# EFFECT OF DIETARY SOY PROTEIN ON SERUM LIPID CONCENTRATIONS DURING THE GROWTH OF MCA-INDUCED TUMORS IN FISCHER 344 MALE RATS

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BY

ANDREA D. HARRIS, B.S., M.S.

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## DEDICATION

This dissertation is dedicated to my mother,

Norma Jean Chaney Jackson,

(September 1, 1942 - November 23, 1993)

## ACKNOWLEDGEMENT

Although I can not possibly name everyone, I would like to thank all those who helped me complete this degree. I would like especially to thank my major professor, Dr. Andie Hsueh, who without her longstanding encouragement and support, I doubt that I would have continued. Thank you for your untiring assistance in completing this project and dissertation.

## ABSTRACT

Effects of Dietary Soy Protein on Serum Lipid Concentrations During the Growth of MCA-Induced Tumors in Fischer 344 Male Rats

Andrea D. Harris

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Eighty male, Fischer 344 rats weighing 210-220 grams (9 weeks old) were randomly assigned to either a 20% casein-based (C) or a 20% soy protein-based (S) diet with 40 rats per dietary group. One week later, one half of the rats in each dietary group were implanted with a piece (2 $mm^3$) of MCA-induced sarcoma (T) and the other half of the rats were sham-operated (N). Ten rats in each subgroup (CT, CN, ST, SN) were killed at 10 days and the remaining 10 rats were killed at 20 days after implantation. The average tumor growth of the rats were not statistically different at either 10 days (CT=3.4 g; ST=2.5 g) or 20 days (CT=35 g; ST=43 g) after implantation. Tumor-bearing rats fed casein had significantly higher HDL-cholesterol (HDL) than the other experimental groups at 10 days and 20 days after implantation. During the first 10 days of tumor growth, serum total cholesterol (TC) concentrations were significantly higher in animals fed casein (56.1 mg/dl) in comparison to animals fed soy protein (43.2 mg/dl) and in animals with tumors (54.8 mg/dl) in comparison to animals without tumors (44.6 mg/dl). After 20 days of tumor growth, casein fed rats with tumors had significantly higher serum total

cholesterol than any of the other groups. There were no significant differences in triglyceride (TG) concentrations after 10 days of tumor growth. However, after 20 days of tumor growth, TG concentrations were significantly higher in tumor-bearing rats regardless of the type of dietary protein. These data indicate an increase in the serum lipid levels during the growth of MCA-induced sarcoma.

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# CHAPTER 1

## INTRODUCTION

Numerous studies have suggested that the consumption of soy protein may have a number of health benefits (Messina, 1995) including changing serum lipid concentrations and suppressing cancer development. Soy protein has been shown to alter serum lipids by lowering cholesterol levels in hypercholesterolemic individuals (Bakhit et al., 1994) and rabbits (Kanazawa et al., 1993). Soy protein also reportedly protects against tumor development in laboratory animals and may account for the relatively low rates of hormone-dependent and colon cancer development in Asian countries in comparison to Western countries (Messina and Barnes, 1991).

Changes have been noted in concentrations of serum total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in cancer patients in comparison to non-cancer patients. For example, Sharp and Pocock (1997) reported that in individuals with cancer, serum total cholesterol concentrations were low when compared to controls and decreased even further as the disease progressed. Low serum cholesterol levels were observed in colon cancer patients prior to the development, or at least at the time of the diagnoses of cancer (Miller et al., 1981). It is unclear whether or not these alterations are a result of carcinogenesis or whether or not these changes may contribute to the development of cancer.

In order to study the effect of a diet on tumor growth or on changes in serum lipid levels during tumor growth, it is necessary to design a study for which the time that the tumors are established is known. Several chemical carcinogens have been used to study effects of diet on tumor development. However, the exact time when a tumor is established is difficult to determine in chemically-induced tumors. In contrast, it is well documented that methylcholanthrene (MCA)-induced tumor cells can be transplanted directly into Fischer 344 rats (Grant and Wells, 1974; Popp et al., 1981; Popp et al., 1984). A transplantable tumor can provide the time when the tumor is established. It has been reported that Fischer 344 rats bearing MCA-induced sarcomas that were fed a casein-based diet, exhibited severe anorexia and cachexia during the late stage of tumor growth. In addition, these rats underwent changes in serum lipid concentrations. Radcliffe (1989) stated that these changes include hypercholesterolemia, hypertriglyceridemia, and eventually the depletion of body lipid stores. Animal studies in the literature involving F344 rats and MCA-induced sarcomas were all fed casein-based diets. None of the studies used a soy protein diet.

Since soy protein can affect both serum lipid concentrations (Sirtori et al., 1997) and cancer incidence (Lee et al., 1991; Messina et al., 1994), it would be reasonable to study the effects of a soy protein diet on both tumor growth and changes in serum lipids on rats bearing tumors.

The purpose of this study was to compare the effect of a soy protein-based diet to

that of a casein-based diet on the growth of MCA-induced sarcomas and the concentrations of serum high density lipoproteins, total cholesterol, and triglycerides of Fischer 344 rats at 10 days and 20 days post-implant.

The null hypotheses are:

(1) There is no difference between feeding soy protein diet and casein diet on the concentrations of serum high-density lipoprotein cholesterol, total cholesterol, or triglycerides in Fischer 344 male rats bearing MCA-induced sarcoma at either 10 days or 20 days after tumor implantation.

(2) There is no difference between feeding soy protein diet and casein diet on MCA-induced tumor growth in Fischer 344 male rats bearing MCA-induced sarcoma at either 10 days or 20 days after tumor implantation.

(3) There is no difference between rats bearing MCA-induced tumors and sham-operated rats on the concentrations of serum high-density lipoprotein cholesterol, total cholesterol, or triglycerides in Fischer 344 male rats.

# CHAPTER 2

# LITERATURE REVIEW

Soy protein has been shown to protect against tumor development (Barnes et al., 1990; Messina and Barnes, 1991; Messina et al., 1994) and decrease serum lipid concentrations (Kanazawa et al., 1993; Sirtori et al., 1993) in both animal and human studies; however, the mechanisms for these effects are not clear. Additionally in some studies, serum lipid levels change during human carcinogenesis (Dilman et al., 1981; Gercel-Taylor et al., 1996; Sharp and Pocock, 1997). It is unknown whether these changes are a result of carcinogenesis or whether carcinogenesis occurs as a result of the changes in serum lipids.

## Dietary Role in Cancer

Diet plays an important role in the incidence of cancer in humans. Mortality and incidence rates for major cancer types are significantly different between countries (Wynder and Gori, 1977). Many of these differences have been attributed to diet. Japanese have higher gastric cancer rates, which is highly correlated to the high intake of smoked, pickled and salt-cured foods when compared to the American white population. On the other hand, the incidence and mortality rates of colon, breast, uterine, ovary, and prostate cancers in Japan are lower than those of the United States (Dunn Jr., 1975). Many attribute this difference in the cancer rate to the consumption of low fat, high fiber

diets, and the high intake of soybean foods by the Japanese (Adlercreutz, 1990). For example, there is a strong correlation between geographic distribution and breast cancer incidence, with lower rates in countries where diet is primarily vegetarian (Adlercreutz, 1984). The risk of breast cancer is six times greater in North America and Northern Europe than in Asian countries (Boyd and McGuire, 1990). In these western countries, the diet is high in animal fat and protein, and low in fiber. In Asian countries, the diet is high in fiber and soybean foods but low in fat (Adlercreutz, 1990; Boyd and McGuire, 1990).

Changes in dietary intake and cancer incidence in immigrants support this theory, as well. Immigrants generally adopt the diet of their new country (Adlercreutz, 1990). Studies of cancer incidence rates among Japanese migrants in the United States provide further evidence that diet plays a role in the differing cancer rates among countries (Haenszel and Kurihara, 1968; Kelsey, 1979; Rose and Connolly, 1992). Breast cancer incidence rates gradually increase among Japanese descendents of migrants to the United States and began to approach those of white Americans after two or three generations.

In addition to inter-country differences, intra-country differences are also seen in cancer rates. For example, the Seventh-Day Adventists have the lowest cancer rates in the U.S., attributed in part to their vegan or lacto-ovo vegetarian diet (Kelsey, 1979). Jewish-Americans have a lower rate of upper alimentary tract cancer, which is believed related to their lack of alcohol consumption (Wynder and Gori, 1977). Ethnic differences within the

U.S. have also been noted and many of these differences could be a result of different dietary practices. African-American males have a higher incidence of prostate and colon cancer than white American males (Rose and Connolly, 1992). Large differences in cancer incidence are also seen among Jewish populations in Israel. Breast cancer rates per 100,000 for Jews born in Europe or the U.S., for Jews born in Israel, and for Jews born in Africa or Asia are 61.7, 54.0, and 34.3 respectively (Boyd and McGuire, 1990).

Several theories suggest that the typical "Western", high fat, low fiber diet is the main factor contributing to the high incidence of hormone-dependent cancers such as breast, prostate, and endometrial cancers and two other non-hormone dependent western diseases, colon cancer and coronary artery disease (Adlercreutz, 1990). Individuals on a low fiber, high fat diet have high plasma sex hormone levels and low sex hormone binding globulins when compared to populations which consume a diet high in fiber and low in fat. These individuals also have low fecal excretion of estrogens. Higher testosterone levels have also been found to correlate with increased risk of prostate cancer. Rose and Connolly (1992) noted that African-American males have a higher prostate cancer rate and higher testosterone levels in comparison with white Americans. This difference can not be wholly attributable to race but appear to be a result of environmental influences such as diet, because Nigerian males have lower testosterone levels and a lower rate of prostate cancer when compared to African-American males. In addition, high fat diets promote plasma lipid levels, which increase risk of heart disease. Low high-density lipoproteins

(HDLs) and high low-density lipoproteins (LDLs) have been associated with an increased risk for coronary artery disease (Stampfer et al., 1991; NIH Consensus Conference, 1993). Furthermore, high fat diets promote high intestinal bile acids, which may be co-carcinogenic and contribute to the development of colon cancer (Adlercreutz, 1984; Adlercreutz, 1990). Plasma hormone and lipid levels change in migrant families toward that of people in their new country. This may account for the increased risk of developing hormone-dependent cancers, such as breast cancer, and other diseases such as coronary artery disease, in families which migrate from Japan to the U.S. (Kelsey, 1979; Adlercreutz, 1990).

As a result of many of these studies, the American Cancer Society has taken the position that diets high in fruits, vegetables, and fiber may reduce the risk of some cancers and that Americans may reduce their cancer risk by following the 1995 Dietary Guidelines.

## Changes in Serum Lipids During Cancer Development

### Serum Lipids and Cancer

Many cancer patients have altered serum lipid levels when compared to non-cancer patients. Various studies have documented abnormal levels of one type of lipid or in a combination of triglycerides, fatty acids, cholesterol, and lipoproteins. However, lipid profiles may vary depending on the nature of the disease, the individual patient, and the study design. Furthermore, there is still some debate as to the cause of these lipid changes. The question as to whether alteration of lipid metabolism precedes or

contributes to the development of cancer, or is a result of tumor formation and growth, remains unanswered. Dilman (1981) reported elevated serum triglyceride concentrations in breast and lung cancer patients. Whereas, more recently, Prisco et al. (1995) reported no differences in triglyceride levels in male lung cancer patients when compared to controls. Gercel-Taylor (1996) reported that triglyceride levels were reduced in patients with ovarian cancer when compared with controls. This experiment also examined mono-, diglycerides, and free fatty acids. The results showed an elevation of all three fat types. In addition, when they examined each of these three fat fractions, they found alterations of the fatty acids present. In the monoglyceride and diglyceride fractions of cancer patients, all of the fatty acids present were elevated. Also in the diglyceride fractions, 20:1 and 20:3 fatty acids could only be found in the cancer patients. In the triglyceride fraction, all the fatty acids were reduced in the cancer patients except 20:1, 20:3, and docosahexaenoic acid (22:6). Other alterations of fatty acids reported by Prisco et al. (1995) included lower levels of linoleic acid (18:2), an n-6 fatty acid and n-3 fatty acids, 20:5, 22:5, and 22:6. Dietary fat intake was not examined in any of these experiments.

