LIPIDS IN HYPERCHOLESTEROLEMIC POSTMENOPAUSAL WOMEN

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by Antonella Dewell

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ABSTRACT

Effects of phytoestrogens on lipids in hypercholesterolemic postmenopausal women

by Antonella Dewell

Phytoestrogens (PE) are hypothesized to be hypocholesterolemic. The present study investigated the effects of PE on serum lipids in 36 moderately hypercholesterolemic, postmenopausal women. Subjects were randomized into two groups and received either a 150-mg PE supplement per day (n=20) or a placebo (n=16). Lipid analysis was performed after two and six months of treatment using standard Lipid Clinic Procedures. Non-paired t test and ANOVA were employed to compare the two groups. The results (mean ± SEM) indicated no significant differences in total triglyceride (1.3 ± 0.2 vs 1.2 ± 0.2 mmol/L), total cholesterol (6.4 ± 0.4 vs 6.5 ± 0.2 mmol/L), or HDL cholesterol (1.0 ± 0.1 vs 1.0 ± 0.1 mmol/L) between the placebo and the PE groups, respectively, after two months of treatment. Moreover, total triglyceride and cholesterol remained unchanged after six months.

These results suggest that PE do not significantly alter serum lipids in postmenopausal women.

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PREFACE

The following is a publication style thesis. Chapter I and III are written according to guidelines outlined in the *Publication Manual of the American Psychological Association, 4th edition, 1994.* Chapter II is written in journal format for submission to *The Journal of Clinical Endocrinology & Metabolism.*

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Postmenopausal women are a population at increased risk for coronary artery disease (CAD) (Witteman, Grobbee, Kok, Hofman, & Valkenburg, 1989). This increase is believed to be due to changes in lipoprotein metabolism that accompany the loss of endogenous estrogen secretion, including elevated plasma cholesterol, elevated low-density lipoprotein (LDL) cholesterol, and decreased high-density lipoprotein cholesterol (HDL) (Nasr & Breckwoldt, 1998). There is evidence in the current literature suggesting that dietary intake of soy protein may contribute to lowering these risk factors in a variety of populations (Potter et al., 1993; Crouse et al., 1999), including postmenopausal women (Baum et al., 1998).

Although it has been proposed that the phytoestrogen (PE) content of soy may be the responsible agent of this beneficial effect (Setchell & Cassidy, 1999), few studies have been published on the effect of PE supplementation on these risk factors (Hodgson, Puddey, Beilin, Mori, & Croft, 1998; Nestel et al., 1997; Nestel et al., 1999; Simons, von Konigsmark, Simons, & Celermajer, 2000). Results have suggested a decreasing trend of LDL cholesterol, but have failed to reach statistical significance (Nestel et al., 1999). However, the amount of phytoestrogens (PE) used was limited to a maximum of 80 mg, a quantity that approximates the level reachable with soy-based diets (Simons et al., 2000). Although this is of practical importance, higher levels available through supplementation may be effective in decreasing the risk for CAD (Simons et al., 2000).

The purpose of the present study was to assess the role of supplementation with higher amounts of PE (150 mg per day) on serum lipoprotein triglyceride and cholesterol

concentrations in moderately hypercholesterolemic postmenopausal women. The significance of this study lies in contributing to the increased understanding of the role of PE in the cholesterol-lowering properties of soy and in evaluating the potential benefit of PE supplementation in reducing the risk for CAD in postmenopausal women.

Review of Literature

Phytoestrogens

Definition and sources. Phytoestrogens are a group of non-steroidal plant chemicals that can behave as estrogen mimics (Setchell & Cassidy, 1999). The PE present in edible plants can be categorized into three main classes: isoflavones, lignans and coumestans (Davis, Murkies, & Wilcox, 1998). Whereas lignans are found predominantly in flax seeds, and do not contribute significantly to dietary phytoestrogen intake, isoflavones are widespread in leguminous plants and are present in highest amounts in soybeans (Tham, Gardner, & Haskell, 1998). On the other hand, coumestans are associated with germination and found in highest concentrations in the soybean sprout and alfalfa (Davis et al., 1998).

Isoflavone content of soy. Isoflavones are concentrated in the soybean hypocotyl with lesser amounts in the cotyledon (Wang & Murphy, 1994). Soybeans contain three types of isoflavones - genistein, daidzein, and glycitein - occurring in four chemical forms: as unconjugated aglycones or conjugated as glucosides, acetylglucosides, or malonylglucosides (Wang & Murphy, 1994). Because isoflavones are associated with the protein fraction of the soybean (Setchell & Cassidy, 1999), they are present only in

the whole soybean and other high-protein secondary products. Consequently, soy oils and soy lecithin, which are contained in a high proportion of foods typical of the Western diet, are devoid of isoflavones (Setchell & Cassidy, 1999). The isoflavone content of soy and soy products varies substantially according to the variety of soybean, location and/or harvest year, and degree of processing (Wang & Murphy, 1994). In 1994, Wang and Murphy quantified the amounts of isoflavones available in typical soybeans and soy foods and found the highest concentrations of isoflavones in soybeans (995-1636 μg/g), texturized vegetable protein (1342-1382 μg/g), soy flour (1124 μg/g), and soy protein isolate (466-610 μg/g). Traditional nonfermented soy foods - roasted soybeans (1625 μg/g), and instant soy beverage powder (1001-1183 μg/g) - showed a 2-3 times greater isoflavone content as compared with fermented soy foods - tempeh, bean paste, miso, and fermented bean curd (294-625 μg/g). An exception was represented by tofu, which contained significantly lesser amounts of isoflavones (337 μg/g) than the other nonfermented soy foods, due to the aqueous processing used during manufacture (Anderson & Wolf, 1995). Second-generation soy foods, prepared by adding soy ingredients to a variety of foods - soy hot dogs, tempeh burger, tofu yogurt, soy cheese and soy noodles - contained isoflavones ranging from 34 to 289 μg/g, or 6-20% of the total content of soybeans (Wang & Murphy, 1994). The reduced isoflavone content in these products reflects the presence of soy as only one of several ingredients. Finally, soy protein concentrates were found to contain insignificant amounts of isoflavones (56 μg/g). These products were obtained through alcohol extraction, a technique that removes most of the isoflavones from soy (Wang & Murphy, 1994).

