SERUM ESTROGEN CONCENTRATION OF RATS FED SOY PROTEIN DIET AND SUBJECTED TO 20% ENERGY RESTRICTION

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BY

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DEDICATION

To my husband, Farzin,

because of his love and continuous encouragement and support

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ABSTRACT

Serum estrogen concentration of rats fed soy protein diet and subjected to 20% energy restriction. Mehran, Shiva. A Dissertation, Department of Nutrition and Food Sciences, Texas Woman's University, Denton, Texas. December 1996.

Forty-eight 4-week-old female Sprague-Dawley rats were fed one of the four experimental diets for four weeks. Groups C-AL and SPI-AL were fed casein or soy protein isolate diets, respectively, ad libitum. The 20% energy restricted groups, C-R and SPI-R were fed casein or soy protein isolate diets at 80% energy intake of C-AL and SPI-AL, respectively. Serum estradiol concentration of these rats was determined at the end of feeding period and no significant differences were detected among the four groups. However, the 20% energy restricted groups had 77%-88% of serum estradiol concentration of their ad libitum counterparts.

In the second study, 81 sera from 26-week-old rats that had the same four dietary treatments and received either 7,12-Dimethylbenz(a)anthracene (DMBA) or sesame oil at the age of 8 weeks were analyzed for estradiol concentration. No significant differences in serum estradiol concentrations were detected among the groups. DMBA-treated groups had serum estradiol concentration of 79%-94% of their sesame

oil-treated counterparts. Except for the rats in the sesame oil-treated group which were fed casein ad libitum, all ad libitum groups had higher serum estradiol concentration than their respective restricted groups. The type of the dietary protein did not influence the serum estradiol concentration in these rats.

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CHAPTER I

INTRODUCTION

Breast cancer is the most common form of cancer among American women. One in every eight American women will develop breast cancer, about 182,000 women developed breast cancer in 1995, of which 46,000 died (Cancer Facts & Figures 1995). The magnitude of the problem is more astonishing when breast cancer incidence in the U.S. is compared to that of the Asian countries where the incidence of breast cancer is much lower. The possibility of involvement of the genetic factors was weakened by the results of migration studies which indicated breast cancer incidence and mortality are mostly related to environmental factors (Committee on diet, Nutrition, and Cancer, 1982).

Lengthy exposure to cyclical estrogen is included among the risk factors for breast cancer (Cancer facts & figures 1995). Estrogens are a family of steroid hormones synthesized mainly by ovaries. The principal functions of the estrogen are cellular proliferation and growth of tissues of the sex organs and of other tissues related to reproduction (Guyton 1991). The most abundant estrogen

secreted by ovaries is ß-estradiol. It has been proposed that elevated free blood estrogen concentration is associated with increased breast cancer risk (Key et al. 1988).

Tamoxifen is a potent synthetic antiestrogen drug. It decreases the circulatory concentration of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn leads to a reduction in the secretion of estrogens by ovaries (Golder et al. 1976). Some of these effects can be mimicked by dietary factors such as isoflavonoids from soybean. Isoflavonoids whose structures are similar to estrogens are nonsteroidal estrogens and are believed to compete with estrogens for the binding to estrogen receptors in target cells (Setchell et al. 1984). Isoflavonoids could be the contributing factors for the anti-carcinogenic effect of soybean (Cassidy et al. 1994). Lower incidence of breast cancer in Asian women is thought to be related to their high intakes of soy products in combination with low fat diets (Lee et al. 1991).

Epidemiologic evidence has shown a strong positive correlation between fat consumption and incidence of breast cancer (Armstrong et al. 1975). Animal studies have supported the promoting effect of high fat diets on mammary tumorigenesis (Welsch et al. 1985, Carroll et al. 1991). Recently, the influence of caloric intake on cancer

development is more emphasized. Caloric restriction was shown to inhibit chemically-induced mammary tumorigenesis in rats, even when the rats were fed a high fat diet (Klurfeld et al. 1989). A recent study from our laboratory (Ong 1995) has shown that rats fed a soy protein diet with 20% energy restriction had lower incidence of 7, 12-Dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis than those who consumed casein or soy protein at ad libitum level. The hormonal status of the animals was not examined. Blood samples from these rats were being stored at -70 °C and were available for analysis.

Available data on serum estrogen are mostly comparisons between vegetarian and omnivorous women (Shultz et al. 1983), or between caucasian American and oriental immigrant women (Goldin et al. 1986). Cassidy et al. (1994) reported significant suppression of midcycle surges of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and delayed menstrual cycle in premenopausal women who had a constant soy protein diet. Feeding soy protein diet to rats was shown to reduce the incidence of mammary tumorigenesis by Barnes et al. (1991) and Hawrylewicz et al. (1995). In these studies, serum concentration of estrogen of rats was not determined. To the knowledge of this investigator, there are no available data on the serum concentration of estradiol from animals consuming soy protein diet either

before or after the mammary tumor formation. Nor are there any data on the relationship between energy restriction and serum estradiol concentrations.

The purpose of this study was to determine serum estrogen concentrations of rats fed a soy protein diet with or without 20% energy restriction during the time period prior to (day 28-56) or post carcinogen administration which occurred at day 56.

Research Questions

1. What is the effect of feeding soy protein before DMBA administration on serum free estradiol concentrations in rats?
2. What is the effect of feeding soy protein on serum free estradiol concentration in rats which developed DMBA-induced mammary tumors?
3. What is the effect of 20% energy restriction before DMBA administration on serum estradiol concentrations in rats?
4. What is the effect of 20% energy restriction on serum free estradiol concentration in rats which developed DMBA-induced mammary tumors?

CHAPTER II

REVIEW OF LITERATURE

Cancer, a disturbance in the rate of cellular growth and differentiation, is a multi-stage disease. Two distinct stages, initiation and promotion, have been identified. Initiation is the phase in which genetic information of the cell has been altered as the result of carcinogen interaction with the cell. Promotion is when initiated cells show an altered pattern of cellular proliferation (Hick 1983). Each of these stages may be affected by diet (Poirier 1987).

Diet plays an important role in the etiology of cancer, especially hormone-related cancers, or colon cancer (Armstrong et al. 1975, Gori 1978). Dietary fat is the most studied food component in relation to breast cancer and evidence supporting its involvement in cancer risk is overwhelming in both epidemiological studies and animal experimentations. Tannenbaum (1942) was the first to show that spontaneous mammary tumors developed more rapidly in mice fed a high fat diet. In 1991 Carroll et al. suggested that high fat diets tend to promote mammary cancer by

increasing the amount of adipose tissue in the gland . This adipose tissue appears to exert a strong influence on growth and development of the glandular parenchymal tissue, in which cancers originate. This hypothesis could also explain the effects of dietary restriction because restriction would be expected to result in a marked reduction in the fat content of the mammary gland.

The influence of caloric restriction on the outcome of mammary tumorigenesis has also been studied in rodents. Kritchevsky et al. (1984) reported that 40% caloric restriction completely inhibited chemically-induced mammary tumorigenesis in rats. Klurfeld et al. (1989) further showed that caloric restriction could inhibit DMBA-induced mammary tumorigenesis in rats even when the animals were fed a high fat diet. Recently, Zhu et al. (1991) showed that a 30% caloric reduction after tumor formation could inhibit the growth of chemically-induced mammary tumors in rats.

The effect of the quantity or the quality of dietary protein on breast cancer incidence is the subject of ongoing researches. Although many studies have been done, the results are inconclusive. As early as 1949, Tannenbaum reported no effect of increasing the amount of dietary protein from 9% to 45% on spontaneous mammary tumor incidence in mice.

Clinton et al. (1979) demonstrated that increasing the

level of protein intake from 7.5% to 15% during the initiation phase of mammary tumorigenesis decreased the incidence of 7,12-Dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. These authors further demonstrated that the decreased incidence of mammary tumors in rats was related to the increased activity of hepatic xenobiotic metabolizing enzymes. Clinton and his colleagues (1984, 1986, 1988) later showed that the protective effect of high protein intake against mammary tumorigenesis was only effective during the initiation phase, not the promotion phase of carcinogenesis.

On the other hand, in a multi-generation study by Hawrylewicz et al. (1982) increased chemically induced mammary tumor incidence was shown in rats fed high amount of protein (31%) throughout the initiation and promotion stages of tumorigenesis.

As far as the quality of dietary protein is concerned, a few studies are available. Lubin et al. (1986) indicated that increased intake of animal protein is associated with higher breast cancer incidence. Hawrylewicz et al. (1991) showed that rats treated with N-methylnitrososurea (NMU) and fed a soy protein isolate diet had lower mammary tumor incidence than those fed a casein diet. Supplementing soy protein isolate with methionine increased the NMU-induced mammary tumor incidence in rats by 50%. In 1991 Hsueh et

al. reported that soy protein diet supplemented with methionine increased the total number of DMBA-induced mammary tumors in rats. The tumors in methionine-supplemented soy group were 100% adenocarcinoma as opposed to only 20% in the soy protein group. These data support that the difference in amino acid composition of proteins is a contributing factor in tumor growth rate and histopathology of the mammary tumor.