The majority of studies reporting on the differences in lipid profiles of cancer patients have examined cholesterol levels. High serum cholesterol levels have been positively correlated with increased risk of cancer development, particularly cancers associated with high fat diets (Rose et al., 1974), as evidenced by the higher incidence of hormone-dependent cancers seen in certain populations (Aldercreutz, 1990). However,

some studies have reported low cholesterol levels in individuals at high risk for developing some cancers based on risk for coronary heart disease and fat intake (Rose et al., 1974; Williams et al., 1981; Sharp and Pocock, 1997; Verschuren, 1997; Zureik et al., 1997). Low serum cholesterol levels have been reported in many individuals with cancer with a decline becoming more pronounced as the disease progresses (Rose et al., 1974; Williams et al., 1981; Sharp and Pocock, 1997; Verschuren, 1997; Zureik et al., 1997). Miller et al. (1981) observed low serum cholesterol levels in colon cancer patients. This group believed that this was the result of the metabolic influence of advanced tumors and did not believe the drop in serum cholesterol levels preceded tumor formation. It is possible that in many of the reported cases the low blood cholesterol concentration may have resulted from already existing or pre-clinical cancer, which had not been diagnosed at the time cholesterol levels were first measured. However, this has not been documented. Negative correlation was not seen in other cancers such as stomach, pancreas, rectum and anal, liver and bile duct cancers, that have not been linked with high fat diets (Rose et al., 1974).

**Serum Lipids and Breast Cancer**

Researchers have reported changes in serum lipid levels after the development of breast cancer, one of the cancers associated with diet. These changes are not well understood and studies have reported different conclusions. For example, Barclay (1970) measured three different fractions of high-density lipoprotein cholesterol ($HDL_1$, $HDL_2$,

$HDL_3$) and reported that breast cancer patients had lower $HDL_2$ levels than those without either breast cancer or a family history of cancer. Furthermore, subjects with low $HDL_2$ values had an unusually high incidence of cancer in their families. Men and women without cancer and with lower values of $HDL_2$ and higher levels of very low-density lipoprotein cholesterol VLDL, tended to have a family history of cancer. Dilman (1981) reported relatively high blood triglyceride levels and low HDL in patients with breast cancer and that the removal of the breast tumor did not change the lipid profile. In 1986, Bani et al. found that concentrations of plasma phospholipids, triglycerides, cholesterol and free fatty acids were all higher in breast cancer patients and that HDL cholesterol concentration was lower when compared to non-cancer patients. Both Potischman, (1991) and Kokoglu (1994) found higher triglyceride levels and lower cholesterol levels in breast cancer patients when compared to controls. Plasma cholesterol levels continued to decline with more advanced stages of cancer. Evidence up to this point has suggested lower than normal HDL levels and higher triglyceride and LDL cholesterol levels may be a risk factor for cancer. However, two reports by Boyd and associates (1989, 1990) suggest otherwise. The first (Boyd et al., 1989) was a study that compared women with extensive mammographic dysplasia (at least 75%) and those with little dysplasia (25% or less). Mammographic dysplasia is an abnormality of the breast, which is believed to be a risk factor for breast cancer. It was found that total plasma cholesterol concentration was not significantly different between the two groups and that HDL-cholesterol concentration

was higher while LDL-cholesterol and triglyceride levels were lower in women with extensive dysplasia when compared to women without. In the second study, Boyd and McGuire (1990) reported that both breast cancer risk and HDL-cholesterol levels are higher in women who live in northern European countries than in women who live in Asia. Increases in dietary fat intake, alcohol consumption, and levels of endogenous hormones are associated with an increase in the levels of HDL cholesterol. HDL cholesterol concentration was found to be higher in subjects with a family history of breast cancer. The data is incomplete on the risk of obesity and the use of exogenous hormones (i.e. birth control pills and postmenopausal estrogens). Both of these components have been associated with increased risk of breast cancer. Data on HDL levels of women with breast cancer appears inconsistent. Only one study looked at the different HDL fractions and conflicting data on HDL levels may be due in part to differences between the different fractions. Clearly, more studies are needed to help delineate the relationship between breast cancer and serum lipids.

**Soy Protein**

Messina et al., (1991, 1994) suggested that the contribution of soybeans to the diet of oriental countries such as Japan and China might contribute to the relatively low rates of cancer in these countries, particularly those cancers associated with diet. Soybeans and a number of its components may either inhibit or enhance carcinogenesis or mutagenesis in experimental animals (Persky and Horn, 1995; Liener, 1995). These components include

saponins, phytic acid, protease inhibitors, and phytoestrogens. A number of mechanisms have been proposed for the anticarcinogenic properties of each of the above mentioned components.

**Anticarcinogenic Components of Soy Protein**

Saponins

Saponins are thought to have anticarcinogen properties through four different mechanisms summarized below (Rao & Sung, 1995). First of all, they are believed to have direct cytotoxic and growth inhibitory effects against cells including tumor cells. Saponin extracts from the Chinese herbal drug Yunnan Bai Yao were shown to inhibit tumor cells in vitro (Ravikumar et al. 1979) and to promote wound healing (Wu et al. 1990,). Saponins may modify immune cells and increase the activity of natural killer cells. Yunnan Bai Yao antitumor activity may be attributed to its ability to modify the immune system (Wu et al. 1990). Saponins found in ginseng were shown to enhance immunity by increasing natural killer cells and interferon (Kenarova, 1990). Saponins may also normalize epithelial cell proliferation. Rao and Sung (1995) reported that abnormal cell proliferation occurring in the colon was stopped by a one-percent saponin solution. And finally, saponins may delay initiation and progression of cancer by binding to bile acids. Saponins reduce the ability of intestinal microflora to form secondary bile acids and as a result, may prevent the development of colon cancer (Sidhu and Oakenfull, 1986). Direct evidence of this has not been reported but there is a high correlation between colon

cancer, a high concentration of cholesterol metabolites, and bile acids in the feces (Reddy and Wynder, 1973; Mower et al., 1979).

Phytic Acid (Inositol Phosphates)

Phytic acid is believed to control cell division including the division of cancer cells, by chelating cations such as iron, magnesium, and zinc (Shamsuddin, 1995). It prevents the generation of the hydroxyl radicals by chelating iron and thus preventing lipid peroxidation and DNA damage. Additionally, magnesium and zinc are essential for cell proliferation of tumors, which are also chelated by phytic acids.

Protease Inhibitors

Initially, protease inhibitors were thought to be the sole anticarcinogenic component of soybeans. The primary protease inhibitors found in soybeans are the Kunitz trypsin inhibitor and Bowman Birk trypsin and chymotrypsin inhibitors (Messina and Barnes, 1991). Troll et al. (1980) examined the role of protease inhibitors on the reduction of mammary cancer by feeding a raw soybean diet (50% by weight) containing high levels of protease inhibitors to female Sprague Dawley rats. The content of trypsin inhibitor in the diets was 4.75% in the raw soybean, 0.23% in the Purina Rat Chow, and 0.0% in the casein. The study found that 44% of the irradiated rats on the soybean diet developed either an adenocarcinoma or a fibroadenoma as compared to 70% of those fed rat chow and 74% of those fed casein. The incidences of fibroadenomas (with or with out adenocarcinomas) were 22%, 44%, and 50%, respectively. The incidence of

adenocarcinomas (with or without fibroadenomas) was 29, 40, and 42%, respectively. In the non-irradiated animals, the number and type of tumors found varied with diet. There were no mammary neoplasms found in those animals eating the raw soybean diet. One fibroadenoma and one adenocarcinoma were found in those animals eating rat chow. In those animals eating a casein diet, two rats developed a single fibroadenoma, one rat developed two fibroadenomas, and one rat developed one adenocarcinoma. These investigators assumed that the cancer suppression was due to the amount of protease inhibitors in the soybean. St. Clair, et al. (1990) showed that the Bowman-Birk protease inhibitor (BBI) could suppress dimethylhydrazine induced carcinogenesis in male Cd-1 mice. Diets supplemented with pure and semipure BBI significantly reduced the incidence of liver tumors, whereas diets supplemented with either partially or totally inactivated BBI had no influence on the incidence of liver tumors. Squamous tumors of the anal gland were not affected by any diet. Protease inhibitors have also been shown to have an adverse effect. Liener (1995) stated that raw soybeans cause a hypertrophic and hyperplastic enlargement of the pancreas and an increased secretion of the sulfur rich enzymes trypsin, chymotrypsin and elastase. As a result, amino acids, primarily sulfur containing ones, are taken from body tissue protein to resynthesize these enzymes that are lost in the feces. Weight loss occurs because these are the same amino acids that are limiting in soy protein. Furthermore, an inverse relationship exists between the trypsin inhibitor activity and the protein efficiency ratio (PER) in rats. Additionally, trypsin

inhibitors have been shown to magnify the carcinogenic effect of azaserine (Morgan et al. 1977) and di(hydroxypropyl) nitrosamine (Levison et al. 1979) on the pancreas of the rat. However, studies with mice and hamsters showed pancreatic hypertrophy but no lesions (Liener and Hasdai, 1986). These studies suggested that the effect of protease inhibitors might be species specific. Since heat processing destroys most of the protease inhibitor activity, any anticarcinogenic benefit or adverse effects from these components is probably minimal (Anderson and Wolf, 1995). Protease inhibitors would not be present in appreciable amounts in soy foods (Messina & Barnes, 1991; Anderson and Wolf, 1995).

Phytoestrogens

Soybeans contain phytoestrogens. One of the most studied phytoestrogens is the isoflavone, genistein. Genistein, which has been shown to inhibit different cancers (Lamartiniere et al., 1995; Hoffman, 1995) binds to the receptors for many hormones and growth factors which influence the growth of many normal and neoplastic cells (Molteni, et al., 1995). However, the effect of these receptors have on the regulation of tumor growth is not known. Since isoflavones bind to many of these receptors, they compete with the physiologic hormones and/or growth factors, thus may play a role in the modulation and evolution of carcinogenesis (Molteni et al. 1995). Isoflavones stimulate sex hormone binding globulin synthesis in the liver and may reduce the biological effects of sex hormones (Adlercreutz, 1990). Genistein, was shown to inhibit tyrosine protein kinases (Akiyama et al., 1987; Dean et al., 1989; Huang et al., 1992). These protein

kinases are associated with cellular receptors for epidermal growth factor, insulin, insulin-like growth factor, platelet derived growth factor and mononuclear phagocyte growth factor, as well as oncogene expression. Adlercreutz (1990) reported different plasma hormonal patterns in vegetarians, semivegetarians and omnivores, and suggested that this may be due to dietary differences including high intake of phytoestrogen containing foods.

**Soy and Serum Lipids**

The components of soybeans that are considered anticarcinogenic are also considered to be hypocholesterolemic. The effect of soy protein on cholesterol was first noted when rabbits fed cholesterol free, semipurified diets containing casein, developed hypercholesterolemia. Feeding the animals soy protein in place of casein (Hamilton and Carroll, 1976) could prevent this hypercholesterolemia. Investigators then demonstrated that substituting soy protein for animal protein in the diet of hypercholesterolemic individuals reduces the concentrations of total and low density lipoprotein cholesterol in serum and plasma (Sirtori et al., 1977; Sirtori et al., 1993). Potter reviewed several mechanisms in 1995. First, soy protein may reduce cholesterol levels by enhancing bile acid excretion. This effect may be species specific and has been demonstrated primarily in rabbits and rats. Second, soy protein may directly affect the hepatic metabolism of cholesterol. There may be an increased removal of LDL and VLDL by hepatocytes, an increase in the activity of HMG CoA reductase, or an increase in apolipoprotein B and E

receptor activities. Finally, soy protein may alter hormone concentrations such as the thyroid hormones, which are involved in cholesterol metabolism.