Metabolism and bioavailability of isoflavones. The predominant isoflavones in soy, genistein and daidzein, are usually present in soybeans as glycoside conjugates (Wang & Murphy, 1994). In humans, the glycosides are poorly absorbed but are readily hydrolyzed by gastric hydrochloric acid and bacteria present in the small intestine (Xu, Harris, Wang, Murphy & Hendrich, 1995). Isoflavones are inactive in their bound form but, when the sugar residue is removed, become activated (Tham et al., 1998). The released aglycones can either be absorbed as such or undergo fermentation in the colonic microflora (Xu et al., 1995), where genistein may be metabolized to p-ethyl phenol and daidzein to equol or to O-demethylangolensin (O-DMA) (Tham et al., 1998). In addition, because isoflavones undergo enterohepatic circulation, bacteria in the distal small intestine, as well as colonic bacteria, can liberate the aglycones from conjugates of the liver and greatly influence the extent of their reabsorption (Xu et al., 1995). On the other hand, intestinal bacteria can also metabolize and degrade isoflavones, and promote their excretion, which is partitioned between urinary and biliary routes (Xu et al., 1995). Hence, intestinal microflora play an essential role in determining the magnitude of the bioavailability of isoflavones.

Investigations on the absorption of isoflavones have established a dose-dependent relationship between dietary intake and plasma concentrations (Xu et al., 1995). Others have demonstrated a strong correlation between isoflavone intake and urinary excretion, (Slavin, Karr, Hutchins, & Lampe, 1998; Xu et al., 1995) whereas, in general, fecal estrogens are negatively associated with urinary and plasma estrogens (Tew, Xu, Wang, Murphy, & Hendrich, 1996). On average, 15 to 35% of isoflavones ingested are

absorbed, depending on individual intestinal microflora. It has been suggested that isoflavones in soy protein are bioavailable at intakes as low as 9 mg/day, or the amount present in one ounce of tofu. However, the optimal intake necessary to exert beneficial biological effects has not yet been established (Slavin et al., 1998).

Isoflavone metabolism is highly variable among individuals and is influenced by intestinal microflora, transit time (Slavin et al., 1998), as well as other components of the diet (Setchell, 1998). Slavin et al. (1998) observed that higher intakes of dietary fiber and carbohydrate are associated with greater equol excretion and suggested that a high carbohydrate environment may promote the growth of bacteria responsible for equol production and increase the extent of isoflavone metabolism. On the other hand, high intakes of insoluble fiber (40 g/day) interfere with the absorption of isoflavones by binding to these compounds in the intestines, as is known to happen with estrogens (Tew et al., 1996). Moreover, the bulking effect of insoluble fiber can decrease the concentration of intestinal microflora, thus reducing the reabsorption of isoflavones excreted in the bile (Tew et al., 1996).

The importance of the role of colonic microflora in phytoestrogen metabolism is evidenced by the absence of urinary phytoestrogen recovery in individuals receiving antibiotics and in 4-month-old infants fed soy infant formulas (Setchell, 1998). The extent to which isoflavones are metabolized is an important determinant of their physiologic effects (Setchell & Cassidy, 1999). Equol is known to be more estrogenic than both daidzein and O-DMA and to remain in the circulation longer than genistein or daidzein (Slavin et al., 1998). As a result, the physiologic effect of isoflavone

consumption may be higher in individuals with greater equol production (Slavin et al., 1998).

Pharmacokinetics and plasma concentrations of isoflavones. Genistein and daidzein were shown to have a half-life of 7.9 h in adults, as measured from their plasma appearance and disappearance, with peak concentrations occurring between 6 and 8 hours after ingestion of the pure compounds (Setchell, 1998). Because maintenance of steady-state plasma concentrations are necessary for longer-term efficacy of the isoflavones in soy foods or supplements, these data suggest that divided doses, rather than single daily intakes, are the optimal regimen of administration (Setchell & Cassidy, 1999).

In adults consuming approximately 50 mg/d of total isoflavones, or the amount present in the traditional Japanese diet (Setchell, 1998), plasma concentrations of daidzein, genistein and equol range between 80 and 800 ng/mL (Adlercreutz, Markkanen, & Watanabe, 1993). These levels are 100 times higher than the average concentration of endogenous estrogens (40-80 pg/mL) (Setchell & Cassidy, 1999).

Chemical structure and biological activity of isoflavones. The chemical structure of isoflavones is very similar to those of endogenous estrogens and allows them to bind to the estrogen receptor (Tham et al., 1998). Common structural features include a polycyclic hydrocarbon backbone, at least one aromatic ring containing a free phenolic hydroxyl group, and a second oxygen, usually an hydroxyl group, situated approximately 12 Å from the phenolic oxygen (Miksicek, 1994) (Figure1). The presence of the phenolic ring and the distance between the hydroxyl groups are considered prerequisites for binding to the estrogen receptor (Tham et al., 1998). In 1994, Miksicek assessed the

estrogenicity of isoflavones using the human estrogen receptor expressed in cell culture. He determined the estrogenic activity of genistein and daidzein to be 10^{-2} to 10^{-3} that of 17β-estradiol, the major endogenous estrogen. Although phytoestrogens are less potent than 17β-estradiol, they can still achieve maximal stimulation of the estrogen receptor, with the exception of daidzein, which acts only as partial agonist (Miksicek, 1994). In addition, both genistein and daidzein exhibit strong specificity for the estrogen receptor (Miksicek, 1994).

Coronary Artery Disease (CAD) and Plasma Lipids

The pathology of CAD. The most common cause of CAD is coronary atherosclerosis, a disease initially characterized by lipid deposition in the intimal layer of the artery (Price & Wilson, 1997). A series of pathologic processes - calcification, fibrosis, thrombosis, hemorrhage and necrosis of smooth muscle cells - can follow and progressively lead to the occlusion of the vessel lumen and the weakening of the arterial wall (Price & Wilson, 1997).