The combined effect of reduced dietary intake and type of dietary protein on mammary tumorigenesis was studied by Hsueh et al. (1992) who showed when dietary intake was reduced by 20%, a diet containing casein (good quality protein) was more beneficial than a diet containing wheat gluten (poor quality protein) on DMBA-induced mammary tumor incidence. A more recent study from our laboratory (Ong 1995) indicated that the DMBA-induced mammary tumorigenesis in rats could be reduced by feeding soy protein diet and 20% energy restriction. The rats which consumed soy protein at 20% energy restriction had the lowest tumor incidence after DMBA administration.

Much attention has been focused on soy consumption and breast cancer risk in recent years (Messina et al. 1994). Lee et al. (1991) proposed that a high intake of soy products may protect against breast cancer in premenopausal women. Comparing the dietary habits of 200 Singapore

Chinese women with breast cancer to 420 control subjects, these authors observed a decreased breast cancer risk with the intake of soy products.

Hawrylewicz et al. (1991) indicated that methionine-deficiency of soy protein may contribute to the tumor-suppressing activity of soy products in rats. These authors showed that the casein-fed rats had the highest number of NMU-induced mammary tumors followed by rats fed soy protein supplemented with methionine. The rats which been fed soy protein had the lowest number of NMU-induced mammary tumors.

Barnes et al. (1990) reported chemopreventive effect of soy bean diets on NMU-induced mammary tumorigenesis in rats. In their study, soy protein concentrate at all levels (5%-20%), both autoclaved and nonautoclaved, inhibited the appearance of NMU-induced mammary tumors. These authors also suggested that the antiestrogenic compounds, phytoestrogens, in soy beans are responsible for the inhibiting effect on tumorigenesis. Later in 1995, Barnes showed that isoflavone-depleted soy was inactive against rat mammary tumorigenesis. Further, he showed that genestein, the major isoflavonoid in soy, delayed the appearance and growth of tumors rather than preventing their formation.

Isoflavones have been indicated as the most active anti-tumor constituent in soy. Molteni et al. (1995) defined isoflavones as weak estrogens which may function

against or with estrogen depending on the animal species, hormonal status of the subject, type of the isoflavone and its concentration. Isoflavones are capable to bind to estrogen receptors in the mammary cells, thus competing with estrogen to bind to its receptors. By this competition, isoflavones may contribute to the preventing of neoplastic growth.

Phytoestrogens have shown estrogenic or antiestrogenic activity, depending on the level of endogenous estrogens (Adlercruetz 1990) and the level of exogenous estrogens (Zava et al. 1995). Zava et al. (1995) found that adding low amounts of genestein (1-100 nM) to an estrogen-free serum medium promotes the growth of a human breast cancer cell line, but at a higher concentration (1 μM) it inhibits the growth. These authors concluded that genestein appears to act as estrogen agonist at physiological concentration.

Several other possible mechanisms of tumor-suppressing effects of genestein have also been proposed. It has been reported by Akiyama (1987) that genestein is an inhibitor of protein tyrosine kinase (PTK), an enzyme whose action promotes cell proliferation. Increased activity of PTK is known to give cells a proliferative advantage (LeCam 1991).

Genestein is also an effective inhibitor of eukaryotic DNA topoisomerase II, an enzyme involved in cellular replication (Markovits et al. 1989). Decreased activity of

either topoisomerases or tyrosine kinases has been implicated in the differentiation of a number of cell types.

Genestein inhibits malignant angiogenesis which could be a protection against cancer. However, for genestein to block the angiogenesis, this vital part of tumor growth and metastasis, its serum level must be in an excessive amount (Fotsis et al. 1993).

Genestein is believed to have biological antioxidant properties (Wei et al. 1993) which inhibits the production of hydrogen peroxide in human and skin cells. Reactive oxygen species play an important role in mutagenesis and carcinogenesis, particularly tumor promotion (Frenkel 1992).

Constantinou et al. (1995) showed that genestein, in a dose dependent manner, triggered cellular differentiation, which led to the inhibition of cell proliferation. These authors showed that genestein, first, interacted with topoisomerase II and stabilized the complex between DNA and the enzyme. Second it affected the phosphorylation of protein substrates, as a PTK inhibitor. Both of these events led the cell to become more differentiated rather than proliferated.

In 1995 Lamartiniere et al. hypothesized that genestein exerts its chemoprevention action by acting directly to enhance maturation of terminal ductal structures and by altering the endocrine system to reduce cell proliferation

in the mammary gland. In their study, female Sprague-Dawley rats which were given genestein neonatally had increased latency and reduced incidence and multiplicity of DMBA-induced mammary tumors compared with vehicle-treated animals. In 21-day old genestein-treated rats, mammary glands were larger and there were more terminal end buds and terminal ducts. These authors concluded that the maturation of terminal end buds to lobules appeared to provide a basic protective mechanism against chemical carcinogenesis. The same conclusion was drawn from another study done by Thodarson et al. (1995) who showed that parity could totally inhibit the formation of NMU-induced mammary tumors in female sprague-dawley rats. They hypothesized that parity resulted in permanent changes in the mammary gland that prevent cancer development.

Adlercreutz et al. (1992) showed a positive correlation between urinary excretion of phytoestrogens and plasma concentrations of sex hormone binding globulin (SHBG) in Finnish omnivorous and vegetarian women. These authors suggested that isoflavonoids increased SHBG synthesis in hepatocytes. The elevated SHBG could then cause a decrease in the concentration of free estradiol, thereby lowering tissue exposure to estrogen. In regard to the effect of isoflavonoids on serum SHBG concentrations more controversies exist. In 1995, Baird et al. reported no

increase in serum SHBG levels in post menopausal women on a four week soy product diet. Although urinary isoflavone level increased markedly in these women, no changes in serum SHBG and estradiol concentrations were reported. Loukovaara (1995) showed that genestein increases SHBG concentration significantly only within the hepatocytes without considerable impact on SHBG secretion, possibly by regulating the production of nonsecreted nonsteroid-binding form of the protein. Other isoflavonoids such as daidzein and equol increased the level of SHBG both intra- and extracellularly. These authors concluded that isoflavonoids stimulate the production of SHBG in hepatocytes but their effect on the secretion of SHBG is compound-specific.

The female breast is exposed to a lifetime of hormonal controls, which is more evident at the time of menarche, during the menstrual cycle, pregnancy and lactation (Hulka et al. 1994). Estrogens, the steroid hormones synthesized mainly by ovaries, increase the mitotic rate in the terminal duct of mammary glands (Guyton 1991).

Estradiol induces DNA synthesis in quiescent cells, increases the expression of oncogenes, and functions as a potent mitogen in estrogen responsive tissues (Pick et al. 1993). These observation support the hypothesis that estrogens, because of their mitogenic property, can increase the susceptibility of the target tissue to initiation of

carcinogenesis (Fishman et al. 1995). The 16α-hydroxylated metabolite of estradiol (16α-OHE_1) induces genotoxic damage and aberrant hyperproliferation similar to that induced by chemical carcinogens in the rodent cell culture model. In initiated or fully transformed rodent or human cells, 16α-OHE_1 promotes the expression of the transformed phenotype (Fishman et al. 1995).

In an epidemiological study, Key et al. (1988) showed a higher incidence rate of breast cancer in women with elevated free blood estrogen concentration. Henderson et al. (1985) believed that early removal of the ovaries, the primary source of estrogen and progesterone production, may be beneficial in reducing the risk of breast cancer by a reduction in the life-time number of menstrual cycles. Women with shorter cycles spend more of their life in the luteal phase in which the mitotic activity of the breast reaches its peak (Ferguson et al. 1981).

According to American Cancer Society (Cancer Facts & Figures-1995), long exposure to cyclical estrogen is a risk factor for breast cancer, and among the possible therapies, hormonal status alteration would be a practical choice. Drugs are the usual breast cancer therapeutic means. If changes in sex hormone metabolism can be induced by diet, it would be a more natural way of providing therapeutic means

in relation to breast cancer. Several investigators have shown changes in serum hormone concentrations when diets of women were manipulated. Bennett et al. (1990) showed that change to vegetarian diet for three months could decrease the concentration of serum estradiol in postmenopausal women. Goldin et al. (1994) reported a reduction in serum concentrations of estrone and sex hormone binding globulins (SHBG) in premenopausal women who consumed a low fat/high fiber diet. Earlier, Goldin et al. (1986) reported that premenopausal Caucasian women had 30-75% higher plasma estrone and estradiol levels than their age-matched cohorts in Hawaii. Analysis of the dietary components showed a positive correlation ($r=0.65$, $p<0.001$) between total dietary fat intake and plasma estradiol concentrations of those women. Rose et al. (1993) did not find any significant change in the serum estradiol levels of postmenopausal breast cancer patients after being on a low-fat diet (15-20% of total calorie) for 18 months.