Several constituents of soy have been suggested as hypocholesterolemic agents. Amino acids and peptide patterns found in soy have been shown to lower cholesterol in various studies (Huff et al., 1977). Saponins increase bile excretion and therefore have been suggested as being hypocholesterolemic (Sidhu and Oakenfull, 1986; Oakenfull et al., 1984). Phytic acid binds minerals and thus may modulate the Zn:Cu ratio (Klevay, 1975). A high Zn:Cu ratio has been associated with high plasma cholesterol concentrations. However, Jariwalla et al. (1990) reported that Zn:Cu ratios were decreased only in rats consuming diets supplemented with cholesterol and phytic acid and not those supplemented with phytic acid alone. This suggested a role of phytic acid that was independent of the Zn:Cu ratio. In addition, the binding of iron may prevent lipid peroxidation which has been implicated as a contributing factor in the development of atherosclerosis (Potter, 1995). Both soy fiber and protein have been reported to lower blood lipid concentrations. In the study by Bakhit (1994), soy protein lowered total cholesterol concentrations and soy fiber actually lowered HDL-cholesterol concentrations in hypercholesterolemic individuals but had little effect on those with normal blood cholesterol levels. Low HDL-cholesterol concentration is considered to be a risk factor for cardivascular disease (Stampfer et al., 1991). Isoflavones have also been suggested to have an effect on serum lipid levels but the evidence is inconclusive (Potter, 1995).

## Animal Tumor Models

### Chemical Carcinogens Used in Animal Studies

Several chemical carcinogens have been used to study the effects of diet on tumor development. Kritchevsky, et al., (1984) and Klurfield, et al., (1989) studied the effect of caloric restriction and dietary fat on dimethylbenzaldehyde (DMBA)-induced mammary tumors in female Sprague Dawley rats. More recently, genistein was found to suppress DMBA-induced mammary tumorigenesis in Sprague Dawley rats (Lamartiniere et al., 1995). Other chemical carcinogens such as methylnitrosourea (MNU) and azomethane (AOM) have been used to examine the effects of dietary fat on the development of breast cancer and colon cancer, respectively, in Sprague Dawley rats (Tang et al., 1996). As stated previously, none of these experiments examined the serum lipid profiles during tumor development. Another chemical carcinogen, methylcholanthrene (MCA), was painted on the backs of albino mice to study the effect of dietary fat and caloric restriction on skin tumors in mice (Lavic and Bauman, 1943). This experiment examined the effect of diet on tumor burden and no mention of serum lipids was made. In the early 70's, Grant and Wells were successful in propagating MCA-induced transplantable tumors in Fischer 344 rats (Grant and Wells, 1974). These investigators used MCA to initially induce a fibrosarcoma by subcutaneous injection. They were then able to take pieces of the tumor and transplant it into tumor-free rats where it would grow. Since then, several studies have shown that MCA tumor-bearing Fischer 344 rats exhibit severe anorexia and

cachexia in the advanced stages of cancer and in addition undergo changes in serum lipid levels similar to those seen in human cancers (Popp et al., 1981; Radcliffe et al., 1986; Radcliffe, 1989). Cancer induced anorexia and cachexia is characterized by weight loss and depletion of adipose tissue and skeletal mass (Tisdale, 1997). Cancer induced cachexia appears irreversibly to increase lipolysis thus contributing to altered serum lipids.

**Effect of MCA tumors on serum lipids**

The MCA tumor causes hypercholesterolemia and especially hypertriglyceridemia, and eventually depletion of body lipid stores in Fischer 344 rats. Radcliffe (1989) reported significantly higher concentrations of serum cholesterol, triglycerides, free fatty acids and total lipids in animals bearing an MCA tumor. In a 1996 abstract, Kavanaugh et al. reported a decreased hepatic synthesis of the fatty acids, palmitate and stearate, but no changes in cholesterol synthesis in animals bearing MCA tumors. Most studies to date using this tumor model report severe anorexia and wasting in rats between 13-16 days after transplantation with death occurring no later than 30 days (Popp et al., 1984; Popp, Brennan, and Morrison, 1982).

**Soy Protein and MCA tumors**

The literature indicates that including soy protein in the diet can affect both chemically induced cancer development in animals and serum lipids in humans. None of the studies using methylcholanthrene (MCA)-induced tumors have included soy protein in the experimental diets.

## Rat Serum Lipid Concentrations

Most human cholesterol is carried in the low-density lipoprotein fraction (Fried, et al., 1968). Rats, on the other hand, have similar lipid and density classes of lipoproteins as humans (Fried, et al., 1968). However, high-density lipoproteins (HDLs) are the main carrier of cholesterol and relatively little low-density lipoproteins are found in rat serum (Lasser, et al., 1973). Therefore, HDL-C is often used to examine changes in the serum cholesterol concentrations of rats.

# CHAPTER 3

# MATERIALS AND METHODS

## Diet and Animals

The AIN-76 diet and the diet ingredients for the experimental diets were purchased from BioServe, Frenchtown, NJ. The soy protein isolate and the casein contained 93% and 88% protein, respectively (Appendix B). The investigator formulated both experimental diets according to the composition of AIN-76 diet with modification on the amount of corn oil to 10 %. The modified diet was used in the current study for two reasons. First of all, AIN-93 diet uses soybean oil as the source of fat and it was not clear whether soybean oil in the AIN-93 diet would have an effect on the outcome of the experiment (Reeves et al., 1993). Secondly, experiments in chemical carcinogenesis conducted in our laboratory used AIN-76 diet. In order to compare the results of this experiment to that of the previous experiments, the same modified AIN-76 diet was used. The composition of the diet is shown in Table 1. Pope Testing Laboratories, Inc., Dallas, TX analyzed the diets and the results are shown in Appendix C.

Ninety, 8-week old Fischer F344 male rats (weighing 190-195 grams) were purchased from Harlan Sprague Dawley, Indianapolis, IN. The rats were given food and water ad libitum throughout the experiment. Each animal was weighed upon its arrival at

**Table 1**

**Composition and caloric density of the diets**

| Ingredients* (g/kg diet) | Casein Diet | Soy Diet |
|---|---|---|
| Casein** | 227 | |
| Soy Protein Isolate*** | | 215 |
| Sucrose | 420 | 432 |
| Cornstarch | 150 | 150 |
| Cellulose | 50 | 50 |
| Corn Oil | 100 | 100 |
| DL-Methionine | 3 | 3 |
| AIN-76 Mineral Mix | 38 | 38 |
| AIN-76 Vitamin Mix | 10 | 10 |
| Choline Bitartrate | 2 | 2 |
| Caloric Density (kcal/g diet) | 3.98 | 4.03 |

*Purchased from BioServe Inc., Frenchtown, NJ

**Protein=88%; ***Protein=93%

eight weeks of age. In addition, the animals were weighed at the time of tumor implantation, at which time they were 9 weeks old, and again the day before sacrifice, which was 9 days or 19 days after implantation. Food intake was also recorded at 9 weeks and again at 9 and 19 days after implantation.

**Experimental Design**

At 8-weeks of age, the animals were assigned to one of nine experimental groups using a randomized block design. Ten rats were assigned to each experimental group. The first experimental group was the baseline group that was fed the AIN-76A diet. These rats were killed one week later at 9-weeks old for the determination of serum lipid concentrations. The remaining rats were divided into groups based on their diet, tumor status, and time killed. The animals were first divided into two dietary groups, 20% casein (C) and 20% soy protein (S). Each dietary group was further divided into two groups based on their designated tumor status. Rats that were sham-operated or did not receive tumor implants were identified with the letter N. Animals receiving tumor implants were identified with the letter T. Finally, in order to examine serum lipid concentrations at two stages of tumor growth, animals were divided into two groups based on the number of days after implant that they were killed. Rats killed at 10 days after implant were labeled 10 and those killed at 20 days after implant were labeled 20. As a result, there were eight treatment groups (CN10, CN20, CT10, CT20, SN10, SN20, ST10, and ST20) with ten rats per group (Table 2).

**Table 2**

**Experimental design**

| Group | Diet | n | Tumor Implantation | Time of Sacrifice: 0, 10 or 20 days after implant procedure (age of rats) |
|---|---|---|---|---|
| AIN | AIN-76A | 10 | No | 0 (9 weeks old; before implant procedure) |
| CN10 | Casein | 10 | No | 10 days (10 ½ weeks old) |
| CN20 | Casein | 10 | No | 20 days (12 weeks old) |
| CT10 | Casein | 10 | Yes | 10 days (10 ½ weeks old) |
| CT20 | Casein | 10 | Yes | 20 days (12 weeks old) |
| SN10 | Soy Protein | 10 | No | 10 days (10 ½ weeks old) |
| SN20 | Soy Protein | 10 | No | 20 days (12 weeks old) |
| ST10 | Soy Protein | 10 | Yes | 10 days (10 ½ weeks old) |
| ST20 | Soy Protein | 10 | Yes | 20 days (12 weeks old) |

A diagram of the experimental design is shown in Figure 1.

According to Popp et al.(1981), anorexia and cachexia would develop in the Fischer 344 rat bearing MCA-induced sarcomas after 13-16 days and death would occur in 35-45 days. In our experience, our tumor-bearing rats could live to at least 21 days after tumor implant without the symptoms of anorexia or cachexia. We, therefore, designed our study to terminate the experiment at 20 days post-implant.

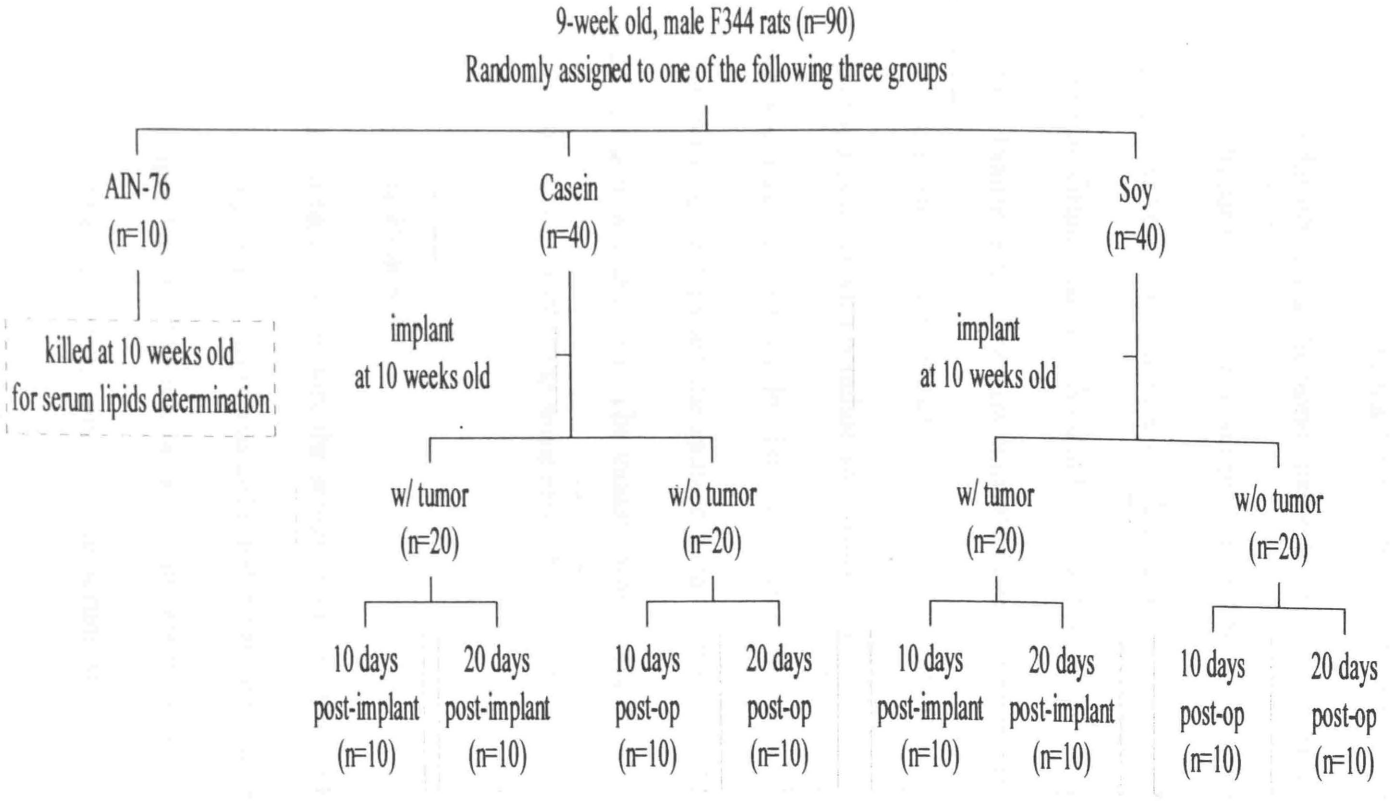
**Tumor Tissue Preparation**

One F344 rat bearing an established MCA-induced sarcoma was purchased from Dr. L. Byerley at the University of Chicago. After receiving the rat, successive transplanting procedures were carried out approximately every three weeks to test the success in transplanting procedure and to maintain several rats with tumors in the animal facility for providing tumor tissues for later experiments. The tumor tissue used in this experiment was from several F344 male rats that had successfully established MCA-induced sarcomas. These rats were designated as "tumor donors" for the present study.