Risk factors. Several nonmodifiable and modifiable risk factors are known to increase the possibility of developing coronary atherosclerosis in a given individual (Price & Wilson, 1997). The first class comprises age, male gender, and family history (Price & Wilson, 1997). The second includes elevated plasma lipids, hypertension, cigarette smoking, diabetes mellitus, sedentary life-style, and obesity (Price & Wilson, 1997). Among the modifiable risk factors, elevated plasma lipids are the major cause of cardiovascular mortality (O'Brien & Nguyen, 1997).

The role of plasma lipids in the development of CAD. Cholesterol and triglyceride (TG) are the plasma lipids of major clinical significance in relation to coronary atherosclerosis (Price & Wilson, 1997). Because they are insoluble in an aqueous environment, cholesterol and TG are found in plasma bound to proteins, with which they form four major classes of lipoproteins: chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Whereas chylomicrons and VLDL are TG-rich lipoproteins, LDL contains mostly cholesterol and HDL is highest in protein (Price & Wilson, 1997).

Elevated plasma levels of LDL-cholesterol are associated with an increased risk of CAD (Ginsberg & Karmally, 2000). In normal cholesterol transport, VLDL carry endogenous TG from the liver to peripheral tissues and are converted to LDL, as they lose their TG content through the interaction with lipoprotein lipase (Ginsberg & Karmally, 2000). LDL are then taken up by specific receptors, expressed mainly in the liver, through an endocytotic process. However, when low levels of LDL receptors are present, LDL accumulate in plasma and undergo chemical modification in the form of oxidation of phosholipid (Ginsberg & Karmally, 2000). Modified LDL particles do not bind to the LDL receptor, but are liable to aggregate in the subendothelial space and be internalized by macrophages via a scavenger pathway in the arterial wall. These macrophages further oxidize the retained lipoproteins and develop into foam cells, which are chemotactic and stimulate proliferation and migration of smooth muscle cells and calcification of plaque, key components of the advanced lesion that characterizes the atherogenic process (Ginsberg & Karmally, 2000).

High concentrations of postprandial TG-rich VLDL are also positively related to CAD risk (Semenkovich, 1999). When elevated, these particles exchange their TG content for cholesterol with HDL (Semenkovich, 1999). The resulting cholesterol-rich VLDL are abnormal lipoproteins that follow the same metabolic pathway as modified LDL and contribute to the development of atherogenesis.

On the other hand, elevated plasma levels of HDL-cholesterol are inversely related to the risk of CAD (Semenkovich, 1999). Although the exact reason for this epidemiological observation is still unknown, high HDL levels may be beneficial because of HDL's role in reverse cholesterol transport, the process by which excess cholesterol is transported from peripheral tissues to the liver for excretion (Semenkovich, 1999). A second explanation involves their inverse relationship to the atherogenic TG-rich lipoproteins (Semenkovich, 1999).

CAD in postmenopausal women. CAD is the leading cause of mortality among women in the United States (Nasr & Breckwoldt, 1998). Postmenopausal women are at increased risk for developing CAD, as compared with premenopausal women (Nasr & Breckwoldt, 1998). This is attributed to the protective effect of endogenous estrogen during the premenopausal years (Nasr & Breckwoldt, 1998). The estrogen deprivation experienced with menopause, when ovaries stop producing steroid hormones, is accompanied by the major risk factors for CAD: elevations in plasma cholesterol, LDL-cholesterol and triglyceride, and reduction in HDL-cholesterol (Nasr & Breckwoldt, 1998). Decreased LDL-cholesterol catabolism and HDL-cholesterol production results in

a greater influx of cholesterol to the arterial wall and a diminished capacity for its removal (Nasr & Breckwoldt, 1998).

The Effect of Estrogen on Plasma Lipids

Endogenous estrogen is known to affect cholesterol homeostasis by stimulating the synthesis of LDL receptors and thereby increasing the clearance of cholesterol from plasma (Grundy, 1999). Several studies have reported the beneficial effects of estrogen administration on plasma lipids (Nasr & Breckwoldt, 1998). A large-scale clinical trial showed a significant reduction in LDL-cholesterol (10%) and a significant increase in HDL-cholesterol (9%) in postmenopausal women taking unopposed estrogen (estrogen without progestogen), compared to the placebo group; similar results were shown in women taking an estrogen/progestin regimen (The PEPI Trial, 1995). Higher reductions in LDL-cholesterol (22%) and increments in HDL-cholesterol (21%) have been reported with the use of estradiol, whereas a combination of estrogen and progestogen showed a 10-18% lowering of total cholesterol (Hirvonen, Mälkönen, & Manninen, 1981). Thus, women who choose estrogen replacement therapy (ERT) after menopause can reduce their risk for CAD by approximately 50% and estrogen's effects on the lipid profile is estimated to account for 25-50% of the total reduction (Nasr & Breckwoldt, 1998).

The Effect of Soy on Plasma Lipids

In mixed populations. The hypocholesterolemic effect of soy was first observed in 1940, when rabbits fed soybean flour did not develop hypercholesterolemia compared to rabbits fed the same amount of casein (Meeker & Kesten, 1940). It is important to note that rabbits do not habitually eat casein, which may have had a hypercholesterolemic

effect in the controls. Whereas these findings have been consistent in other animal species, studies in human populations have given variable results (Carrol, 1991). The effect of soy on plasma lipids has been shown to depend on the subjects' initial cholesterol concentration, with the greatest decline occurring in individuals with hypercholesterolemia (Carrol, 1991). In general, reductions are seen in the LDL fraction, whereas HDL is not significantly affected; TG concentrations tend to decrease, especially in individuals with hypertriglyceridemia (Carrol, 1991). More recent studies have reported significant, but quantitatively small (2.5-8%) reductions in LDL-cholesterol and/or plasma cholesterol in men (Potter et al., 1993; Teixeira et al., 2000) and mixed populations (Crouse et al., 1999). Moreover, the results of a meta-analysis of 38 studies, performed from 1967 to 1994, showed significant reductions in plasma cholesterol (9.3%), LDL-cholesterol (12.9%) and TG (10.5%) and a nonsignificant increase in HDL-cholesterol (2.4 %) (Anderson, Johnstone, & Cook-Newell, 1995). Initial cholesterol concentrations were confirmed to be strongly related to the decreases in plasma cholesterol and LDL-cholesterol (Anderson et al., 1995). However, a major limitation of this approach to data analysis lies in summarizing data from studies with different research designs and populations. The studies evaluated in this meta-analysis involved mostly men, and some premenopausal women and children. Moreover, alterations in the content of soy-protein were not the only dietary changes made in many of these studies. Changes in other dietary components such as saturated fat and cholesterol could also have explained the lower plasma cholesterol concentrations reported in this meta-analysis.