Plasma levels of estradiol were significantly lower in vegetarians when compared to non-vegetarians by Shultz et al. (1983).

The biological effect of soy protein diet on the menstrual cycle of six healthy, non-vegetarian premenopausal women was studied by Cassidy et al. (1994). A daily intake of 60 g soy protein for one month significantly ($p<0.01$)

lengthened the follicular phase. Concentrations of plasma estradiol were also significantly ($p<0.02$) higher during the follicular phase of the menstrual cycle. Midcycle peaks of Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) were significantly ($p<0.05$ and $p<0.01$, respectively) suppressed. The authors commented that dietary modification can lead to significant changes in the regulation of the menstrual cycle and that such changes may be beneficial to the risk factors of breast cancer. In a subsequent follow-up study, Cassidy et al. (1995) reported that when the data from both of their studies (1994, 1995) were combined ($n=9$) there was no significant increase in the follicular-phase plasma estradiol concentration during soy consumption periods.

CHAPTER III

MATERIALS and METHODS

Animals and diets

Female, 21-day old, Sprague-Dawley rats purchased from Sasco Co. Inc., (Omaha, NE) were used in all studies. A copy of the approval of the study protocol from the Animal care and User Committee of Texas Woman's University is included in Appendix A. The rats were individually housed in stainless steel cages with suspended wire-bottom in the animal facility of Texas Woman's University. The animal room was maintained at a temperature of 22±2 °C, a relative humidity of 55%±5%, and a 12 hr light/dark cycle. All the rats were given free access of AIN-76A diet and water for one week of acclimation.

Four semi-purified experimental diets (C-AL, C-R, SPI-AL, SPI-R) modeled after the AIN-76A diet were formulated by BioServ (Frenchtown, NJ). The dietary compositions are shown in Table 1 and the proximate analysis of the diets provided by Bioserv is included in Appendix B. These four diets were isocaloric (4.0 kcal/g of diet). Diets C-AL and

Table 1

Dietary composition (g/kg diet) and caloric density

Ingredient	Diet			
	C-AL	C-R	SPI-AL	SPI-R
Casein[1]	216	270	-	-
Soy Protein Isolate[2]	-	-	226	283
DL-Methionine	3	4	-	-
Sucrose	435	355	430	348
Corn Starch	145	118	143	116
Corn Oil	100	125	100	125
Cellulose	51	64	51	64
AIN-76 Mineral Mix	38	48	38	48
AIN-76A Vitamin Mix	10	13	10	13
Choline Bitartrate	2	2	3	3
Caloric Density (Kcal/g diet)	4.0	4.0	4.0	4.0

[1] High nitrogen casein (BioServ., Frenchtown, NJ) Protein, 91.20%; Carbohydrate, 3.10%; Fat 0.00%.

[2] Soy Protein Isolate (BioServ., Frenchtown, NJ) Protein, 87.06%; Carbohydrate, 0.79%; Fat 0.45%.

SPI-AL contained same amount of protein (20%). The amount of protein, fat, and micronutrients in diets C-R and SPI-R were adjusted so that when 20% energy reduction was imposed on the rats, all four groups of rats consumed the same amount of these nutrients except that they took in a reduced amount of energy.

Feed intakes were monitored by measuring the food offered, food left, and the spillage. An orange color construction paper placed under each individual cage was used to collect the spillage. Orange color is the easiest color to observe spillage. Rats in C-AL and SPI-AL were fed ad libitum. The intakes of the rats in C-R and SPI-R groups were reduced to 80% of the group average intake of C-AL and SPI-AL, respectively. For example, if the average intake of C-AL was 20 g on one day, the rats in the C-R group was given 16 g of the C-R diet on the following day. Body weights were recorded weekly.

Preparation of serum

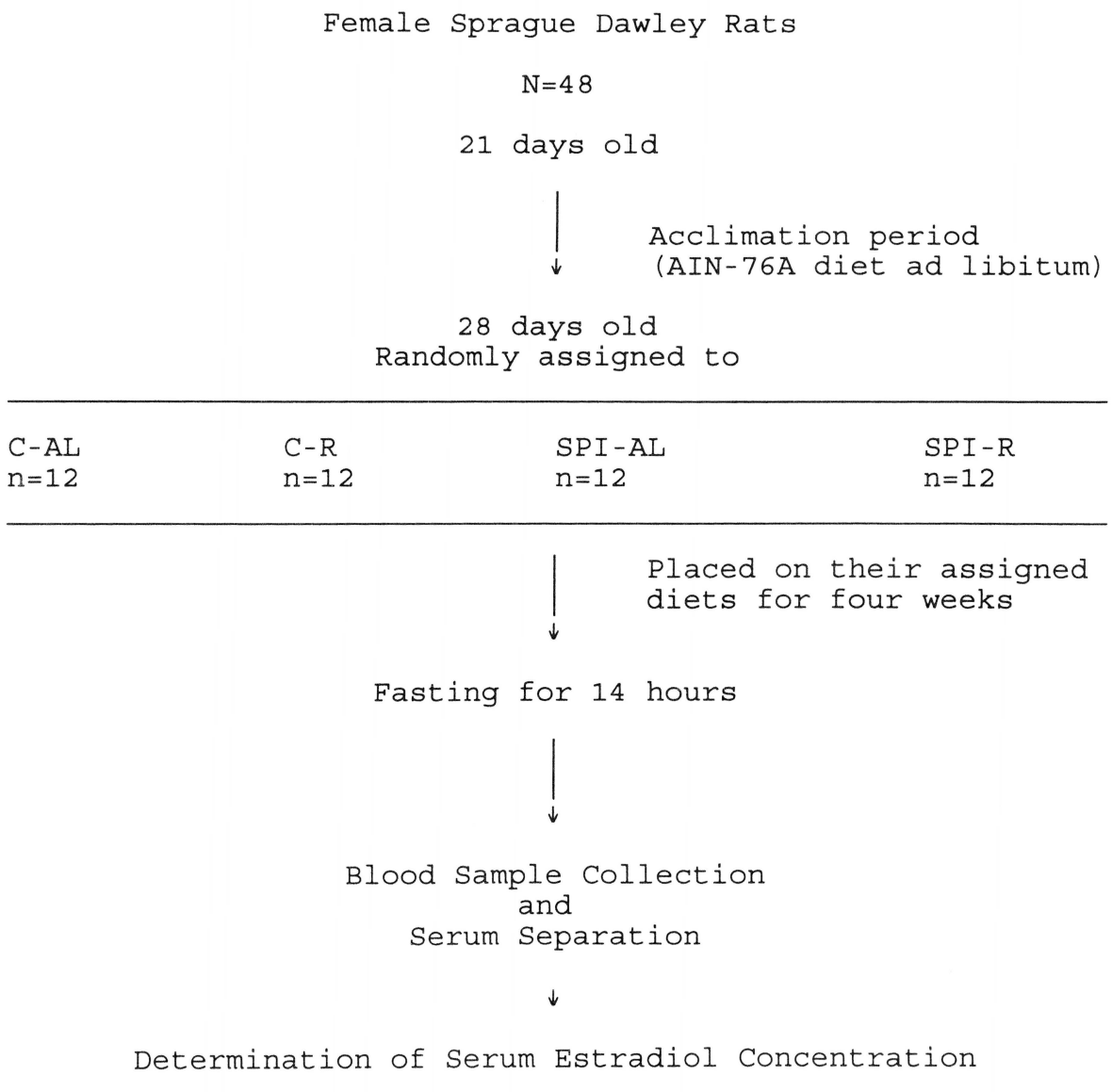
Animals were fasted for 14 hr and anesthetized with Metofane. Blood was drawn from the rats via cardiac puncture and collected in test tubes. Blood samples were allowed to stand at room temperature for 30 min. Serum was separated from the whole blood by centrifuging for 20 min at

1500 x g (IEC, B-22M digital-programmable floor model, International Equipment Company, Needham Heights, MA) and was kept at -70 °C until analysis was done. Figure 1 is a schematic diagram of the study.