Approximately 20 days after implant, each "tumor donor" was asphyxiated with $CO_2$. The tumor tissue from the dead "tumor donor" rat was excised. Strips of tissue approximately 2 mm wide by 2 mm thick were cut off. The strips of tumor tissue were placed into tissue culture media (RPM1 1640 with L-glutamine, Sigma Chemical Co., St. Louis, MO), cleaned of all blood vessels, and cut into cubes of 2-$mm^3$. The cubes of tumor tissue were maintained in the same tissue culture media until implant.

**Figure 1**

**Diagram of experimental design**




Rats killed for tumor growth measurements and serum lipid determinations at 10 days and 20 days post-implant.

**Surgical Procedure**

At 10 weeks of age, half of the animals from each diet group (20 rats per group) were either sham-operated or implanted with a 2-$mm^3$ MCA- induced tumor tissue in the left flank. The procedure for obtaining the tumor tissue for implant is detailed in the section of Tumor Tissue Preparation. Prior to surgery, all of the animals were anesthetized by injecting (i.m.) their right hind leg with 0.068 mg/100 g body weight ketamine (Butler Chemical, Grand Prairie, TX) and 0.45 mg/100 g body weight xylazine (Butler Chemical, Grand Prairie, TX). Once the animals were in deep sleep (no reflex by lightly pinching the paw), an area of approximately 4 $cm^2$ on the left hind flank was shaved. The area was then cleaned with betadine and alcohol swabs. A small (approximately 4 mm wide) incision was made. For tumor implanted rats, a micro straight tip hemostat was used to tunnel underneath the incision as far as possible (approximately 1 cm) and a 2-$mm^3$ tumor tissue was inserted. The incision was closed with wound clips. The sham-operated animals went through the same procedure except no tumor tissue was inserted.

**Blood Collection and Tumor Removal**

Twelve hours prior to blood collection, the animals were weighed and their food was removed. After the fasting period, blood was collected from each animal via cardiac puncture and kept on ice immediately. The whole blood samples were centrifuged at 4 °C for 10 minutes at 1000 x g. After centrifugation, the clear serum was transferred to

smaller vials and stored in the refrigerator until analysis. Tumors were removed, cleaned of excess fat and connective tissue, weighed and their weights were recorded.

**Serum Lipid Determination**

At nine weeks of age, ten rats fed the AIN-76A diet were sacrificed for baseline determination of serum concentrations of HDL-cholesterol, total cholesterol and triglycerides. HDL-cholesterol concentration was determined within four days of the collection of blood and the concentrations of serum total cholesterol and triglyceride were determined within seven days of collection. Serum HDL-cholesterol concentration was determined using Stanbio HDL-Cholesterol Procedure No. 0599 kit (San Antonio, TX). Serum total cholesterol concentration was determined using Stanbio Enzymatic Cholesterol Procedure No. 1010 kit (San Antonio, TX). Serum triglyceride concentrations were determined using Stanbio Enzymatic Triglycerides Procedure No. 2000 kit (San Antonio, TX).

**Statistical Analysis**

Body weight, food intake, tumor weight, and serum lipid levels were analyzed using a two-way analysis of variance. The program used was version 6.1of SPSS (Statistical Package for Social Sciences, Cary, NC). Tukey's post hoc test was used to determine statistical differences among and between the dietary and surgical treatment groups. Significance was determined at the 5% level.

# CHAPTER 4

# RESULTS

## Food Intake

Data on food intake during the first week are presented in Table 3. The first week of the experiment represents the week before tumor implant. The animals in the baseline group (AIN) consumed significantly less AIN-76 diet ($p<0.05$) than those animals fed either a soy protein or a casein diet. There were no significant differences in food intake among the remaining four treatment groups. One of the tumor-bearing animals in the soy protein group designated to carry a tumor for 20 days after implant, never woke up from anesthesia after transplant, consequently, there were only 9 rats in that subgroup.

After being on the experimental diets for one week, the animals in the baseline group were killed and the remaining rats were either implanted with tumors or sham-operated. Food intake data during the first 10 days after tumor implant are presented in Table 4. There were no significant differences in food intake among the four groups of rats regardless of their tumor status or diet.

Food intake data for the second ten days or 20 days after implant are shown in Table 5. Twenty days after the implantation procedure, food intake was significantly lower ($p<0.05$) in animals with tumors than those without tumors regardless of diet (Tables 6 and 7).

**Table 3**

**Total food intake of male F344 male rats during the first week of experiment[1,2]**

| Group[3] | Diet | n | Total Food Intake (g/ 7 days) |
|---|---|---|---|
| | | | g |
| AIN | AIN-76A | 10 | $97.6 \pm 2.0^{a}$ |
| CN | Casein | 20 | $118.6 \pm 3.3^{b}$ |
| SN | Soy protein | 20 | $119.1 \pm 4.0^{b}$ |
| CT | Casein | 20 | $118.7 \pm 2.1^{b}$ |
| ST | Soy protein | 19 | $113.2 \pm 3.1^{b}$ |

[1]Values are mean ± SEM

[2]Values not sharing the same superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]N=Sham-Operated; T=Tumor Implant

**Table 4**

**Total food intake of male F344 rats during the first 10 days after tumor implant[1,2]**

| Group[3] | Diet | n | Total Food Intake (g/10 days) |
|---|---|---|---|
| | | | g |
| CN | Casein | 20 | 124.6 ± 2.7 |
| SN | Soy protein | 20 | 128.8 ± 2.8 |
| CT | Casein | 20 | 130.6 ± 3.4 |
| ST | Soy protein | 19 | 126.1 ± 2.9 |

[1]Values are mean ± SEM

[2]No statistically significant differences were seen among the experimental.

[3]N=Sham-operated; T=Tumor Implant

**Table 5**

**Total food intake of male F344 rats during the second 10 days after tumor implant[1,2]**

| Group[3] | Diet | n | Total Food Intake (g/10 days) |
|---|---|---|---|
| | | | g |
| CN | Casein | 10 | 139.9 ± 7.5 |
| SN | Soy protein | 10 | 136.7 ± 8.9 |
| CT | Casein | 10 | 115.9 ± 8.7 |
| ST | Soy protein | 9 | 120.2 ± 7.1 |

[1]Values are mean ± SEM

[2]There were no significant interactions between diet and tumor groups $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]N=Sham-Operated; T=Tumor Implant

**Table 6**

**Two-factor analysis of variance summary table for food intake at 20 days post-implant**

| Overall Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 22603.53 | 35 | 645.82 | | |
| **Diet (D)[1]** | 2.94 | 1 | 2.94 | .00 | .947 |
| **Tumor (T)[2]** | 3982.14 | 1 | 3982.14 | 6.17 | .018 |
| **D x T** | 136.06 | 1 | 136.06 | .21 | .649 |
| **Total** | 26773.97 | 38 | | | |

**Table 7**

**Comparison of food intake of sham-operated and tumor-bearing F344 male rats at 20 days after implant[1,2]**

| Group[3] | n | Total Food Intake (g/10 days) |
|---|---|---|
| | | g |
| Sham-Operated (CN + SN) | 20 | 138.3 ± 5.7[a] |
| Tumor-Bearers (CT + ST) | 19 | 117.9 ± 5.5[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

There were no significant differences in food intake between animals fed soy protein (SN, ST) and animals fed casein (CN, CT) regardless of tumor status.

**Body Weight**

Body weights of the rats immediately after random assignment to dietary treatments (initial) and at time of implant are presented in Table 8. There were no significant differences in initial body weights among the 5 groups of rats. At time of tumor implant (one week after being on their respective experimental diet) animals in the AIN group weighed significantly less ($p<0.05$) than the other four groups of animals. Table 9 shows the body weights of rats at 10 days and 20 days after tumor implant. No significant differences in body weight were found among the treatment groups at either 10 days post-implant or 20 days post-implant. These body weights were measured before the tumors were excised.

**Tumor Weights**

Tumor weights at 10 days and 20 days after implant are shown in Table 10. Tumor weights were significantly higher ($p<0.05$) at twenty days after implant then at ten days. No significant differences in the tumor weights are observed between animals fed soy protein and casein diets at 10 or 20 days after tumor implant.

Tumor weights expressed as percent of body weight are presented in Table 11 and Table 12. At 10 days post-implant the (Table 11) average tumor weight in the rats fed the casein diet was 1.5% of the total body weight whereas it was 1.1% of the total body

**Table 8**

**Total body weight in grams at the start of the experiment and at time of implant of male F344 rats[1,2]**

| Group[3] | Diet | n | Total Body Weight (g) | |
|---|---|---|---|---|
| | | | Initial | At implant |
| | | | g | g |
| AIN* | AIN-76 | 10 | 190.1 ± 1.4[a] | 207.0 ± 2.2[a] |
| CN | Casein | 20 | 194.6 ± 1.5[a] | 222.9 ± 1.7[b] |
| SN | Soy protein | 20 | 194.6 ± 1.4[a] | 222.3 ± 1.8[b] |
| CT | Casein | 20 | 194.6 ± 1.4[a] | 221.0 ± 1.7[b] |
| ST | Soy protein | 19 | 195.0 ± 1.5[a] | 224.6 ± 1.7[b] |

[1]Values are mean ± SEM

[2]Values not sharing a common letter superscript in a given parameter are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]N=Sham-operated; T=Tumor Implant

**Table 9**

**Total body weight in grams of male F344 rats**

**at 10 days and at 20 days after tumor implant[1,2]**

| Group[3] | Diet | 10 days (n=10) | 20 days (n=10) |
|---|---|---|---|
| | | g | g |
| CN | Casein | 238.3 ± 2.8 | 260.1 ± 3.3 |
| SN | Soy protein | 232.0 ± 2.5 | 257.4 ± 5.1 |
| CT[4] | Casein | 233.5 ± 2.4 | 252.7 ± 5.3 |
| ST[4] | Soy protein | 234.8 ± 2.3 | 264.5 ± 5.4* |

[1]Values are mean ± SEM

[2]No statistically significant differences were seen among the experimental groups.

[3]N=Sham-operated; T=Tumor Implant

[4]Body weight before tumor was excised.