In postmenopausal women. There appears to be only one study, published twice, investigating the effect of dietary soy protein on blood lipids in postmenopausal women (Baum et al, 1998; Potter et al., 1998). This 6-month trial involved 66 hypercholesterolemic women who were studied according to a parallel-group design with three interventions. The subjects were randomly assigned to receive 40g of test protein derived either from isolated soy protein (ISP) with moderate amounts of isoflavones (56 mg), ISP with higher amounts of isoflavones (90 mg) or casein and nonfat dry milk. The analyses of plasma lipids showed a significant decrease in non-HDL cholesterol and a significant increase in HDL-cholesterol in both intervention groups, compared to the controls. In contrast, dietary soy protein did not affect plasma cholesterol or TG concentrations. Although the changes observed were statistically significant, they were quantitatively small (5-7%). Indeed, the differences reported as statistically significant were adjusted mean differences, whereas the actual means at the end of the treatment periods were essentially identical. More recently, reductions of similar magnitudes (6-7%) have been reported in normocholesterolemic perimenopausal women taking 20 g of soy-protein with 34 mg of isoflavones (Washburn, Burke, Morgan, & Anthony, 1999). Again, the changes were quantitatively small. Collectively, these studies show that, although statistically significant reductions in plasma cholesterol in postmenopausal women can be obtained with soy protein intake, quantitatively, these changes are of no clinical significance.

The Effect of Phytoestrogens on Plasma Lipids

Proposed mechanism of action. Although isoflavones have a weaker estrogenic activity than endogenous estrogens, their concentration in plasma may be 100-fold higher (Tham et al., 1998). This observation led to the hypothesis that isoflavones may have similar hormonal effects (Setchell & Cassidy, 1999) and, therefore, may be accountable for the hypocholesterolemic effect of soy protein. Anthony, Clarkson, Bullock, and Wagner (1997) reported significantly lower LDL-cholesterol levels in male cynomolgus monkeys fed intact soy protein compared to monkeys fed soy protein from which the phytoestrogens had been extracted.

The investigation of the relative importance of isoflavones versus soy protein in altering plasma lipids in human subjects confirmed these results (Crouse et al., 1999). In this study a group of 156 men and women were randomly divided to receive an ethanol-extracted soy protein or soy protein with increasing amounts of isoflavones. Analyses of plasma lipids showed a significant reduction in plasma cholesterol and LDL-cholesterol only in the group fed soy protein with the highest isoflavone content. In addition, the results of a trend test showed a significant dose response-effect of increasing concentrations of isoflavones, suggesting that they may be the responsible agents of the cholesterol-lowering effect of soy. However, because alcohol extraction deprives soy protein of other compounds known to affect cholesterol concentrations (saponins, phytic acid and fibers) (Wang & Murphy, 1994), one cannot exclude the possibility that they may have a role in the hypocholesterolemic effect of soy.

Isoflavone supplements. The difficulty in differentiating the effects of isoflavones from those of other compounds present in soy, or the soy protein itself, prompted the use of an isoflavone extract in subsequent investigations of the effects of isoflavones on plasma lipids. Hodgson et al. (1998) examined the effect of a phytoestrogen tablet containing 55 mg of isoflavones in 59 men and women, but failed to detect a significant effect of isoflavone supplementation.

Studies on postmenopausal women produced similar results (Nestel et al., 1997; Nestel et al., 1999; Simons et al., 2000). Nestel and colleagues studied the effects of a pure soybean extract in 21 postmenopausal women in a placebo-controlled, crossover trial over 5- to 10-week periods (Nestel et al., 1997). The subjects had baseline cholesterol concentrations within the normal range (mean ± SD, 5.54 ± 0.94 mmol/L). The results showed that daily supplementation with 40 or 80 mg of isoflavones did not significantly alter lipid or lipoprotein profiles. The authors reported equivalent findings in a second, similar study using an isoflavone extract from red clover, a plant which contains the isoflavones present in the soybean (genistein and daidzein) as well as their precursors (biochanin A and formononetin) (Nestel et al., 1999). Although the 19 participants had slightly higher baseline cholesterol concentrations (5.96 ± 0.98 mmol/L), supplementation had no significant effect. The authors observed a downward trend in LDL (6%) and an upward trend in HDL (4%), resulting in an apparent reduction (10%) in the LDL/HDL cholesterol ratio between the placebo and treatment values. However, none of these differences, including total cholesterol (3%), were significant and all were quantitatively very small. More recently, similar findings were reported in a placebo-

controlled, crossover study of 20 postmenopausal women (Simons et al., 2000). In this study, after 8 weeks of supplementation with 80 mg of soy isoflavones, cholesterol concentrations were essentially identical (≤1%) when compared with placebo values.

In conclusion, investigations of the effect of isoflavones on plasma lipids in postmenopausal women have failed to demonstrate that these components of soy are responsible for the hypocholesterolemic effect observed with soy-based diets (Baum et al., 1998; Potter et al., 1998; Washburn et al., 1999). However, the participants of these studies had average initial cholesterol concentrations. Because of the strong correlation between baseline cholesterol concentrations and reductions in plasma lipids, it is plausible that isoflavone supplementation may be effective in hypercholesterolemic subjects (Hodgson et al., 1998). In addition, the doses of isoflavones administered were limited to the amounts that approximate the average dietary intake in the traditional Japanese diet (50-80 mg/d) (Nestel et al., 1997). Higher doses of isoflavones may be required to significantly lower plasma lipids and lipoproteins and decrease the risk for CAD (Simons et al., 2000). The conditions of the present study allow us to examine the effect of a PE supplement with a higher isoflavone content (150 mg/day) than used in previous investigations in a population of moderately hypercholesterolemic postmenopausal women and, therefore, are optimal for detecting any hypocholesterolemic effect.