Biochemical determination of serum ß-estradiol (E_2)

Serum E_2 concentration was determined by radioimmunoassay described by Xing et al. (1983). Briefly, the serum sample and ^{125}I-labeled estradiol were both added to an antibody-coated tubes. After 3 hr of incubation at room temperature, estradiol that was not bound to the antibody was discarded by decanting. The tube was then counted in a gamma counter (Model 5410, Packard Instrument Company, Meriden, CT). The radioactivity counts were inversely related to the amount of estradiol present in the samples. A set of standards containing estradiol ranging from 0 to 3600 pg/mL were carried out at the same time when the serum samples were analyzed. The quantity of estradiol in the samples was determined by comparing the counts to a standard curve. Coat-A-Count estradiol kits from Diagnostic Products Corporation (Los Angeles, CA) were used. Detailed procedure is included in Appendix C.

Figure 1. Experimental design of study in part I



Serum ß-estradiol concentration of rats during initiation

Forty-eight rats at 28 days of age were randomly assigned to each diet (Table 2) and fed for 28 days. Thereupon, sera were collected and serum ß-estradiol concentration were determined.

Serum ß-estradiol concentration of rats during promotion

One hundred and sixty rats were fed AIN-76A diet ad libitum until 56 days of age. Thereupon, a single dose of 7, 12-Dimethylbenz(a)anthracene (DMBA) was given intragastrically (5 mg/100 g body weight) to each of 120 rats after a 14 hr fast. The concentration of DMBA solution was 5 mg in 0.1 mL sesame oil. The remaining forty rats received sesame oil (0.1 mL/100 g body weight). All the rats were deprived of food for 4 hr before and 4 hr after the administration of DMBA to minimize any interference in the absorption of DMBA by food. During the week immediately following DMBA or sesame oil administration, the animals were housed in the biohazard area of the animal facility and fed AIN-76A diet.

At 63 days of age, the rats were returned to the regular animal room and were randomly assigned to the four dietary treatment groups (Table 2). The end point of the

Table 2

Feeding patterns during initiation and promotion phases

Group	Protein source	Feeding regimen
C-AL	Casein	Ad Libitum
C-R	Casein	80% intake of C-AL
SPI-AL	Soy Protein Isolate	Ad Libitum
SPI-R	Soy Protein Isolate	80% intake of SPI-AL

feeding period was 18 weeks after DMBA administration.

All the rats had free access to water throughout the study. Six weeks after DMBA administration, the rats were examined once weekly until necropsy. Tumor development was monitored. Blood samples were collected at necropsy. A total of 81 serum samples were available for the determination of ß-estradiol concentration. Of which, 41 were from the animals treated with DMBA and 40 were from the animals treated with sesame oil.

Statistical analysis

Body weights, food intakes and serum estradiol concentrations were analyzed using one-way analysis of variance (ANOVA). When statistical significance was detected by ANOVA, Tukey post-hoc test was used to identify the groups that were significantly different. The level of significance was ≤5%.

CHAPTER IV

RESULTS

Body weights and feed intake

Table 3 shows the effect of diets on body weight and feed intake after 28 days of feeding. Rats fed either the casein or the soy protein isolate diet ad libitum (C-AL and SPI-AL) gained similar amounts of weight after consuming the diets for 28 days. Rats in the groups of C-R (Casein diet with 20% energy restriction) and SPI-R (Soy protein isolate diet with 20% energy restriction) also gained similar amount of weight after being on their respective diets for 28 days. Rats in the two ad libitum groups (C-AL and SPI-AL) had significantly ($p<0.05$) gained more body weight than the two restricted groups (C-R, and SPI-R). In addition, rats from the C-R group also gained significantly ($p<0.05$) more weight than those in the SPI-R group for the first two weeks of the feeding. The intake of the C-R and SPI-R groups was significantly ($p<0.05$) lower than the C-AL and SPI-AL groups. Mean weekly weight gains of the rats are included in Appendix D. Neither the data on the body weight and

Table 3

Mean weight gain and feed intake of rats fed casein or soy protein isolate containing diet with or without 20% energy restriction for 28 days

	C-AL	C-R	SPI-AL	SPI-R
Initial body weight (g)	72.0±4.3[a]	71.5±4.2[a]	70.8±3.8[a]	72.1±4.4[a]
Final body weight (g)	180.4±14.7[a]	162.0±10.3[b]	176.9±14.0[a]	151.6±5.1[b]
Weight gain (g)	108.4±10.4[a]	90.5±6.1[b]	106.1±10.2[a]	79.5±0.7[c]
Feed intake (g/day)	14.0±1.5[a]	11.1±1.5[a]	14.5±1.9[a]	11.3±0.3[b]
Feed efficiency of the diet (b.wt gain (g)/ g feed intake)	0.2770	0.2925	0.2618	0.2507

1 Values are Mean ± S.D. Groups not sharing the same letter superscript in a row are significantly different at $p<0.05$ using Tukey post-hoc test.

feed intake, nor the results of tumor development of the rats in the promotion study are reported here. These results were presented in another study by another graduate student. Those rats that received DMBA had all developed mammary carcinomas and the 41 serum samples were from these rats.

Serum estradiol (E_2) concentrations

Table 4 shows the serum estradiol concentrations of the rats fed experimental diets from 28 days to 56 days of age. No statistically significant differences (at $p<0.05$) were found among the four groups. Mean serum estradiol concentrations of the restricted groups (C-R, SPI-R) were 77% and 88% of their respective ad libitum counterparts (C-AL, SPI-AL) as also illustrated in figure 2.

The value from one of the C-R rats was excluded since the Z score was greater than 3 and therefore it was considered as a statistical outlier . The serum estradiol concentrations of individual rats are included in Appendix E.

Serum estradiol (E_2) concentrations of the rats fed casein diets during the 18 weeks after DMBA administration (9-26 weeks of age) are shown in Table 5. No significant differences were found in the serum E_2 concentrations of the

Table 4

Serum estradiol concentration of rats fed experimental diets from 28 days to 56 days of age[1]

Group(n)	Protein Source	Energy Restriction	Serum Estradiol Concentration
			pg/mL
C-AL(12)	Casein	None	25.0 ± 12.3 (100%)
C-R(11)	Casein	20%	19.2 ± 8.8 (77%)
SPI-AL(12)	Soy Protein	None	28.5 ± 14.0 (100%)
SPI-R(12)	Soy Protein	20%	24.7 ± 12.3 (88%)

[1] Values are Mean ± S.D. No significant differences were detected among the four groups at $p<.05$ using Tukey post-hoc test.

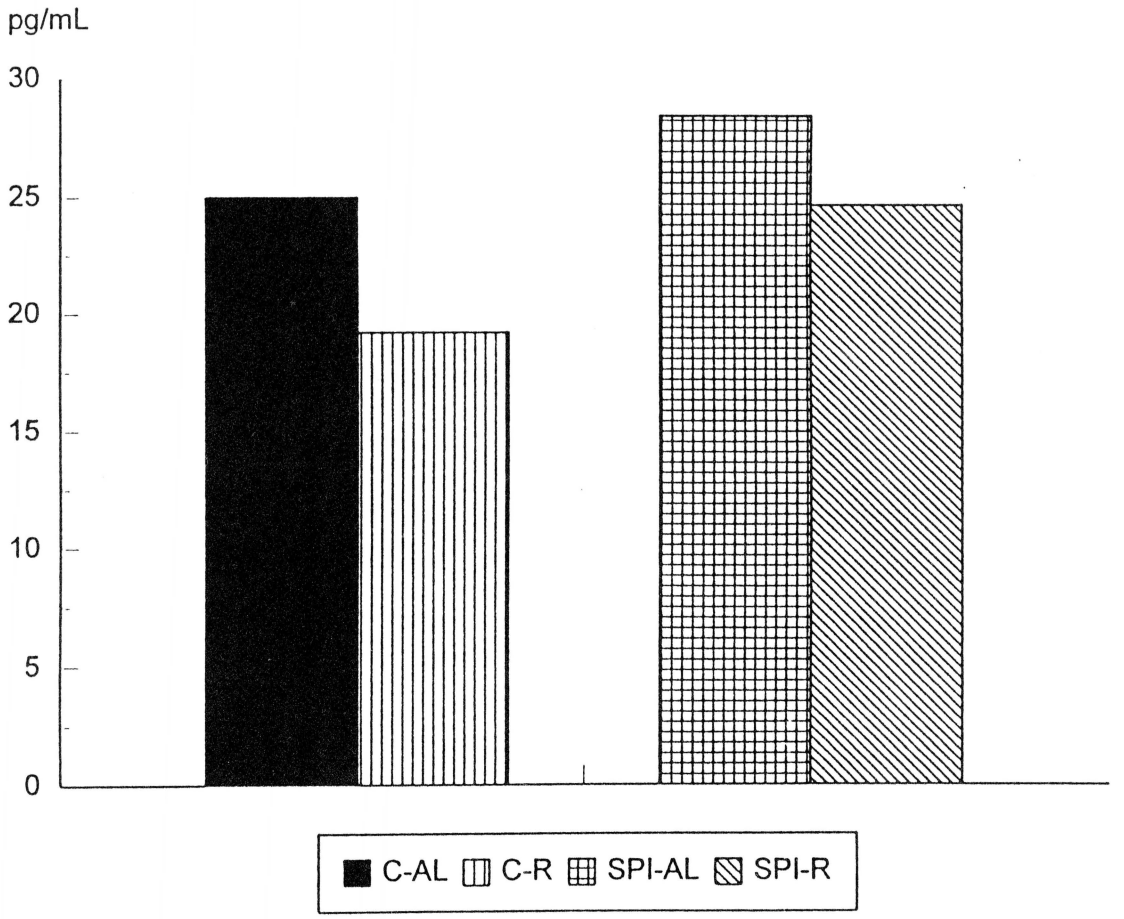


Figure 2. Mean serum estradiol concentrations (pg/mL) of rats fed experimental diets for 28 days