*n=9

**Table 10**

**Tumor weight in grams of male F344 rats at 10 days and 20 days post-implant[1,2]**

| Group[3] | Diet | 10 days | 20 days | P[4] |
|---|---|---|---|---|
| | | g | g | |
| CT | Casein | $3.4 \pm 0.3^{a}$ | $34.8 \pm 4.5^{b}$ | <0.05 |
| | | (n=10) | (n=10) | |
| ST | Soy | $2.5 \pm 0.4^{a}$ | $42.8 \pm 4.3^{b}$ | <0.05 |
| | | (n=10) | (n=9) | |

[1]Values are mean ± SEM

[2]Values not sharing a common letter superscript in the same column are not significantly different.

[3]C=Casein diet; S=Soy diet; T=Tumor Implant

[4]Comparison between 10 days and 20 days in rats with the same dietary treatment.

**Table 11**

**Tumor weight expressed as percent of body weight in male F344 tumor-bearing rats at 10 days after implant [1,2]**

| Group[3] | n | Diet | Total Body Weight | Tumor Weight | Tumor Weight / Body Weight |
|---|---|---|---|---|---|
| | | | g | g | % |
| CT | 10 | Casein | 233.5 ± 2.4 [a] | 3.4 ± 0.3 [a] | 1.5 ± 0.4 [a] |
| ST | 10 | Soy | 234.8 ± 2.3 [a] | 2.5 ± 0.4 [a] | 1.1 ± 0.6 [a] |

[1]Values are mean ± SEM.

[2]No statistical significant differences were seen between CT and ST rats in any given parameter.

[3]T=Tumor Implant (Body weights inclusive of tumor weight)

**Table 12**

**Tumor weight expressed as percent of body weight in male F344 tumor-bearing rats at 20 days after implant[1,2]**

| Group[3] | n | Diet | Total Body Weight | Tumor Weight | Tumor Weight / Body Weight |
|---|---|---|---|---|---|
| | | | g | g | % |
| CT | 10 | Casein | 252.7 ± 5.3 [a] | 34.8 ± 4.5 [a] | 13.9 ± 1.9 [a] |
| ST | 9 | Soy | 264.5 ± 5.4 [a] | 42.8 ± 4.3 [a] | 16.0 ± 1.5 [a] |

[1]Values are mean ± SEM.

[2]No statistically significant differences were seen between CT and ST in any given parameter.

[3]T=Tumor Implant (Body weight inclusive of tumor weights)

weight for the rats that were fed the soy protein diet. No significant difference in the tumor weight expressed as percentage of total body weight was present. Tumor weight expressed as percentage of total body weight at 20 days post-implant, had increased to 13.9% and 16.0% for rats fed casein and soy protein diet, respectively. However, these values are again not statistically significantly different (Table 12).

**Serum Lipids**

Two-factor ANOVAs were performed on serum lipid concentrations obtained from all dietary groups, excluding the baseline group, and the tumor groups at each time period. The baseline group was compared separately to the sham-operated group and to the tumor-bearers at 10 days and 20 days after implant using a three way analysis of variance and Tukey's post-hoc test.

**Serum HDL-Cholesterol**

The ANOVA summary table for serum HDL-cholesterol concentrations at 10 days post-implant is presented in Table 13. At 10 days post-tumor implant, there was a significant interaction (D x T, Table 13) between diet and tumor status for HDL-cholesterol concentration (Table 13 and Figure 2). The tests of simple main effects showed a significant difference in serum HDL-cholesterol concentration between tumor-bearing animals fed soy protein and those fed casein. Tumor bearing rats fed casein had significantly higher ($p<0.05$) HDL-cholesterol levels than tumor bearing rats fed soy protein diet. No differences in the HDL-cholesterol levels were observed between sham-

**Table 13**

**Two-factor analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations at 10 days post-implant**

| Overall Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 1743.09 | 36 | 48.42 | | |
| **Diet (D)[1]** | 2753.94 | 1 | 2753.94 | 56.88 | .000 |
| **Tumor (T)[2]** | 2661.79 | 1 | 2661.79 | 54.97 | .000 |
| **D x T** | 924.48 | 1 | 924.48 | 19.09 | .000 |
| **Total** | 8083.30 | 39 | | | |

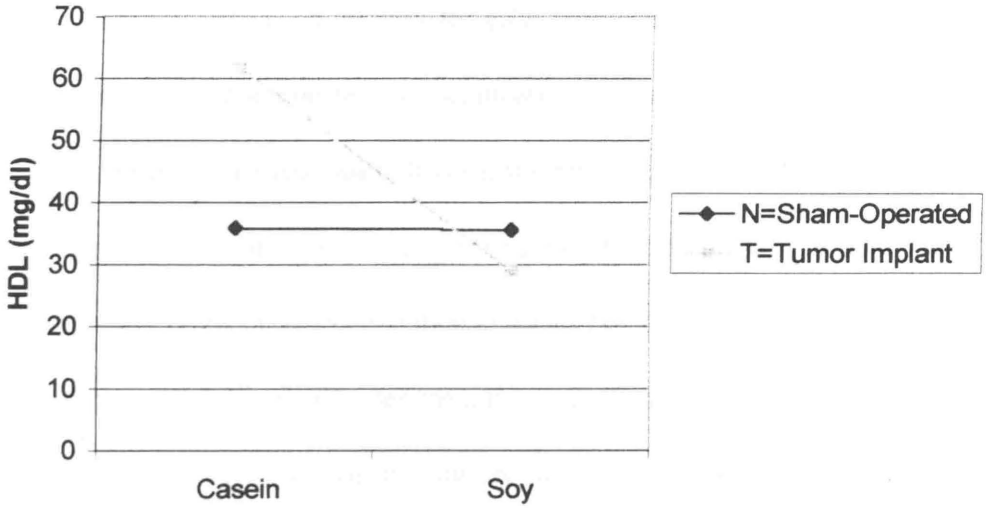
| Simple Main Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 4404.88 | 37 | 119.05 | | |
| **D w/o tumor** | 243.60 | 1 | 243.60 | 2.05 | .161 |
| **D w/ tumor** | 3434.82 | 1 | 3434.82 | 28.85 | .000 |
| **Total** | 8083.30 | 39 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer

**Figure 2**

**Interaction plot of serum high-density lipoprotein cholesterol (HDL-C) concentrations in male F344 rats at 10 days post-tumor implant**



operated animals fed soy or casein diets 10 days after implant. Serum HDL-cholesterol concentrations at 10 days post-tumor implant for each experimental group are presented in Table 14.

The two factor ANOVA table on HDL-cholesterol concentration of rats at 20 days post-implant can be found in Table 15. There was a significant interaction (D x T) between diet and tumor status at 20 days for HDL-cholesterol concentration (Figure 3). Differences in serum HDL-cholesterol concentrations occurred between animals with tumors. Tumor bearing rats fed casein had significantly higher ($p<0.05$) HDL-cholesterol levels at 20 days post-implant than tumor-bearing rats fed soy protein. There were no differences in serum HDL-cholesterol concentrations between sham-operated animals fed soy protein and casein. Individual experimental group means of serum HDL-cholesterol concentration at 20 days post-tumor implant are presented in Table 16.

**Serum Total Cholesterol Concentrations**

A two-factor analysis revealed that there was no significant interaction between diet and tumor at 10 days post-implant, but there was a significant effect of diet (Table 17). Therefore, statistical analysis was carried out by comparing animals fed soy protein and those fed casein regardless of their tumor status. At 10 days after implant there was a significant difference in total cholesterol concentration between rats fed soy protein and rats fed casein regardless of tumor status. Rats fed casein had significantly higher

**Table 14**

**Serum concentrations of high-density lipoprotein cholesterol (HDL-C) in male F344 rats at 10 days post-tumor implant[1,2]**

| Group[3] | Diet | n | HDL-C |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | $35.9 \pm 1.6^{a}$ |
| SN | Soy protein | 10 | $28.9 \pm 1.6^{a}$ |
| CT | Casein | 10 | $61.8 \pm 2.8^{b}$ |
| ST | Soy protein | 10 | $35.6 \pm 2.4^{a}$ |

[1]Values are mean ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 15**

**Two-factor analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations at 20 days post-implant**

| Overall Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 6557.84 | 35 | 187.37 | | |
| **Diet (D)[1]** | 4104.00 | 1 | 4104.00 | 21.90 | .000 |
| **Tumor (T)[2]** | 7295.22 | 1 | 7295.22 | 38.94 | .000 |
| **D x T** | 2477.68 | 1 | 2477.68 | 13.22 | .001 |
| **Total** | 20798.92 | 38 | | | |

| Simple Main Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 13853.06 | 36 | 384.81 | | |
| **D w/o tumor** | 104.88 | 1 | 104.88 | .27 | .605 |
| **D w/ tumor** | 6840.98 | 1 | 6840.98 | 17.78 | .000 |
| **Total** | 20798.92 | 38 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer.

**Figure 3**

**Interaction plot of serum high-density lipoprotein cholesterol (HDL-C) concentrations in male F344 rats at 20 days post-tumor implant**

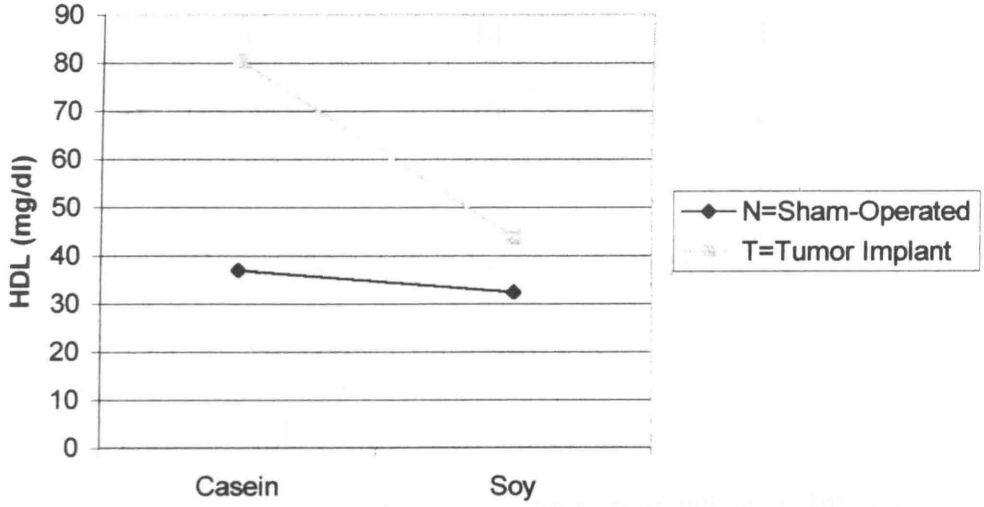


**Table 16**

**Serum concentrations of high-density lipoprotein cholesterol (HDL-C) in male F344 rats at 20 days post-tumor implant[1,2]**

| Group[3] | Diet | n | HDL-C |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | $37.0 \pm 1.7^{a}$ |
| SN | Soy protein | 10 | $32.4 \pm 2.2^{a}$ |
| CT | Casein | 10 | $80.3 \pm 6.4^{b}$ |
| ST | Soy protein | 9 | $43.8 \pm 5.4^{a}$ |

[1]Values are mean ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 17**

**Two-factor analysis of variance summary table for serum total cholesterol (TC) concentrations at 10 days post-implant**

| Overall Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 1938.42 | 36 | 53.84 | | |
| **Diet (D)[1]** | 1670.56 | 1 | 1670.56 | 31.03 | .000 |
| **Tumor (T)[2]** | 1037.34 | 1 | 1037.34 | 19.27 | .000 |
| **D x T** | 73.71 | 1 | 73.71 | 1.37 | .250 |
| **Total** | 4720.03 | 39 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer

($p<0.05$) total cholesterol concentrations than those rats fed soy (Table 18). There was also a significant effect of tumor status. Therefore, statistical analysis was also carried out by comparing tumor-bearing animals to non-tumor bearing animals regardless of diet consumed. At 10 days after implant, animals bearing MCA-induced sarcomas had significantly higher ($p<0.05$) total cholesterol concentrations than those animals that were sham-operated regardless of the type of dietary protein consumed (Table 19). Individual group means comparing baseline serum total cholesterol concentrations to each of the other diet and tumor groups are presented in (Table 20).