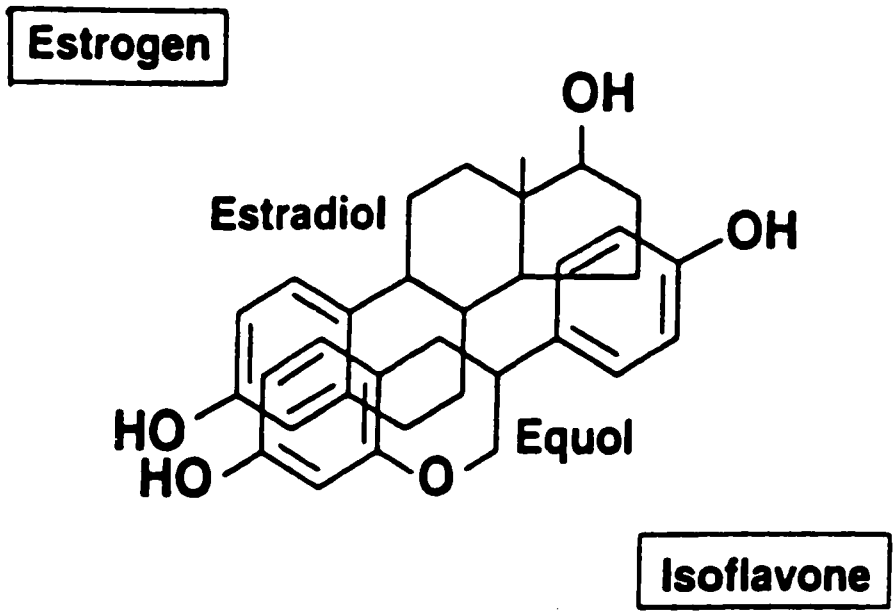

Figure 1. Chemical structures of estrogen and isoflavone.

CHAPTER II

JOURNAL ARTICLE

Title: The effects of soy-derived phytoestrogens on serum lipids and lipoproteins in moderately hypercholesterolemic postmenopausal women

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PHYTOESTROGENS AND POSTMENOPAUSAL WOMEN

ABSTRACT

The effects of soy-derived isoflavones on serum lipids and lipoproteins in moderately hypercholesterolemic postmenopausal women

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Postmenopausal women are at an increased risk of developing coronary artery disease (CAD). This increase is due primarily to elevated cholesterol concentrations accompanying the loss of endogenous estrogen secretion. Recently, the consumption of soy foods has been shown to reduce serum cholesterol concentrations. Phytoestrogens (PEs) have been proposed as the responsible agents of the hypocholesterolemic effect of soy foods. However, few studies have investigated the effect of phytoestrogen (PE) supplementation on serum lipoproteins. The purpose of the present study is to investigate the effects of PE supplementation (150 mg) on serum lipids and lipoproteins in moderately hypercholesterolemic, elderly postmenopausal women. Thirty-six subjects were randomized into two groups and received either a 150-mg PE supplement per day (n=20) or a placebo (n=16). Serum samples obtained at baseline and 2 months were analyzed for total triacylglycerol, total cholesterol and HDL cholesterol using standard Lipid Clinic Procedures. In addition, total triacylglycerol and cholesterol were measured following 6 months of treatment. Student's t test and ANOVA were employed to compare the two groups. The results (mean ± SEM) indicated no significant differences in total triacylglycerol (1.3 ± 0.2 vs. 1.2 ± 0.2 mmol/L), total cholesterol (6.4 ± 0.4 vs. 6.5 ± 0.2 mmol/L), or HDL cholesterol (1.0 ± 0.1 Vs 1.0 ± 0.1 mmol/L) between the placebo and the PE groups, respectively, after 2 months of treatment. Moreover, total triacylglycerol and cholesterol remained unchanged after 6 months. Our findings suggest that PE supplementation with 150 mg/day over a 6-month period does not significantly alter serum lipoproteins in postmenopausal women and, therefore, may not effectively reduce the risk of CAD in this population.

KEY WORDS: Postmenopausal women, phytoestrogens, isoflavones, genistein, coronary artery disease, CAD, lipids

Introduction

Postmenopausal women are a population at increased risk for coronary artery disease (CAD) (1). This increase is believed to be due to changes in lipoprotein metabolism that accompany the loss of endogenous estrogen secretion, including elevated plasma cholesterol, elevated low-density lipoprotein (LDL) cholesterol, and decreased high-density lipoprotein (HDL) cholesterol (2). There is evidence in the current literature suggesting that dietary intake of soy protein contributes to lowering these risk factors in a variety of populations (3, 4), including postmenopausal women (5, 6).

It has been proposed that phytoestrogens (PE) may be the component in soy responsible for this beneficial effect because of their similarity to endogenous estrogen in chemical structure and biological activity (7). The structures of estradiol and equol, a PE metabolite, are virtually superimposable (Figure 1). Specifically, the presence of the phenolic ring and the distance between the hydroxyl groups, which is nearly identical in the two molecules, are considered prerequisites for estrogen binding. Despite this hypothesis, few studies have been published on the effect of PE supplementation on these risk factors (8, 9, 10). Results have suggested a decreasing trend of LDL cholesterol but have failed to reach statistical significance (9). However, the amount of PE used was limited to a maximum of 80 mg, a quantity that approximates the level obtainable with soy-based diets (10). Although this is of practical importance, higher levels available through supplementation may be effective in decreasing the risk for CAD (10). The purpose of this study is to assess the role of supplementation with higher amounts of PE

(150 mg per day) on serum lipoprotein triacylglycerol and cholesterol concentrations in moderately hypercholesterolemic, elderly postmenopausal women.