Table 5

Serum estradiol concentrations of rats fed casein diet with or without 20% energy restriction from 9-26 weeks of age[1]

Treatment	Serum estradiol concentration		% of C-AL
	Ad libitum (C-AL)	20% energy restriction (C-R)	
	pg/mL		
DMBA	26.0±20.4 (n=12)	20.6±8.4 (n=10)	79%
Sesame oil (Control)	27.7±11.8 (n=9)	34.1±12.4 (n=9)	123%
% of control	94%	60%	-

[1] Values are Mean ± S.D. No significant differences was detected among the groups at $p<0.05$ using Tukey post-hoc test.

rats among the four groups. Regardless of the dietary treatments, in the ad libitum groups (C-AL and SPI-AL) that rats received DMBA had an E_2 concentration of 94% of those which had received sesame oil. In the two restricted groups (C-R, and SPI-R), the mean E_2 concentration of the DMBA administered group was 64% of the sesame oil administered group. Regarding 20% energy restriction, rats that were fed the casein diet (C-R) reduced their average E_2 concentration by 21% as compared to those received which had DMBA but were not energy restricted. In contrast to the other groups, sesame oil administered rats with 20% energy restriction had elevated E_2 concentration (by 23%).

When the rats were fed the soy protein isolate based diet, energy restriction reduced the serum E_2 concentrations to 75% or 86% for DMBA or sesame oil treated rats (Table 6). A reduction in the serum E_2 concentration was observed when the rats received DMBA rather than sesame oil (Table 6), irrespective of the feeding condition (whether the rats were fed ad libitum or were 20% energy restricted).

Two rats from sesame oil treated groups (one each from casein-fed restricted and soy protein isolate-fed restricted groups) had a Z score > 3. These data excluded in the statistical analysis. Data on serum E_2 concentration from each rat are included in Appendix E.

Table 6

Serum estradiol concentrations of rats fed soy protein isolate diet with or without 20% energy restriction from 9-26 weeks of age[1]

Treatment	Serum estradiol concentration		% of SPI-AL
	Ad libitum (SPI-AL)	20% energy restriction (SPI-R)	
	pg/mL		
DMBA	32.1±18.7 (n=11)	24.2±10.6 (n=11)	75%
Sesame oil (Control)	40.7±21.4 (n=8)	34.8±14.4 (n=9)	86%
% of control	79%	70%	-

[1] Values are Mean ± S.D. No significant differences was detected among the groups at $p<0.05$ using Tukey post-hoc test.

CHAPTER V

DISCUSSION

The present study investigated the effect of soy protein at two levels of energy intake on the serum estradiol concentrations in female Sprague-Dawley rats either before or after 7,12-Dimethylbenz(a)anthracene (DMBA) administration (representing the initiation or promotion of carcinogenesis). This effect was compared to that of the rats fed a casein-containing diet. Feeding during the initiation period lasted for 4 weeks while feeding during the promotion period was for 18 weeks.

In the study in which the experimental diets were fed to the rats for 4 weeks (from 4 weeks to 8 weeks of age), the quality of the casein diet was superior over the soy protein isolate diet. Feed efficiency (defined as body weight gain per one gram of feed consumed) was higher for rats fed the casein diet than those fed the soy protein isolate-diet, irrespective of total energy consumption (Table 3). Both Carroll (1995) and Hsueh et al. (1991) had reported similar findings on feeding soy protein diets to rats.

No significant differences in the serum estradiol (E_2) concentrations were detected among the four groups of rats in the present study regardless of whether the rats were fed the experimental diets for 4 weeks or 18 weeks. Lamartiniere et al. (1995) did not find any significant difference in serum E_2 concentrations of 50-day-old female Sprague-Dawley rats that were neonatally treated with genestein in comparison to those that were in the control group. The genestein-treated rats did show a longer latent period and a reduced DMBA-induced mammary tumor incidence than those that were not treated with genestein. Baird et al. (1995) did not find any significant change in serum E_2 levels in post-menopausal women who consumed a soy supplemented diet for four weeks. Cassidy et al. (1994), on the other hand, found a significantly higher plasma E_2 level during the follicular phase in six pre-menopausal women who consumed soy-base diet for one month. However, in a subsequent study of nine pre-menopausal women who had consumed a soy diet for one month (including the six women previously studied) no significant increase in the follicular-phase plasma E_2 concentration was found.

Although the present study did not find any statistically significant difference of serum estradiol levels among the experimental groups, there are several

interesting points worthy of mentioning. When the rats were fed ad libitum, the serum E_2 concentrations were higher (ranged from 12% to 25% higher) than those of the rats had a 20% energy restriction (Tables 4, 5, and 6). This was true for all but one group of rats. The length of the feeding period or the type of protein in the diet did not have much influence on the outcome. The 20% energy restricted rats had a 12% to 25% lower serum E_2 concentrations than those fed ad libitum. No data on serum E_2 concentration from either animal or human studies related to dietary or energy restriction are available in the literature. It seems reasonable to assume that reduced intake of energy can diminish the synthesis of E_2, thus lower the circulating E_2 in the serum. The one exception was when casein was the diet and the rats had received sesame oil. These 20% energy restricted rats had an increase of 23% in their serum E_2 concentrations over that of their ad libitum counterpart. The reason for the increase is unknown.

Rats that were given DMBA, consistently showed a lower serum E_2 concentration than those which received sesame oil (Table 6). These rats were given either DMBA or sesame oil at the age of 56 days and at that time their dietary treatments began. The serum E_2 concentrations were determined after 18 weeks of dietary treatment. It seems

that either the treatment of DMBA or the presence of the tumor can depress serum E_2 level.

These results may suggest that treatment with DMBA or bearing mammary tumors could either decrease the biosynthesis of E_2 or increase the metabolism of this hormone. There is some evidence to support the latter. Adams (1991) has suggested that microsomal hydroxy steroid dehydrogenases and p450-dependent steroid hydroxylases are critical for the biotransformation of highly estrogenic estradiol to less estrogenic metabolites. Fishman et al. (1995) showed that elevated C16α-hydroxylation of estradiol is associated either with increased risk for breast cancer or with the presence of breast cancer. It was suggested that serum E_2 concentration may represent a useful endocrine biomarker for mammary carcinogenesis. These investigators further showed that the mammary tissue exhibits cancer-risk dependent alteration in estradiol metabolism, indicating that estradiol metabolites may directly influence the mammary epithelium. A consistent lower concentration of serum estradiol in DMBA-treated rats in the present study supports the hypothesis of an increased rate of estradiol metabolism. The clinical assay used in our study was designed to detect specifically 17ß-estradiol (E_2). Since other metabolites of E_2 are also present in the circulation,

it is not possible at this time to confirm whether there is a change in the concentration of other metabolites of E_2. Key et al. in 1988 suggested that higher concentration of serum estrogen is associated with higher incidence rate of breast cancer in women. From animal experimentations, Fishman et al. (1995) concluded that natural estrogen-estradiol, is a well known promoter of rodent mammary carcinogenesis.

Lower concentration of serum estradiol, prior to the administration of a carcinogen (initiation of carcinogenesis) may influence the outcome of tumorigenesis. It is possible that by imposing a 20% energy restriction throughout the entire period of initiation and promotion of chemically-induced tumorigenesis, the level of serum estradiol is suppressed to the point where it becomes insufficient for mammary tumor to develop. Several investigators reported the beneficial effect of energy restriction on chemically-induced mammary tumorigenesis (Kritchevsky et al. 1984, Klurfeld et al. 1989, Ong 1995). However, the mechanism by which energy restriction lowered the incidence of chemically-induced mammary tumorigenesis was not investigated.