The ANOVA summary table for serum total cholesterol concentrations at 20 days post-implant can be found in Table 21. There was a significant interaction seen at 20 days post-tumor implant between diet and tumor status ( Figure 4). Significant differences were present between soy protein and casein fed animals that had been implanted with a tumor 20 days after implant. Casein fed tumor-bearing rats had significantly higher ($p<0.05$) total cholesterol concentrations than tumor bearing rats fed soy protein. Both tumor-bearing groups had significantly higher serum total cholesterol concentrations than the non-tumor bearing animals. No significant dietary differences occurred in sham-operated rats. Group means for total cholesterol concentration at 20 days after the tumor implantation are presented in Table 22.

**Table 18**

**Comparison of serum total cholesterol concentrations (TC) of male F344 rats fed either soy protein or casein at 10 days after implant[1,2]**

| Group[3] | n | TC |
|---|---|---|
| | | mg/dL |
| Casein (CN + CT) | 20 | 56.1 ± 2.2[a] |
| Soy (SN + ST) | 20 | 43.2 ± 1.8[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 19**

**Comparison of serum total cholesterol concentrations (TC) of sham-operated and tumor-bearing F344 male rats at 10 days after implant[1,2]**

| Group[3] | n | TC |
|---|---|---|
| | | mg/dL |
| Sham-Operated (CN + SN) | 20 | $44.6 \pm 1.9^{a}$ |
| Tumor-Bearers (CT + ST) | 20 | $54.8 \pm 2.5^{b}$ |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 20**

**Serum concentrations of total cholesterol (TC)**

**in male F344 rats at 10 days post-tumor implant[1,2]**

| Group[3] | Diet | n | TC |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | 49.7 ± 2.0 |
| SN | Soy protein | 10 | 38.4 ± 2.0 |
| CT | Casein | 10 | 62.6 ± 2.5 |
| ST | Soy protein | 10 | 47.0 ± 2.5 |

[1]Values are mean ± SEM.

[2]There were no statistically significant interactions between diet and tumor status at $p<0.05$ using ANOVA.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 21**

**Two-factor analysis of variance summary table for serum total cholesterol (TC) concentrations at 20 days post-implant**

| Overall Effects | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 3319.52 | 35 | 94.84 | | |
| **Diet (D)[1]** | 2144.18 | 1 | 2144.18 | 22.61 | .000 |
| **Tumor (T)[2]** | 17785.85 | 1 | 17785.85 | 187.53 | .000 |
| **D x T** | 759.48 | 1 | 759.48 | 8.01 | .008 |
| **Total** | 24490.44 | 38 | | | |

| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
|---|---|---|---|---|---|
| **Within +Residual** | 21105.37 | 36 | 586.26 | | |
| **D w/o tumor** | 180.60 | 1 | 180.60 | .31 | .582 |
| **D w/ tumor** | 3204.47 | 1 | 3204.47 | 5.47 | .025 |
| **Total** | 24490.44 | 38 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer

**Figure 4**

**Interaction plot of serum total cholesterol (TC) concentrations in male F344 rats at 20 days post-tumor implant**

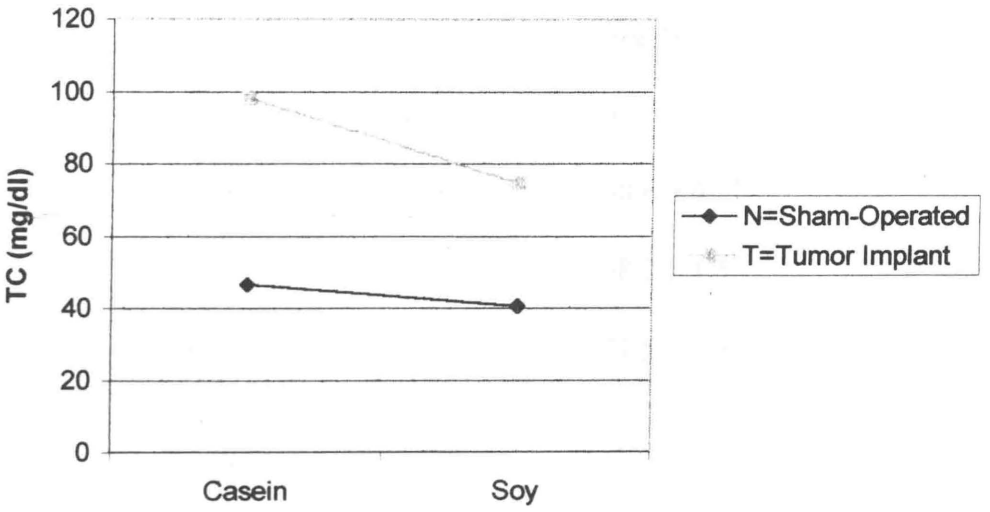


**Table 22**

**Serum concentrations of total cholesterol (TC) in male F344 rats at 20 days post-tumor implant[1,2]**

| Group[3] | Diet | n | TC |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | $46.6 \pm 2.1^{a}$ |
| SN | Soy protein | 10 | $40.6 \pm 4.2^{a}$ |
| CT | Casein | 10 | $98.2 \pm 3.5^{b}$ |
| ST | Soy protein | 9 | $74.5 \pm 1.9^{c}$ |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[2]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Serum Triglyceride Concentration**

There were no significant interactions between diet and tumor status at 10 or 20 days post-tumor implant for triglyceride concentrations (Table 23 and Table 24). Therefore, statistical analysis was carried out by comparing the effect of diet regardless of tumor status and tumor status regardless of dietary protein.

Ten days after implant, triglyceride concentrations were significantly higher ($p<0.05$) in animals fed casein than in animals fed soy protein regardless of tumor status (Table 25). However, at 20 days after implant, no significant difference was observed in TG concentration between groups (Table 26).

Ten days after tumor implant, triglyceride concentrations were significantly lower ($p<0.05$) in tumor bearing animals than sham-operated ones regardless of the dietary protein consumed (Table 27). However, at 20 days after tumor implant the serum triglyceride concentrations in tumor-bearing rats had increased to significantly higher levels ($p<0.05$) than the triglyceride concentrations in sham-operated animals (Table 28). Individual group means for serum triglyceride concentrations at 10 days and 20 days post-implant are shown in Table 29 and Table 30 respectively.

**Table 23**

**Two-factor analysis of variance summary table for serum triglyceride (TG) concentrations at 10 days post-implant**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 12503.04 | 36 | 347.31 | | |
| **Diet (D)[1]** | 2544.02 | 1 | 2544.02 | 7.33 | .010 |
| **Tumor (T)[2]** | 1974.03 | 1 | 1974.03 | 5.68 | .023 |
| **D x T** | 229.44 | 1 | 229.44 | .66 | .422 |
| **Total** | 17250.53 | 39 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer

**Table 24**

**Two-factor analysis of variance summary table for serum triglyceride (TG) concentrations at 20 days post-implant**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Within +Residual** | 40428.97 | 35 | 1155.11 | | |
| **Diet (D)[1]** | 206.28 | 1 | 206.28 | .18 | .675 |
| **Tumor (T)[2]** | 24358.93 | 1 | 24358.93 | 21.09 | .000 |
| **D x T** | 1570.68 | 1 | 1570.68 | 1.36 | .251 |
| **Total** | 66424.23 | 38 | | | |

[1] Diet (D) variable has two levels: soy protein and casein.

[2] Tumor (T) variable has two levels: sham-operated and tumor-bearer

**Table 25**

**Comparison of serum triglyceride (TG) concentrations of male F344 rats fed casein or soy protein at 10 days after implant[1,2]**

| Group[3] | n | TG |
|---|---|---|
| | | mg/dL |
| Casein (CN + CT) | 20 | 71.0 ± 5.3[a] |
| Soy (SN + ST) | 20 | 55.0 ± 3.2[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 26**

**Comparison of serum triglyceride (TG) concentrations of male F344 rats fed casein or soy protein at 20 days after implant[1,2]**

| Group3 | n | TG |
|---|---|---|
| | | mg/dL |
| Casein (CN + CT) | 20 | 86.1 ± 9.8[a] |
| Soy (SN + ST) | 19 | 79.9 ± 9.2[a] |

[1]Values are means ± SEM.

[2]No statistically significant differences were seen between casein and soy.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 27**

**Comparison of serum triglyceride (TG) concentrations of sham-operated and tumor-bearing F344 male rats at 10 days after implant[1,2]**

| Group[3] | n | TG |
|---|---|---|
| | | mg/dL |
| Sham-Operated (CN + SN) | 20 | 70.0 ± 5.5[a] |
| Tumor-Bearers (CT + ST) | 20 | 56.0 ± 3.1[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 28**

**Comparison of serum triglyceride (TG) concentrations of sham-operated and tumor-bearing F344 male rats at 20 days after implant[1,2]**

| Group[3] | n | TG |
|---|---|---|
| | | mg/dL |
| Sham-operated (CN + SN) | 20 | 58.8 ± 5.4[a] |
| Tumor- Bearers (CT + ST) | 19 | 108.6 ± 9.6[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 29**

**Serum concentrations of triglycerides (TG) in male F344 rats at 10 days post-tumor implant[1,2]**

| Group[3] | Diet | n | TG |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | 80.4 ± 8.7 |
| SN | Soy protein | 10 | 54.7 ± 5.9 |
| CT | Casein | 10 | 61.6 ± 4.8 |
| ST | Soy protein | 10 | 50.4 ± 3.3 |

[1]Values are means ± SEM.

[2]There were no statistically significant interactions between diet and tumor status at $p<0.05$ using ANOVA.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Table 30**

**Serum concentrations of triglycerides (TG) in male F344 rats at 20 days post-tumor implant[1,2]**

| Group[3] | Diet | n | TG |
|---|---|---|---|
| | | | mg/dL |
| CN | Casein | 10 | 67.5 ± 8.9 |
| SN | Soy protein | 10 | 50.2 ± 5.0 |
| CT | Casein | 10 | 104.4 ± 2.5 |
| ST | Soy protein | 9 | 112.9 ±10.8 |

[1]Values are means ± SEM.

[2]There were no statistically significant interactions between diet and tumor status at $p<0.05$ using ANOVA.

[3]C=Casein diet; S=Soy diet; N=Sham-operated; T=Tumor Implant

**Effect of Time on Serum Lipid Concentrations for Sham-Operated Rats**

The ANOVA summary tables comparing serum lipid concentrations of baseline rats to sham-operated rats at 10 days and 20 days are presented in Tables 31 through 36. Data comparing serum lipid concentrations in sham-operated rats at time 0, 10 days and 20 days after tumor implant are shown in Table 37. HDL-C concentrations were significantly higher in the baseline rats than in those animals fed soy protein at 10 days but were not significantly different than those animals fed soy protein at 20 days. There were no significant differences between the three time periods (0, 10, or 20 days) in animals fed casein diet.

Rats fed soy protein at 10 and 20 days post-implant had significantly lower total cholesterol concentrations ($p<0.05$) than baseline animals. When casein fed rats were compared to the baseline animals total cholesterol concentration was significantly lower ($p<0.05$) by 20 days.

There were no significant differences in triglyceride levels at any time period (0,10, or 20 days post-implant) regardless of diet.