Subjects and Methods

Subjects and study design

Subjects were recruited initially as part of a larger randomized, double blind, placebo-controlled trial with a parallel design to assess the role of PE supplementation on bone mineral health. Thirty-six healthy, moderately hypercholesterolemic (mean total cholesterol 6.6 ± 1.3 mmol/L) postmenopausal women (mean age 69 ± 4 y), not on hormone-replacement therapy (HRT), were randomly assigned to take either a PE supplement (n = 20) or a placebo (n = 16) three times daily for 6 months. With the exception of mild hypercholesterolemia, all subjects were in good general health with no clinical or biochemical evidence of diabetes, renal, hepatic or cardiovascular disease. With the exception of two individuals, none of the subjects was taking any medication known to affect carbohydrate or lipid metabolism. One subject was taking simvastatin and the other fluvastatin for hypercholesterolemia. Both subjects were on a stable dose for at least 1 year prior to the study, and medications were not altered during the study period. Because the results from these two individuals were indistinguishable from the other 34 participants, they were included in the analyses of the data. All subjects signed a consent form approved by the Administrative Panel on Human Subjects in Medical Research at Stanford University School of Medicine before participating in the study.

The baseline characteristics of the subjects are provided in Table 1. The two groups did not significantly differ in age, weight, body mass index (BMI), total

cholesterol, HDL cholesterol, non-HDL cholesterol or triacylglycerol concentrations. The PE and placebo tablets contained either 150 mg of soy- derived isoflavones or maltodextrin with 10% caramel color, respectively. In the PE tablet, 90mg of the total isoflavones were present as aglycones, of which 45% (40 mg) were genistein and the remaining (55%) daidzein and glycitein (11). Fasting serum was obtained at baseline, 2, and 6 months. The subjects were instructed to maintain their usual diet and lifestyle habits, with the exception of excluding soy-containing foods. Thirty-five of the 36 subjects completed a 3-month, self-administered, semi-quantitative food-frequency questionnaire (National Cancer Institute version FULL 87) (12). Diets were analyzed at baseline and at 3 months using the Dietary Analysis System (Dietsys) version 3.6 (National Cancer Institute, Bethesda, MD).

Lipid analyses

Total serum triacylglycerol and cholesterol concentrations were measured on fasting samples obtained at baseline and again following 2 months and 6 months of treatment. Because of unavailability of serum, determinations of triacylglycerol and cholesterol concentrations at 6 months were performed only on 17 and 18 of the 20 subjects in the PE treated group, respectively. All samples were analyzed in duplicate. Intraassay coefficients of variation were $\leq 2\%$ and interassay coefficients of variation were $\leq 4\%$. Total serum triacylglycerol concentrations were determined using a quantitative enzymatic method (Sigma Diagnostics, St Louis MO) (13). Total serum cholesterol and HDL cholesterol concentrations were measured at the same time for each individual, using standard Lipid Research Clinic Procedures established by the CDC in

Atlanta, Georgia (14, 15). HDL cholesterol concentrations were determined, following selective precipitation of apo B-containing lipoproteins with PTA and $MgCL_2$, at baseline and again at 2 months of treatment. Non-HDL cholesterol concentrations were calculated by subtracting HDL cholesterol from total serum cholesterol.

Statistical analyses

All values are presented as mean ± SEM. Standard statistical techniques for the analysis of two sample means were used (16). Non-paired t test was used to compare differences in dietary intake at baseline and 3 months between the two groups. Non-paired t test was used to compare differences in total triacylglycerol, cholesterol, HDL cholesterol and non-HDL cholesterol between groups, at baseline and 2 months. ANOVA was employed to compare fasting triacylglycerol and cholesterol concentrations between the two groups, at baseline and after 2 and 6 months. The level of statistical significance was set at $P < 0.05$.

Results

The composition of the subjects' diets at baseline and 3 months following supplementation are presented in Table 2. There were no significant differences between the two groups at baseline or at 3 months with respect to total energy intake, the amount of total fat, saturated fat, cholesterol or dietary fiber, but there was a significant difference in protein intake between the two groups at 3 months ($P<0.05$). The changes in dietary protein intake, although statistically significant, were quantitatively very small and resulted from a 1% increase in the PE treated group at 3 months. These differences

are probably of little or no clinical significance, given the semi-quantitative nature of the food-frequency questionnaire.

The results of the lipid analyses are presented in Table 3. The main comparisons in serum lipids were made between the placebo and PE groups at baseline and following 2 months of treatment. The results showed no significant differences between the two groups in total triacylglycerol, total cholesterol, HDL cholesterol or non-HDL cholesterol, at baseline or after 2 months of supplementation with 150 mg of PEs. In addition, comparisons of total serum triacylglycerol and cholesterol concentrations at 6 months continued to show no significant changes between the two groups.

Discussion

The results of the present study demonstrate that PE supplementation with 150 mg of soy-derived isoflavones, for 6 months, was ineffective in reducing total serum triacylglycerol and cholesterol concentrations in these moderately hypercholesterolemic postmenopausal women. In addition, there were no significant differences in the cholesterol concentrations found in either the HDL or non-HDL lipoprotein fractions at 2 months. As such, these data argue strongly against the notion that the hypocholesterolemic effect previously reported with soy-based diets (3, 4, 5, 6) resulted from their PE content.

Phytoestrogens have been promoted as good candidates for the cholesterol-lowering effect of soy because of their similarity to endogenous estrogen in chemical structure and biological activity (7). Genistein, the predominant PE in soy, exhibits a weak estrogenic activity, on the order of 10^{-2} to 10^{-3} that of 17β-estradiol, but can still

achieve maximal stimulation of the estrogen receptor (17). However, our findings, along with previous investigations on the effect of PE supplementation in postmenopausal women (8, 9, 10), do not support this general hypothesis. Nestel and colleagues studied the effects of a pure soybean extract in 21 postmenopausal women in a placebo-controlled, crossover trial over 5- to 10-week periods (8). The results suggested that daily supplementation with 40 or 80 mg of isoflavones did not significantly alter lipid or lipoprotein profiles. The authors reported equivalent findings in a second, similar study using an isoflavone extract from red clover, a plant which contains the isoflavones present in the soybean (genistein and daidzein) as well as their precursors (biochanin A and formononetin) (9). The authors discussed an apparent downward trend in LDL (6%) and an upward trend in HDL (4%), which resulted in an apparent reduction (10%) in the LDL/HDL cholesterol ratio between the placebo and treatment values. However, none of these differences, including total cholesterol (3%), was statistically significant, and all were quantitatively small. More recently, similar findings were reported in a placebo-controlled, crossover study of 20 postmenopausal women (10). In this study, after 8 weeks of supplementation with 80 mg of soy isoflavones, cholesterol concentrations were essentially identical ($\leq 1\%$) when compared with placebo values.