In conclusion, the present study suggests that the influence of energy restriction on serum estradiol

concentration in rats is greater than the influence of the type of dietary protein. In addition, rats that had been treated with DMBA or had developed DMBA-induced mammary tumor also had a lower concentration of serum E_2. Feeding soy protein isolate-base diet did not show an influence on the serum E_2 concentration when compared to the effect of casein-base diet. However, this should not undermine the hypothesis of a beneficial effect of soy protein on suppressing mammary tumor formation as previously reported by other investigators (Barnes et al. 1990, Hawrylewicz et al. 1995). It is known that compounds other than phytochemicals such as trypsin inhibitors, phytic acid, and saponins in soy bean may also have anticarcinogenic activity. Consuming soy protein or soy bean products may still provide favorable approach to cancer prevention.

CHAPTER VI

SUMMARY AND CONCLUSION

The present study investigated the effect of feeding soy protein diet with or without 20% energy restriction on serum estradiol (E_2) concentrations in rats during the time period of before or after 7, 12-Dimethylbenz(a)-antheracene (DMBA) administration.

Forty eight female Sprague-Dawley rats at the age of 28 days, were randomly assigned to one of the four dietary treatment groups. Group C-AL was fed a casein diet, ad libitum. Group C-R was fed a diet containing casein with a reduced energy intake to 80% of the intake of C-AL. The third group, SPI-AL, was fed a soy protein diet, ad libitum. The fourth group of rats (SPI-R) received soy protein diet and their intake was also reduced to 80% of the energy intake of SPI-AL. The feeding period was 28 days. Thereupon, rats were killed and blood samples were drawn. Estradiol concentration of the serum of the rats were determined. Growth of the rats was significantly ($p<0.05$) reduced by 20% energy restriction. The average body weight of the rats that were fed a diet containing soy protein

isolate was not significantly different from that of the rats consumed a casein-base diet at the end of the 28-day feeding period. Feed efficiency of the soy protein isolate diet showed to be lower than the casein diet indicating the slightly lower quality of soy protein. Feed efficiency is defined as body weight gain per one gram of feed consumed. Serum estradiol concentrations of these rats were determined when the feeding was completed.

In the second study, female Sprague-Dawley rats were given either DMBA or sesame oil at the age of 8 weeks and were given the same four dietary treatments as described above from 9 weeks until 26 weeks of age. Forty-one serum samples from the rats received DMBA and 40 sera from those received sesame oil were analyzed for E_2 concentration. Regardless of the type of the dietary protein, the serum E_2 concentrations of rats in the 20% energy restricted groups were about 75% to 86% of those fed ad libitum. Except in one occasion, when the rats fed the casein-base diet received sesame oil and were 20% energy restricted. The average serum E_2 concentration of these rats was 123% of the ad libitum group. None of the comparisons on serum E_2 concentrations were statistically significant.

Rats that were administered with DMBA and developed mammary tumors had serum E_2 concentration about 60%-94% of

those received sesame oil. The source of the dietary protein was not a factor for the change, although in general, the lower percentage was from the groups that were 20% energy restricted rather than the ones that were fed ad libitum.

In conclusion, the present study shows an effect of energy restriction on lowering serum estradiol concentration while the quality of dietary protein had no such effect. High levels of serum estradiol has been associated with higher risk for breast cancer (Key et al. 1988). Lower incidence in chemically induced mammary tumorigenesis has been found in feeding soy protein diet (Hawrylewicz et al. 1995) and in energy restriction (Klurfeld et al. 1989). It seems that the strategy of lower energy intake and increase the use of soy protein/soy products is a favorable approach to cancer prevention.

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APPENDICES

APPENDIX A

Animal Care and Use Committee Approval

FORM 1988A
Animal Project .No 1996 01 (For Office Use Only)

USE OF VERTEBRATE ANIMALS
TWU ANIMAL RESEARCH FACILITY

Project Title: Serum estrogen and lipids concentrations of rats fed soy protein based diet with 20% energy reduction

Investigators/instructors (Indicate Principal Investigator/Instructor with an asterisk)

Andie M. Hsueh* and Shiva Mehran

Department Nutrition and Food Sciences Phone Ext. 2646

Proposed Duration of Project: From 2/1/96 to 8/31/96

Funding Source or Proposed Funding Source Human Nutrition Research Fund

Project Classification (check)
A. Grant Proposal (external source)_____
New Proposal or Pilot Project_____Modification of Ongoing Grant _____
Competitive Renewal _____ Grant Supplement _____
Noncompetitive Continuation (indicate significant changes only)_____
B. Local Research _____funding source _____
C. Thesis/Dissertation Project ____X_____
D. Course _____

By whom was (will) peer review accomplished?

Doctoral Research Committee

Previously assigned Animal Project No. if application is other than a New Proposal or Pilot Project _____
Date Received by ACUC: 1-16-96
Review Board Action : Date 1-19-96
Approved ✓ Approved Contingent ___Disapproved ____
Returned for Revision_____
Remarks:

Additional Review Required? NO ✓
YES_____Safety_____Radiation_____Biohazard_____

Signature of ACUC Representative Lynda Wahowe

Date Received by Safety/Radiation/Biohazard Committee: _____

Review Board Action: Date: _____
Approved _____Approved Contigent_____Disapproved ____Returned for Revision_____

Remarks:

Signature of Safety/Radiation/Biohazard Representative_____

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Animal Purchase Form

Principal Investigator Andie M. Hsueh

Department/College Nutrition & Food Sciences/Health Sciences

Project Title Serum estrogen and lipids concentrations of rats fed soy protien based diet with 20% energy reduction

Animal Project No. 1996-01 **Date of Approval** 1/19/96

Animal Species Rat **Strain** Sprague-Dawley

Number of animals stated in the proposal 48 female weaning rats

Number of animals already purchased _____/Date_____
_____/Date_____
_____/Date_____
_____/Date_____

Number of animals to be purchased now _____

Source Sasco

Intended housing site GRB 148

Name(s) of animal care personnel:
Shiva Mehran
Andie M. Hsueh

Individual authorized to approve purchase for ACUC:

(Name in print) LYNDA Uphouse

Signature Lynda Uphouse Date 2-1-95

APPENDIX B

Proximate Analysis of Diets

BIO-SERV. A Hoffon Industries Co. P.O. Box 450, FRENCHTOWN, NJ 08825
Phone (908) 996-2155
(800) 473-2156
FAX (800)473-2187
Outside USA (908) 996-4123

10/12/94

CERTIFICATE OF ANALYSIS

TEXAS WOMAN'S UNIVERSITY
DR. ANDIE HSUEH
NUTRITION & FOOD SCIENCES
P. O. BOX 24134
DENTON, TX 76204

SALES ORDER NO: 25781
INVOICE NO.: 53468
INVOICE DATE: 10/11/94
P. O. NO.: P33347
PROD. NO.: F3242
LOT NO.: 21142

C-AL

DESCRIPTION: RAT DIET, FORMULA A (MEAL)

PROXIMATE PROFILE	THEORETICAL	ACTUAL	% VARIABILITY
PROTEIN	20.40%	20.10%	<=10 %
FAT	10.01%	9.94%	<=10 %
FIBER	5.25%	4.87%	<=20 %
ASH	3.57%	3.27%	<=10 %
MOISTURE <	10.00%	4.14%	<=10 %
CARBOHYDRATE	55.82%	57.68%	Calculated

CALORIC PROFILE			
PROTEIN	0.871	0.850 kcal/ GRAM	Calculated
FAT	0.884	0.878 kcal/ GRAM	Calculated
CARBOHYDRATE	2.210	2.284 kcal/ GRAM	Calculated
ETHANOL	0.000	0.000 kcal/ GRAM	Calculated
TOTAL	3.965	4.020 kcal/ GRAM	Calculated

	LIMITS (ppm)	RESULTS (ppm)
ORGANOPHOSPHATES		
Carbophenothion (Trithion)	0.300	< 0.025
Diazinon	0.300	< 0.010
Disulfaton	0.300	< 0.015
Endosulfan (Thiodan)	0.300	< 0.012
Ethion	0.300	< 0.040
Malathion	0.500	< 0.015
Parathion (Ethyl)	0.300	< 0.009
Parathion (Methyl)	0.300	< 0.015
Phorate (Thimet)	0.300	< 0.015
PESTICIDES AND PCB'S		
Aldrin	0.030	< 0.005
Benzene Hexachloride (BHC)	0.050	< 0.009
BHC	1.000	< 0.001
Chlordane	0.050	< 0.008
DDT (Total)	0.100	< 0.014
Dieldrin	0.030	< 0.009
Endrin	0.030	< 0.012
Heptachlor	0.030	< 0.004
Heptachlor Epoxide	0.030	< 0.006
Lindane	0.050	< 0.003
PCB	0.050	< 0.035
Toxaphene	0.200	< 0.045
AFLATOXIN	0.005	< 0.001
HEAVY METALS		
Arsenic	1.000	< 1.000
Cadmium	0.200	< 0.060
Lead	1.500	< 0.900
Mercury	0.100	< 0.005

This assay certifies that the above diet is guaranteed to meet the above theoretical parameters as specified by Bio Serv and/or the investigator.