**Table 31**

**Analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations comparing baseline rats to sham-operated rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 475.57 | 2 | 237.78 | 6.68 | .004 |
| **Error** | 961.46 | 27 | 35.61 | | |
| **Total** | 1437.03 | 29 | 49.55 | | |

**Table 32**

**Analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations comparing baseline rats to sham-operated rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 35.51 | 2 | 17.76 | .62 | .547 |
| **Error** | 775.78 | 27 | 28.73 | | |
| **Total** | 811.29 | 29 | 27.98 | | |

**Table 33**

**Analysis of variance summary table for serum total cholesterol (TC) concentrations comparing baseline rats to sham-operated rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 1803.30 | 2 | 901.65 | 9.05 | .001 |
| **Error** | 2690.68 | 27 | 99.65 | | |
| **Total** | 4493.97 | 29 | 154.97 | | |

**Table 34**

**Analysis of variance summary table for serum total cholesterol (TC) concentrations comparing baseline rats to sham-operated rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 443.32 | 2 | 221.66 | 3.92 | .032 |
| **Error** | 1526.90 | 27 | 56.55 | | |
| **Total** | 1970.22 | 29 | 67.94 | | |

**Table 35**

**Analysis of variance summary table for serum triglyceride (TG) concentrations comparing baseline rats to sham-operated rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 1467.83 | 2 | 733.91 | 2.30 | .119 |
| **Error** | 8610.24 | 27 | 318.90 | | |
| **Total** | 10078.07 | 29 | 347.52 | | |

**Table 36**

**Analysis of variance summary table for serum triglyceride (TG) concentrations comparing baseline rats to sham-operated rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 1183.78 | 2 | 591.89 | .92 | .409 |
| **Error** | 17269.56 | 27 | 639.61 | | |
| **Total** | 18453.34 | 29 | 636.32 | | |

**Table 37**

**Comparison of baseline serum lipid concentrations to serum lipid concentrations of F344 male rats fed soy protein or casein diet at 10 days or 20 days after sham-operation[1,2]**

| | Group[3] | 0 days (AIN*) (n=10) | 10 days (n=10) | 20 days (n=10) |
|---|---|---|---|---|
| **HDL-C (mg/dl)** | | | | |
| | SN | 38.6 ± 1.8[a] | 28.9 ± 1.6[b] | 32.4 ± 2.2[ab] |
| | CN | 38.6 ± 1.8[a] | 35.9 ± 1.6[a] | 37.0 ± 1.7[a] |
| **TC (mg/dl)** | | | | |
| | SN | 55.8 ± 2.9[a] | 38.4 ± 2.0[b] | 40.6 ± 4.2[b] |
| | CN | 55.8 ± 2.9[a] | 49.7 ± 2.0[a] | 46.6 ± 2.1[b] |
| **TG (mg/dl)** | | | | |
| | SN | 66.7 ± 5.9[a] | 54.7 ± 5.9[a] | 50.2 ± 5.0[a] |
| | CN | 66.7 ± 5.9[a] | 80.4 ± 8.7[a] | 67.5 ± 8.9[a] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript in the same row are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; N=Sham-operated

**Effect of Time on Serum Lipid Concentrations in Tumor-Bearing Rats**

The ANOVA summary tables for serum lipid concentrations in tumor-bearing rats are shown in Tables 38 through 43. Data comparing serum lipid concentrations in tumor bearing rats at time 0, 10 days and 20 days after tumor implant are shown in Table 44. There were no significant differences between the baseline animals and animals fed soy protein at 10 or 20 days post-implant. Animals fed casein had significantly higher ($p<0.05$) HDL-cholesterol concentrations than baseline animals. Casein fed rats killed 20 days after tumor implant had significantly higher ($p<0.05$) HDL-cholesterol levels than rats killed at 0 or 10 days.

In animals fed soy protein, total cholesterol concentrations were significantly less ($p<0.05$) at 10 days after implant than baseline and 20 day rats. Soy fed rats killed at 20 days had significantly higher ($p<0.05$) TC concentrations than both the baseline and rats killed at 10 days. Total cholesterol was significantly higher ($p<0.05$) in rats fed casein and killed at 20 days post-tumor implant when compared to animals killed at baseline and 10 days. There were no significant differences between the baseline animals and 10 day casein fed animals.

Triglyceride levels were significantly higher ($p<0.05$) in animals killed at 20 days post-tumor implant than in animals killed at any other time regardless of diet.

**Table 38**

**Analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations comparing baseline rats to tumor-bearing rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 324.84 | 2 | 162.42 | 1.43 | .258 |
| **Error** | 2959.53 | 26 | 113.83 | | |
| **Total** | 3284.37 | 28 | 117.30 | | |

**Table 39**

**Analysis of variance summary table for serum high-density lipoprotein cholesterol (HDL-C) concentrations comparing baseline rats to tumor-bearing rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 8761.90 | 2 | 4380.95 | 25.00 | .000 |
| **Error** | 4731.13 | 27 | 175.23 | | |
| **Total** | 13493.04 | 29 | 465.28 | | |

**Table 40**

**Analysis of variance summary table for serum total cholesterol (TC) concentrations comparing baseline rats to tumor-bearing rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 3708.66 | 2 | 1854.33 | 30.46 | .000 |
| **Error** | 1582.79 | 26 | 60.88 | | |
| **Total** | 5291.44 | 28 | 188.98 | | |

**Table 41**

**Analysis of variance summary table for serum total cholesterol (TC) concentrations comparing baseline rats to tumor-bearing rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 10349.62 | 2 | 5174.81 | 58.04 | .000 |
| **Error** | 2407.17 | 27 | 89.15 | | |
| **Total** | 12756.79 | 29 | 439.89 | | |

**Table 42**

**Analysis of variance summary table for serum triglyceride (TG) concentrations comparing baseline rats to tumor-bearing rats fed soy protein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 19653.66 | 2 | 9826.83 | 20.38 | .000 |
| **Error** | 12535.53 | 26 | 482.14 | | |
| **Total** | 32189.19 | 28 | 1149.61 | | |

**Table 43**

**Analysis of variance summary table for serum triglyceride (TG) concentrations comparing baseline rats to tumor-bearing rats fed casein diet over a period of 20 days**

| Overall Effect | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **SS** | **DF** | **MS** | **F** | **Significance of F** |
| **Model** | 11149.28 | 2 | 5574.64 | 5.41 | .010 |
| **Error** | 27821.27 | 27 | 1030.42 | | |
| **Total** | 38970.55 | 29 | 1343.81 | | |

**Table 44**

**Comparison of serum lipid concentrations during MCA sarcoma growth to serum lipid concentrations at baseline (0 days) in male F344 rats fed casein or soy protein at 10 or 20 days after tumor implant[1,2]**

| | Group[3] | 0 days (AIN*) (n=10) | 10 days (n=10) | 20 days (n=10) |
|---|---|---|---|---|
| **HDL-C (mg/dl)** | | | | |
| | ST | 38.6 ± 1.8[a] | 35.6 ± 2.4[a] | 43.8 ± 5.4[a] |
| | CT | 38.6 ± 1.8[a] | 61.8 ± 2.8[b] | 80.3 ± 6.4[c] |
| **TC (mg/dl)** | | | | |
| | ST | 55.8 ± 2.9[a] | 47.0 ± 2.5[b] | 74.5 ± 1.9[c] |
| | CT | 55.8 ± 2.9[a] | 62.6 ± 2.5[a] | 98.2 ± 3.5[b] |
| **TG (mg/dl)** | | | | |
| | ST | 66.7 ± 5.9[a] | 50.4 ± 3.3[a] | 112.9 ± 10.8[b] |
| | CT | 66.7 ± 5.9[a] | 61.6 ± 4.8[a] | 104.8 ± 15.8[b] |

[1]Values are means ± SEM.

[2]Values not sharing a common letter superscript in the same row are significantly different at $p<0.05$ using ANOVA and Tukey's post hoc test.

[3]C=Casein diet; S=Soy diet; T=Tumor Implant

# CHAPTER 5

# DISCUSSION

In the current study, animals killed for baseline data had significantly ($p<0.05$) lower intake of AIN-76A diet during the first week of the study than any of the other groups that were fed either the casein or the soy protein diet (Table 3). Food intake among the four groups fed the casein or soy protein diet was not significantly different from each other. The significantly lower food intake of AIN-76A diet may have been because these animals initially weighed 4 grams less than the remaining groups of animals. Although the difference in weight was not significant, the small variation may have contributed to the significantly lower food intake after the first week.

As stated previously, there were no significant differences in the initial body weights between any of the experimental groups. However, after one week on their respective experimental diets, animals in the baseline group that were fed the AIN-76A diet weighed significantly ($p<0.05$) less than the remaining groups of animals (Table 6). Several factors may have contributed to this difference. First of all, the baseline animals ate significantly ($p<0.05$) less food than the remaining groups during the first week of the study (Table 3). Secondly, the AIN-76A diet contained less fat than the remaining groups (5% w/w corn oil vs 10% w/w corn oil). Thirdly, they were slightly smaller (by 4 grams) than the remaining groups initially.

In the present experiment, neither food intake nor body weight was significantly

different among the tumor-bearing rats at 10 days after tumor implant indicating that anorexia and/or cachexia had not yet occurred in these rats (Table 4). However, anorexia and/or cachexia appeared to have begun by 20 days after tumor implant. Food intake for tumor-bearing animals at 20 days post-implant, was significantly ($p<0.05$) lower than sham-operated animals (Table 5). In addition, some wasting could have occurred in the tumor-bearing rats. Although the weight of tumor-bearing rats was not significantly different from sham-operated rats, the tumor accounted for over 10% of the tumor-bearing rats weight (Table 8). Bearing 3-methylcholanthrene (MCA) induced sarcoma has been reported to cause anorexia and cachexia in the Fischer 344 rat in 13 to 16 days (Popp et al., 1984; Popp et al., 1981). Radcliffe (1989) reported significantly depressed food intake within 10-14 days in casein fed F344 rats bearing MCA-induced sarcomas. Smith et al. (1993) reported that death occurred in animals bearing 3-MCA -induced sarcomas within 35-45 days. Additionally, previously mentioned studies used animals weighing between 120-150 grams, whereas the animals in this study were approximately 220 grams at the time of implant. Since the current experiment did not extend beyond 20 days after tumor implant, it is unknown whether the animals would develop anorexia/ cachexia.

In the present experiment, we studied the effect of feeding soy protein to rats on the growth of the MCA transplantable tumors and the concentrations of HDL- cholesterol, total cholesterol, and triglycerides in the serum of the rats during tumor growth. Tumor growth was not inhibited by feeding the rats a soy protein diet in comparison to a casein

diet. In fact, 20 days after tumor implantation, tumors weights of animals fed soy protein diet and casein diet were similar (Table 8). There may be a number of explanations for the results obtained in this study. First, consuming soy protein is believed to be associated with the reduction of the incidence of human cancer and chemically induced tumor incidence in animals. However, much of the evidence of soy protein's effect on cancer is in the form of epidemiological studies or experimental studies that examine the effect of specific components in soy protein on tumor growth of animals. No study has examined the effect of soy protein intake on transplantable 3-methylcholanthrene (MCA) tumor growth. Soy protein contains a number of components that may affect tumor growth and some of these components have been shown to enhance rather than suppress tumor growth. For instance, protease inhibitors did not suppress squamous tumors of the anal gland (St. Clair et al., 1990) and raw soy flour increased dihydroxypropyl nitrosamine and azaserine induced carcinogenesis in the pancreas of male Wistar rats (Levison et al., 1979; Morgan et al., 1977). In general, processing would remove much of the protease inhibitors in raw soybean or soy flour; however, soy protein isolate may contain anywhere from 1-30 mg/g protein of protease inhibitors depending on the original content in the soybean and the amount of washing (Anderson and Wolf, 1995). Secondly, soy protein is generally deficient in methionine in comparison to casein and methionine may have a stimulating effect on tumor growth (Hawrylewicz et al., 1991). In the present experiment, 0.3% DL-methionine was added to both the soy protein

and the casein diet. This resulted in a concentration of 14 g/kg of methionine in the soy protein diet and 24 g/kg of methionine in the casein diet. Therefore, any potential effect of stimulating tumor growth from methionine in the soy protein diet is probably negligible. Thirdly, the method used in this experiment for inducing the rats with the MCA sarcoma was different from the method used by Radcliffe (1989), Popp et al. (1981) or Smith et al. (1993). Radcliffe (1989) injected animals with 300 mg of MCA-sarcoma tissue in the right flank whereas Popp et al. (1981) injected the animals with $1 \times 10^6$ viable cells through a skin incision in their flank. Smith et al. (1993) inserted tumor tissue through a skin incision in their flank. We inserted a piece of 2 $mm^3$ MCA-induced sarcoma directly under the skin of the rats on the right flank. Additionally, the rats in our experiment were heavier (220 grams) at the time of implant. Both Radcliffe (1989) and Popp et al. (1981) used 150 gram rats and Smith et al. (1993) used 120 gram rats. The method and time of inducing rats with MCA-sarcoma may account for the differences in the rate of tumor growth among experiments conducted by different investigators.