It has been suggested that the cholesterol-lowering effects of soy protein are strongly associated with baseline serum cholesterol concentrations (18) and may depend on the dose of PE administered (10). All the subjects in the present study were moderately hypercholesterolemic and the dose of isoflavones used (150 mg/day) was 2 to 3 times the levels that could be reasonably expected in soy-based diets. Thus, the

conditions of the present study should have been optimal for the detection of any hypocholesterolemic effect. The results of this study expand upon previous investigations and strongly suggest that PE may play a limited role in modifying serum cholesterol in this population. The fact that total serum triacylglycerol and cholesterol concentrations remained unchanged for 6 months after supplementation with 150 mg of PE suggests that components present in soy, other than PE, such as saponins, phytic acid or the soy protein itself, may contribute to the cholesterol-lowering potential observed with soy (19). It is also possible that the hypocholesterolemic effect observed with soy-based diets may not be attributed specifically to a single factor but result from a synergistic action of several components present in soy. Unfortunately, the experimental design of the present study does not allow us to address this hypothesis, and the nature of the hypocholesterolemic effects of soy remains to be elucidated.

In conclusion, PE supplementation at 150 mg/day, for 6 months, did not significantly change lipid or lipoprotein concentrations in moderately hypercholesterolemic postmenopausal women. Collectively, these results, along with previously published data (8, 9, 10) strongly suggest that PE are not the responsible agent for the hypocholesterolemic effect of soy protein. Moreover, it is unlikely that supplementation would effectively lower CAD risk factors associated with hypercholesterolemia in postmenopausal women. The mechanism for the cholesterol-lowering effect of soy remains to be established and its importance in respect to the risk for CAD in this population may need to be further evaluated.

Acknowledgements

The authors gratefully acknowledge Drs. Robert Marcus, Gail Butterfield, Gene Spiller, and Rosemary Schmele for providing the serum samples and for their invaluable assistance in the completion of this study.

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Table 1

Baseline characteristics of subjects in the placebo and phytoestrogen (PE) supplemented groups[1]

Variable	Placebo (n = 16)	PE (n = 20)
Age (y)	70 ± 1 (65-77)	69 ± 1 (64-83)
Body weight (kg)	66 ± 4 (48-102)	68 ± 2 (50-86)
Body mass index (kg/m^2)	25 ± 1 (19-39)	25 ± 1 (18-32)
Total cholesterol (mmol/L)	6.3 ± 0.5 (4.9-12.3)	6.8 ± 0.2 (5.7-8.2)
HDL cholesterol (mmol/L)	1.2 ± 0.1 (0.7-1.8)	1.2 ± 0.1 (0.7-2.0)
Non-HDL cholesterol (mmol/L)	5.1 ± 0.5 (3.5-11.4)	5.6 ± 0.2 (4.4-7.0)
Triacylglycerol (mmol/L)	1.3 ± 0.2 (0.1-3.0)	0.8 ± 0.1 (0.2-2.4)

[1]Values are expressed as mean ± SEM; ranges are given in parentheses.

Table 2

Dietary intakes in the placebo and phytoestrogen (PE) supplemented groups at baseline and 3 months[1]

	Placebo (n=16)		PE (n=19)	
Variable	Baseline	Post	Baseline	Post
Energy (kJ)	7502 ± 786	6460 ± 531	6870 ± 536	6958 ± 469
Protein (%)	15 ± 1	15 ± 1	16 ± 1	17 ± 1[2]
Fat (%)	35 ± 2	34 ± 2	32 ± 2	34 ± 2
Saturated (%)	10 ± 1	10 ± 1	10 ± 1	11 ± 1
Cholesterol (mg)	258 ± 70	183 ± 20	184 ± 22	210 ± 20
Carbohydrate (%)	49 ± 2	50 ± 3	53 ± 2	48 ± 2
Fiber (g)	17 ± 1	17 ± 2	20 ± 2	19 ± 2

[1]Values are expressed as mean ± SEM.

[2]Significantly different from placebo ($P < 0.05$)

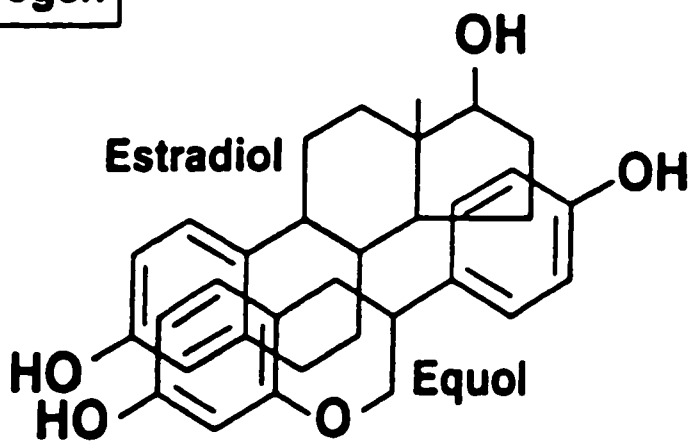
Table 3

Serum lipids and lipoproteins in the placebo and phytoestrogen (PE) supplemented groups at baseline and postintervention[1]

	Placebo (n=16)			PE (n=20)		
Variable	Baseline	2 months	6 months	Baseline	2 months	6 months
Total cholesterol (mmol/L)	6.3 ± 0.5	6.4 ± 0.4	6.0 ± 0.2	6.8 ± 0.2	6.5 ± 0.2	6.4 ± 0.2
HDL cholesterol (mmol/L)	1.2 ± 0.1	1.0 ± 0.1		1.2 ± 0.1	1.0 ± 0.1	
Non-HDL cholesterol (mmol/L)	5.1 ± 0.5	5.3 ± 0.4		5.6 ± 0.2	5.5 ± 0.2	
Triacylglycerol (mmol/L)	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.9 ± 0.1

[1]Values are expressed as mean ± SEM.

Estrogen



Isoflavone

Legend

Figure 1 Comparison of the chemical structures of estradiol and the isoflavone metabolite equol showing their nearly superimposable characteristics.