Analytical variability, sampling variability, and ingredient levels account for overall differences in theoretical and actual figures for assays.

Information contained herein is believed to be correct and reliable. However, Bio Serv does not assume responsibility for it or for recommendations of our representatives inasmuch as conditions and methods of use are beyond our control. Further, we make no warranty, expressed or implied, of any kind regarding these products or their use, and the purchaser assumes all risks of use or handling either in accordance with directions or not.

Assays performed by: Independent Analytical Laboratory
Method of Reference: AOAC-10/92

Penny Mosner
Quality Assurance Manager

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CERTIFICATE OF ANALYSIS

10/12/94

TEXAS WOMAN'S UNIVERSITY
DR. ANDIE HSUEH
NUTRITION & FOOD SCIENCES
P. O. BOX 24134
DENTON, TX 76204

SALES ORDER NO: 25779
INVOICE NO.: 53467
INVOICE DATE: 10/11/94
P. O. NO.: P33637
PROD. NO.: F3243
LOT NO.: 21143

C-R

DESCRIPTION: RAT DIET, FORMULA B (MEAL)

PROXIMATE PROFILE	THEORETICAL	ACTUAL	% VARIABILITY
PROTEIN	20.50%	20.20%	<=10 %
FAT	12.47%	12.32%	<=10 %
FIBER	6.54%	6.03%	<=20 %
ASH	4.36%	4.19%	<=10 %
MOISTURE <	10.00%	3.47%	<=10 %
CARBOHYDRATE	51.14%	53.79%	Calculated

CALORIC PROFILE			
PROTEIN	0.875	0.862 kcal/ GRAM	Calculated
FAT	1.102	1.089 kcal/ GRAM	Calculated
CARBOHYDRATE	2.025	2.130 kcal/ GRAM	Calculated
ETHANOL	0.000	0.000 kcal/ GRAM	Calculated
TOTAL	4.002	4.081 kcal/ GRAM	Calculated

	LIMITS (ppm)	RESULTS (ppm)
ORGANOPHOSPHATES		
Carbophenothion (Trithion)	0.300	< 0.025
Diazinon	0.300	< 0.010
Disulfaton	0.300	< 0.015
Endosulfan (Thiodan)	0.300	< 0.012
Ethion	0.300	< 0.040
Malathion	0.500	< 0.015
Parathion (Ethyl)	0.300	< 0.009
Parathion (Methyl)	0.300	< 0.015
Phorate (Thimet)	0.300	< 0.015
PESTICIDES AND PCB'S		
Aldrin	0.030	< 0.005
Benzene Hexachloride (BHC)	0.050	< 0.009
BHC	1.000	< 0.001
Chlordane	0.050	< 0.008
DDT (Total)	0.100	< 0.014
Dieldrin	0.030	< 0.009
Endrin	0.030	< 0.012
Heptachlor	0.030	< 0.004
Heptachlor Epoxide	0.030	< 0.006
Lindane	0.050	< 0.003
PCB	0.050	< 0.035
Toxaphene	0.200	< 0.045
AFLATOXIN	0.005	< 0.001
HEAVY METALS		
Arsenic	1.000	< 1.000
Cadmium	0.200	< 0.060
Lead	1.500	< 0.900
Mercury	0.100	< 0.005

This assay certifies that the above diet is guaranteed to meet the above theoretical parameters as specified by Bio Serv and/or the investigator.

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Assays performed by: Independent Analytical Laboratory

Method of Reference: AOAC 10/89

Penny Mosner
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CERTIFICATE OF ANALYSIS

10/11-9/94

TEXAS WOMAN'S UNIVERSITY
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SALES ORDER NO: 25778
INVOICE NO.: 53466
INVOICE DATE: 10/11/94
P. O. NO.: P33283
PROD. NO.: F3244
LOT NO.: 21144

SPI-AI-

DESCRIPTION: FAT DIET, FORMULA C (MEAL)

PROXIMATE PROFILE	THEORETICAL	ACTUAL	% VARIABILITY
PROTEIN	20.00%	19.10%	<=10 %
FAT	10.00%	10.02%	<=10 %
FIBER	5.11%	4.70%	<=20 %
ASH	4.30%	3.32%	<=10 %
MOISTURE <	10.00%	2.55%	<=10 %
CARBOHYDRATE	57.34%	60.31%	Calculated

CALORIC PROFILE			
PROTEIN	0.854	0.816 kcal/ GRAM	Calculated
FAT	0.091	0.886 kcal/ GRAM	Calculated
CARBOHYDRATE	2.270	2.388 kcal/ GRAM	Calculated
ETHANOL	0.000	0.000 kcal/ GRAM	Calculated
TOTAL	4.015	4.089 kcal/ GRAM	Calculated

	LIMITS (ppm)	RESULTS (ppm)
ORGANOPHOSPHATES		
Carbophenothion (Trithion)	0.300	< 0.025
Diazinon	0.300	< 0.010
Disulfaton	0.300	< 0.015
Endosulfan (Thiodan)	0.300	< 0.012
Ethion	0.300	< 0.040
Malathion	0.500	< 0.015
Parathion (Ethyl)	0.300	< 0.009
Parathion (Methyl)	0.300	< 0.015
Phorate (Thimet)	0.300	< 0.015
PESTICIDES AND PCB'S		
Aldrin	0.030	< 0.005
Benzene Hexachloride (BHC)	0.050	< 0.009
DDE	1.000	< 0.001
Chlordane	0.050	< 0.008
DDT (Total)	0.100	< 0.014
Dieldrin	0.030	< 0.009
Endrin	0.030	< 0.012
Heptachlor	0.030	< 0.004
Heptachlor Epoxide	0.030	< 0.006
Lindane	0.050	< 0.003
PCB	0.050	< 0.035
Toxaphene	0.200	< 0.045
AFLATOXIN	0.005	< 0.001
HEAVY METALS		
Arsenic	1.000	< 1.000
Cadmium	0.200	< 0.000
Lead	1.500	< 0.900
Mercury	0.100	< 0.005

This assay certifies that the above diet is guaranteed to meet the above theoretical parameters as specified by Bio Serv and/or the investigator.

Analytical variability, sampling variability, and moisture levels account for minor differences in theoretical and actual figures for assays.

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Assays performed by: Independent Analytical Laboratory
Method of Reference: AOAC-10/89

Penny Mosner
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CERTIFICATE OF ANALYSIS

10/12/94

TEXAS WOMAN'S UNIVERSITY
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DENTON, TX 76204

SALES ORDER NO: 55001
INVOICE NO.: 53469
INVOICE DATE: 10/11/94
P. O. NO.: 033774
PROD. NO.: 13245
LOT NO.: 21145

SPI-R

DESCRIPTION: RAT DIET, FORMULA D (MEAL)

PROXIMATE PROFILE	THEORETICAL	ACTUAL	% VARIABILITY
PROTEIN	20.00%	19.80%	<=10 %
FAT	12.54%	11.80%	<=10 %
FIBER	6.41%	5.90%	<=20 %
ASH	5.09%	4.09%	<=10 %
MOISTURE <	10.00%	2.07%	<=10 %
CARBOHYDRATE	52.79%	56.26%	Calculated

CALORIC PROFILE			
PROTEIN	0.054	0.045 kcal/ GRAM	Calculated
FAT	1.100	1.050 kcal/ GRAM	Calculated
CARBOHYDRATE	2.090	2.227 kcal/ GRAM	Calculated
ETHANOL	0.000	0.000 kcal/ GRAM	Calculated
TOTAL	4.052	4.123 kcal/ GRAM	Calculated

	LIMITS (ppm)	RESULTS (ppm)
ORGANOPHOSPHATES		
Carbophenothion (Trithion)	0.300	< 0.025
Diazinon	0.300	< 0.010
Disulfaton	0.300	< 0.015
Endosulfan (Thiodan)	0.300	< 0.012
Ethion	0.300	< 0.040
Malathion	0.500	< 0.015
Parathion (Ethyl)	0.300	< 0.009
Parathion (Methyl)	0.300	< 0.015
Phorate (Thimet)	0.300	< 0.015
PESTICIDES AND PCB'S		
Aldrin	0.030	< 0.005
Benzene Hexachloride (BHC)	0.050	< 0.009
DDE	1.000	< 0.001
Chlordane	0.050	< 0.008
DDT (Total)	0.100	< 0.014
Dieldrin	0.030	< 0.009
Endrin	0.030	< 0.012
Heptachlor	0.030	< 0.004
Heptachlor Epoxide	0.030	< 0.006
Lindane	0.050	< 0.003
PCB	0.050	< 0.035
Toxaphene	0.200	< 0.045
AFLATOXIN	0.005	< 0.001
HEAVY METALS		
Arsenic	1.000	< 1.000
Cadmium	0.200	< 0.060
Lead	1.500	< 0.900
Mercury	0.100	< 0.005

This assay certifies that the above diet is guaranteed to meet the above theoretical parameters as specified by Bio Serv and/or the investigator.

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Assays performed by: Independent Analytical Laboratory

Method of Reference: AOAC 10.09

Penny Mosner
Quality Assurance Manager

APPENDIX C

Procedure for Determination of Serum Estradiol Concentration

Blood samples were collected into the test tubes via cardiac puncture. After standing at room temperature for 30 min, the sera were separated from blood cells by centrifuging at 1500 x g for 20 min and were kept at - 70 °C until assayed.

Basic Radioimmunoassay Procedure (Coat-A-Count Estradiol kit purchased from Diagnostic Products Corporation, Los Angeles, CA)

1. Label four 12 x 75 mm polypropylene tubes T (total counts) and NSB (non specific binding) in duplicate.
2. Label fourteen Estradiol anti body-coated tubes A through G in duplicate. These are the standard tubes ranging from 0 to 3600 pg/mL.
3. Label as many tubes as needed for serum samples.
4. Pipet 100 μL of the zero calibrator A into NSB and A tubes, and 100 μL of each of the calibrators B through G into correspondingly labeled tubes. Pipet 100 μL of each sample into the tubes prepared. Pipet directly to the bottom.
5. Add 1 mL of ^{125}I-Estradiol to every tube and vortex.
6. Incubate for 3 hours at room temperature.
7. Decant thoroughly using a foam decanting rack.
8. Count for 1 minute in a gamma counter.

APPENDIX D

Table D1

Weekly mean body weights in grams
of the rats fed experimental diets for four weeks[1]

Age (wk)	Group[2] (n=12/group)			
	C-AL	C-R	SPI-AL	SPI-R
4	72.0±4.3	71.5±4.2	70.8±3.8	72.1±4.4
5	110.0±5.8	98.2±6.3	106.0±8.3	90.3±3.8
6	141.2±9.2	124.5±6.7	136.4±10.7	115.7±3.3
7	163.9±12.8	146.2±8.1	159.5±13.1	135.8±4.0
8	180.4±14.7	162.0±10.3	176.9±14.0	151.6±5.1

[1] Values are Mean ± S.D.

[2] C-AL=Casein; Ad Libitum, C-R=Casein; 80% intake of C-AL, SPI-AL=Soy Protein Isolate; Ad Libitum, SPI-R=Soy Protein Isolate; 80% intake of SPI-AL.

Table D2

Mean daily and total feed intakes
of the rats fed experimental diets[1] from 28-56 days of age

Feeding Wk	Group[2] (n=12/group)			
	C-AL	C-R	SPI-AL	SPI-R
		g/day		
1	12.1±1.2[a]	9.4±0.3[b]	13.0±1.8[a]	9.8±0.6[b]
2	14.4±1.4[a]	11.4±0.1[b]	15.9±1.6[c]	12.6±0.1[d]
3	14.8±1.8[a]	11.8±0.1[b]	14.5±1.8[a]	11.4±0.2[b]
4	14.6±1.5[a]	11.6±0.1[b]	14.5±2.2[a]	11.5±0.2[b]
		Total Feed Intake(g)		
1-4	391.3	309.4	405.3	317.1

[1] Values are Mean ± S.D. Values not sharing the same letter superscript at the same age are significantly different at $p<.05$, using Tukey post-hoc test.

[2] C-AL=Casein; Ad Libitum, C-R=Casein; 80% intake of C-AL's, SPI-AL=Soy Protein Isolate; Ad Libitum, SPI-R=Soy Protein Isolate; 80% intake of SPI-AL's.

APPENDIX E

Inividual serum E_2 concentration in rats after adminstration of DMBA or sesame oil

Rat's ID	pg/mL	Rat's ID	pg/mL
C-AL/DMBA 1	35.75	C-AL/S 1	48.08
C-AL/DMBA 2	28.59	C-AL/S 2	33.95
C-AL/DMBA 7	1.79	C-AL/S 3	23.72
C-AL/DMBA 9	22.77	C-AL/S 4	43.16
C-AL/DMBA 12	3.13	C-AL/S 6	13.14
C-AL/DMBA 15	12.79	C-AL/S 7	24.40
C-AL/DMBA 16	54.96	C-AL/S 8	22.14
C-AL/DMBA 18	49.50	C-AL/S 9	25.32
C-AL/DMBA 22	11.90	C-AL/S 10	15.66
C-AL/DMBA 26	59.50		
C-AL/DMBA 27	4.56		
C-AL/DMBA 29	26.93		

Rat's ID	pg/mL	Rat's ID	pg/mL
SP-AL/D 2	12.49	SP-AL/S 1	40.12
SP-AL/D 3	6.90	SP-AL/S 3	38.99
SP-AL/D 4	45.07	SP-AL/S 4	22.84
SP-AL/D 5	54.73	SP-AL/S 5	12.75
SP-AL/D 7	19.59	SP-AL/S 6	54.56
SP-AL/D 9	28.57	SP-AL/S 7	80.30
SP-AL/D 16	56.48	SP-AL/S 8	24.62
SP-AL/D 22	32.20	SP-AL/S 9	49.48
SP-AL/D 24	9.94		
SP-AL/D 28	55.61		
SP-AL/D 29	31.33		

Rat's ID	pg/mL	Rat's ID	pg/mL
C-R/DMBA 1	25.39	C-R/S 1	33.53
C-R/DMBA 5	16.32	C-R/S 2	30.55
C-R/DMBA 8	24.72	C-R/S 3	20.84
C-R/DMBA 10	22.62	C-R/S 4	58.95
C-R/DMBA 15	7.31	C-R/S 5	19.96
C-R/DMBA 17	10.30	C-R/S 6	35.55
C-R/DMBA 22	37.40	C-R/S 7	28.11
C-R/DMBA 23	22.63	C-R/S 8	47.34
C-R/DMBA 26	20.24	C-R/S 9	31.69
C-R/DMBA 27	18.57	C-R/S 10	83.70

Rat's ID	pg/mL	Rat's ID	pg/mL
SP-R/D 3	18.08	SP-R/S 1	22.08
SP-R/D 5	29.15	SP-R/S 2	23.10
SP-R/D 6	10.84	SP-R/S 3	35.77
SP-R/D 8	43.98	SP-R/S 4	24.48
SP-R/D 9	14.91	SP-R/S 5	45.57
SP-R/D 12	15.77	SP-R/S 6	58.16
SP-R/D 16	13.01	SP-R/S 7	16.85
SP-R/D 17	29.49	SP-R/S 8	36.29
SP-R/D 19	37.12	SP-R/S 9	87.48
SP-R/D 23	28.67	SP-R/S 10	50.92
SP-R/D 30	25.24		

Individual serum E_2 concentration of rats fed experimental diets from 4-8 weeks of age

Rat's ID	pg/mL	Rat's ID	pg/mL
C-AL 1	32.27	SPI-AL 1	49.43
C-AL 2	16.47	SPI-AL 2	39.22
C-AL 3	27.51	SPI-AL 3	31.09
C-AL 4	30.56	SPI-AL 4	38.01
C-AL 5	28.20	SPI-AL 5	24.66
C-AL 6	27.83	SPI-AL 6	10.14
C-AL 7	19.37	SPI-AL 7	23.80
C-AL 8	22.87	SPI-AL 8	22.11
C-AL 9	35.19	SPI-AL 9	53.03
C-AL 10	48.23	SPI-AL 10	13.79
C-AL 11	11.18	SPI-AL 11	23.73
C-AL 12	0.41	SPI-AL 12	18.71

Rat's ID	pg/mL	Rat's ID	pg/mL
C-R 1	22.00	SPI-R 1	45.45
C-R 2	20.22	SPI-R 2	22.30
C-R 3	30.54	SPI-R 3	38.21
C-R 4	23.06	SPI-R 4	34.57
C-R 5	10.39	SPI-R 5	22.75
C-R 6	37.47	SPI-R 6	10.42
C-R 7	13.27	SPI-R 7	13.40
C-R 8	12.55	SPI-R 8	21.20
C-R 9	13.58	SPI-R 9	12.68
C-R 10	103.9	SPI-R 10	22.12
C-R 11	9.59	SPI-R 11	41.40
C-R 12	18.71	SPI-R 12	11.66