CHAPTER III

SUMMARY AND RECOMMENDATIONS

REFERENCES

Summary and Recommendations

Phytoestrogens are plant compounds, found mainly in soybeans, that possess chemical characteristics and biological activity similar to endogenous estrogens. Estrogen is known to reduce cholesterol levels in postmenopausal women by increasing its clearance from plasma, and estrogen as well as hormone replacement therapies have been effective in reducing this risk factor for CAD. It was hypothesized that PE would alter lipid metabolism by the same mechanism as endogenous estrogen and that supplementation with PE would be associated with lower cholesterol levels in postmenopausal women. However, no effects of PE on serum cholesterol were observed in a group of 36 moderately hypercholesterolemic postmenopausal women. The fact that lipids and lipoproteins remained unchanged after 6 months of supplementation suggests that PE may not play an important role in altering cholesterol metabolism in this population.

These findings are in agreement with previous research on the effects of PE extracts on the lipid profiles in postmenopausal women. In addition, the current study presents two advantages to previous investigations. It has been demonstrated that initial cholesterol concentrations are a strong determinant of the magnitude of the reductions in cholesterol levels observed with soy-based diets, and the participants in the present study were moderately hypercholesterolemic. Secondly, it has been suggested that effects of PE may be dose-dependent. The dosage of PEs used in this study was higher than those previously utilized, and corresponds to 2 to 3 times the levels observed in the typical

Asian diet. Therefore, the present study was conducted in conditions that were optimal for the detection of an effect of PE, if indeed, it were present.

The results of this investigation lead to the conclusion that PE may not effectively modify the risk factors for CAD associated with hypercholesterolemia. However, CAD is a multifactorial disease and PE have demonstrated great potential in reducing its risk by a variety of other mechanisms. Similarly to endogenous estrogen, they have shown to possess antioxidant activity and effectively reduce the rate of LDL-oxidation. Moreover, they have shown to increase the elasticity of main conduit arteries and inhibit coagulation and platelet aggregation by acting as potent tyrosine kinase inhibitors. Interestingly, only 25-50% of estrogen's effect on CAD risk can be attributed to its modulation of lipid metabolism, and estrogen is known to protect from CAD by mechanisms similar to those discussed above. Unlike estrogen, PE exhibit antiproliferative and anticarcinogenic properties, thus reducing the risk for CAD without increasing the risk for hormone-dependent cancers.

Phytoestrogens are becoming increasingly present in the American diet, due to the recent popularity and availability on the market of soy-based products and dietary supplements. Postmenopausal women are attracted to these compounds for their potential in relieving a number of postmenopausal symptoms, and are constantly in search of alternatives to established therapeutic measures. Although the role of PE in reducing cholesterol levels is questionable, these compounds have great potential for decreasing CAD risks factors other than hypercholesterolemia. However, the optimal dosage and the conditions for maximizing these beneficial effects have not yet been

established and further research is warranted to investigate these promising plant estrogens.

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APPENDIX A

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TO: Antonella Dewell
1911 Magdalena Circle, #87
Santa Clara, CA 95051

FROM: Nabil Ibrahim, N
AVP, Graduate Studies & Research

DATE: November 29, 2000

The Human Subjects-Institutional Review Board has approved your request to use human subjects in the study entitled:

"The Effects of Phytoestrogen Supplementation on Serum Lipids and Lipoproteins in Elderly Postmenopausal Women"

This approval is contingent upon the subjects participating in your research project being appropriately protected from risk. This includes the protection of the anonymity of the subjects' identity when they participate in your research project, and with regard to any and all data that may be collected from the subjects. The approval includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Nabil Ibrahim, Ph.D., immediately. Injury includes but is not limited to bodily harm, psychological trauma and release of potentially damaging personal information. This approval is in effect for one-year and data collection beyond November 29, 2001 requires an extension request.

Please also be advised that all subjects need to be fully informed and aware that their participation in your research project is voluntary, and that he or she may withdraw from the project at any time. Further, a subject's participation, refusal to participate, or withdrawal will not affect any services the subject is receiving or will receive at the institution in which the research is being conducted.

If you have any questions, please contact me at (408) 924-2480.

APPENDIX B

NOV. 20. 2000 10:42AM SUMC IMMUNOLOGY 650 723 9656 SITY
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DAVID GABA, M.D.
CHAIR, PANEL ON MEDICAL HUMAN SUBJECTS
c/o Administrative Panels Office
(650) 725-5973
(650) 725-8013 (fax)

CERTIFICATION OF HUMAN SUBJECTS APPROVAL

DATE: August 10, 1999

TO: G. Butterfield, M.D.
R. Marcus, M.D., G. Spiller, Ph.D.
Department of GRECC

FROM: Chairman, Administrative Panel
on Human Subjects in Medical Research

PROTOCOL TITLE:
Randomized, Double-Blind, Placebo Controlled Trial of Phytoestrogen Supplementation on Bone Density in Post Menopausal Women

The Panel approved human subjects involvement in your research project on August 10, 1999.

The expiration date of this approval is August 9, 2000. If this project is to continue beyond that date, please submit an updated proposal in advance for the Panel's re-approval. If this proposal is used in conjunction with any other human experimentation or if it is modified in any way, it must be re-approved for these special circumstances. In addition, the Panel requests prompt notification of any complications which may occur during any experimental procedure.

All continuing projects and activities must be reviewed and re-approved at least annually by the Panel. Panel approval of any project is for a maximum period of one year. It is the responsibility of the investigator to resubmit the project to the Panel for continuing review.

Please remember that all data including all signed consent form documents must be retained for a minimum of three years past the completion of this research. Additional requirements may be imposed by your funding agency, your department, or other entities. (Policy on Retention of and Access to Research Data, Research Policy Handbook, http://www-portfolio.stanford.edu/105793).

This institution is in compliance with requirements of protection of human subjects (45 CFR 46 and 21 CFR 56).

David M. Gaba, M.D., Chairman

Funding Agency: Archer Daniels Midland Co. pending (SPO pending) (R)
Period of Time: 08/10/99 through 08/09/00
Investigational New Drugs: N ,
Investigational New Device: N ,
Cooperating Institution: N
Full Board Review
Assurance Number: M1272 #03