In the present experiment, we have observed significantly ($p<0.05$) lower serum HDL-cholesterol (HDL-C) and total cholesterol (TC) concentrations at 20 days in tumor-bearing animals fed the soy protein diet than those fed the casein diet at both 10 and 20 days after implant (Table 42). However, when HDL-cholesterol concentrations are examined at each time period separately, significant differences in diet were seen only in animals with tumors and no significant differences were seen between the diets of sham-

operated rats (Tables 12 and 14). For TC, there was a significant difference seen between diets at 10 days regardless of tumor status but not at 20 days (Table 16). At 20 days, TC concentration was significantly ($p<0.05$) lower in only tumor bearing rats fed soy protein when compared to tumor bearing rats fed casein (Table 20).

Feeding a soy protein diet to sham-operated animals also lowered serum triglyceride (TG) concentrations in comparison to those on the casein diet. Triglyceride concentrations were significantly ($p<0.05$) lower at 10 days in rats fed soy protein regardless of tumor status (Table 23). By 20 days significant differences due to diet had disappeared (Table 24). When these serum lipid levels are examined over time, significant dietary differences started to appear since serum lipid concentrations of soy fed animals remained lower than those animals fed casein. The findings of this study do not contradict what appears in the literature. Substituting soy protein in the diet for animal protein has been suggested as a way of reducing serum cholesterol levels of hypercholesterolemic animals (Cohn et al., 1984) and individuals as well as a way of maintaining cholesterol levels in normal individuals (Huff et al., 1977; Sirtori et al., 1977; Kanazwa et al., 1993). Cohn et al. (1984) reported that the total cholesterol concentration in Sprague Dawley rats fed a high cholesterol (1.2 %) casein diet had a mean total cholesterol concentration two times higher than those fed a high cholesterol soy protein diet. Kanazwa (1993) reported significantly lower plasma total cholesterol and triglycerides in stroke patients fed soymilk with no change in HDL-cholesterol concentrations. In another study by Bakhit

and associates in 1994, soy protein lowered plasma total cholesterol levels in hypercholesterolemic individuals including lowering HDL-C but had little effect on the plasma total cholesterol levels in normal individuals. These investigators measured HDL-C, TC, and TG in both normal and hypercholesterolemic subjects.

Several changes may occur in serum lipid concentrations as a result of carcinogenesis. In the present study, tumor bearers fed a casein d in tumor bearing rats iet had significantly ($p<0.05$) elevated serum HDL cholesterol levels at 10 days and at 20 days. There were no significant differences in serum HDL cholesterol levels between sham-operated and tumor-bearing rats fed soy protein at 10 days or 20 days. Reports of serum HDL-C levels during cancer have been conflicting. A couple of researchers have reported higher serum HDL-C in post-menopausal women with breast cancer when compared to controls (Rossner and Walgren, 1984; Miller and Erf, 1956). On the other hand, Miller and Erf reported lower HDL-C levels in women with metastatic breast cancer. Most researchers have reported lower serum HDL-cholesterol concentrations in cancer patients than in controls. Dilman (1981) reported low serum HDL-C in breast and lung cancer patients. Barclay et al. (1970) and Bani et al. (1986) also reported lower serum HDL-C in breast cancer patients compared to controls. It does appear that HDL-cholesterol concentrations are influenced by tumorigenesis. HDL-C is responsible for removing lipids and cholesterol from cells and returning them to the liver for recycling or disposal. Based on these reports, it appears that HDL-C levels may be influenced by the

stage of cancer development, but how it is influenced is not clear. During the early stages of cancer, HDL-cholesterol concentrations are high and during the late stages of development the concentrations are low. High serum HDL-C levels in cancer free subjects have been suggested as a risk factor for breast cancer (Boyd and McGuire, 1990), and women with mammographic dysplasia, which is also associated with increased risk for breast cancer, reportedly have higher HDL-C levels than those without (Boyd et al. (1989). However, Dilman (1981) did not find any significant differences in HDL-C concentrations during several stages of cancer development except during rectal cancer. Soy protein appeared to suppress the elevation of HDL-cholesterol in tumor bearing rats in this experiment. Since HDL-cholesterol levels appear to be influenced by tumor development, the question arises as to whether or not the effect that soy protein had on cholesterol levels influenced tumor growth.

In the present experiment, total cholesterol was significantly ($p<0.05$) elevated in animals with tumors when compared to animals without tumors. In several studies of human cancer, there is a decrease in serum total cholesterol levels as the cancer progresses (Sharp and Pocock, 1997; Miller et al., 1981; Rose et al., 1974. Radcliffe (1989) also reported significantly elevated serum cholesterol levels at day 20 post-implant for male Fischer rats fed casein-based diet. On the other hand, Miller et al. (1981) reported no significant differences in serum cholesterol between tumor patients in the early stages of cancer and controls. It could be that once the cancer progresses, cholesterol levels would

begin to decrease. It should also be noted that the previous experiments showing a decrease in cholesterol levels were human studies and not rat studies and differences between them are to be expected.

In the current experiment, triglyceride concentrations in tumor-bearing rats were significantly ($p<0.05$) lower than in non-tumor bearing rats at 10 days regardless of diet in tumor bearing rats in tumor bearing rats (Table 25). But by 20 days, serum triglyceride concentrations had significantly increased in tumor bearing rats and these animals had significantly ($p<0.05$) higher serum triglyceride levels than sham-operated ones (Table 26). During the early stage of tumor development in this experiment (10 days post-implant), triglyceride concentration may have been primarily influenced by soy protein and less affected by tumor development. At 10 days post-implant tumor growth was still small. However, by 20 days, tumor growth appears to be a major factor influencing triglyceride concentrations. High serum TG levels have been reported in breast and lung cancer patients (Dilman et al., 1981). In cancer patients, lipolysis is significantly elevated and in order to fuel tumor growth, mobilization of fat stores, which are primarily triglycerides, may occur thus increasing serum triglycerides (Gercel-Taylor et al., 1996; Drott et al., 1989). In addition, cachectin (tumor necrosis factor or TNF) found in tumor patients was shown to inhibit lipoprotein lipase activity, which hydrolyzes triglycerides (Semb et al., 1987). If in fact TNF inhibits LPL activity, in tumor bearing rats the significant decrease in lipoprotein lipase activity in Fischer 344 rats bearing a Leydig cell tumor reported by

Obeid and Emery (1993) is not surprising. A decrease in lipoprotein lipase activity would result in a decrease in the hydrolysis of triglycerides. As a result, triglycerides would not be hydrolyzed and removed from the serum and elevation of serum triglycerides would occur.

# CHAPTER 6

## SUMMARY

The purpose of this study was to compare the effect of feeding a soy-based diet to that of a casein-based diet on the growth of MCA-induced sarcomas and on the concentrations of serum high density lipoprotein (HDL) cholesterol, total cholesterol (TC), and triglycerides (TG) in Fischer 344 male rats at 10 days (early stage of tumor development) and 20 days (late stage of tumor development) post-implant.

The first null hypothesis is that there is no difference between feeding soy protein diet and casein diet on the concentrations of serum high-density lipoprotein cholesterol, total cholesterol, or triglycerides in Fischer 344 male rats bearing MCA-induced sarcoma at either 10 days or 20 days after tumor implantation. Results of this study show that tumor-bearing animals fed the soy protein diets had significantly lower serum HDL-C concentrations than tumor-bearing animals fed the casein diets at both 10 and 20 days after implant. Serum total cholesterol concentrations were significantly lower in tumor-bearing animals fed soy protein than those fed casein at 20 days after tumor implant but not 10 days post tumor implantation. Dietary protein did not specifically affect serum triglycerides in tumor bearing rats. Both rats fed soy protein and casein had lower serum triglyceride concentrations at 10 days regardless of whether or not they had a tumor. By

20 days post-implant, there were no significant differences between diets. Serum triglyceride concentrations in tumor bearing rats were not affected by feeding soy protein or the casein diet at any time post tumor implantation. Although there was no significant differences in triglyceride concentrations seen between dietary groups at any time and in total cholesterol concentrations at 10 days, there were significant difference seen on the concentrations of HDL-C at both time periods and on the concentration of serum TC at 20 days. Therefore, the first null hypothesis must be rejected.

The second null hypothesis was that there is no difference between feeding soy protein diet and casein diet on MCA-induced tumor growth in Fischer 344 male rats bearing MCA-induced sarcoma at either 10 days or 20 days after tumor implantation. Tumor growth was not significantly affected by diet at 10 days or 20 days. There were no significant differences between animals fed casein or soy protein at 10 days post implant, although soy protein fed animals had slightly depressed tumor weights than the casein fed animals. By 20 days post-implant, there were still no significant differences between dietary groups and tumor weights in the soy fed animals were not significantly different than the tumor weights in the animals fed casein. As a result, the second null hypothesis can be accepted.

The third null hypothesis was that there is no difference between rats bearing MCA-induced tumors and sham-operated rats on the concentrations of serum high-density lipoprotein cholesterol, total cholesterol, and triglycerides in Fisher 344 male rats. The

observed differences in serum concentrations of HDL-C appeared only to be the result of an interaction between diet and tumor growth, but not due to the presence of a tumor. Significant elevation in HDL-C concentrations at 10 and 20 days of tumor growth and TC concentrations at 20 days only in tumor-bearing animals fed casein and not in tumor bearing animals fed soy protein. It appears as if this part of the null hypothesis is true. However, serum total cholesterol at 10 days post-tumor implant and triglyceride concentrations at 20 days post-tumor implant were significantly higher in tumor-bearers when compared to non-tumor bearers. Therefore, this null hypothesis must be rejected.

In conclusion, diet appears to have an effect on serum lipid concentration in animals with tumors but does not appear to be a factor in animals without tumors. Tumor-bearing animals fed soy protein had lower serum lipid levels than those fed casein in most instances. The only exception was that the triglyceride concentrations of the soy fed tumor-bearers after 20 days was significantly higher than those fed casein. Although not always significant, soy protein appears to suppress the elevation of serum lipid concentrations in all cases except in the case of triglyceride levels after 20 days post-implant. Secondly, soy protein does not appear to suppress the growth of MCA-induced sarcoma. Finally, the presence of the MCA-induced sarcoma appears to elevate serum total cholesterol and triglyceride concentrations in male Fischer 344 rats.

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# APPENDICES

# APPENDIX A

# ANIMAL CARE AND USE COMMITTEE APPROVAL

Form 1988A
Animal Project

No 1997-07 (For Office Use Only)



USE OF VERTEBRATE ANIMALS
TWU ANIMAL RESEARCH FACILITY

Project Title: The comparison of serum Lipid concentrations and the growth of MCA-induced sarcoma in rats fed casein-based and soy-based diets

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

Andie Hsueh* and Andrea Harris

Department Nutrition and Food Sciences Phone Extension 2646

Proposed Duration of Project: From 10/1/97 To 8/31/98

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?
Dr. Betty Alford

Previously assigned Animal Project No. If applicable is other than a New Proposal or Pilot Project
N/A

Date Received by ACUC 8-29-97
Review Board Action: Date 9-26-97
Approved ✓ Approved Contingent _____ Disapproved _____ Returned for Revision _____
Remarks:

Additional Review Required? NO ___ Yes ___ Safety ___ Radiation ___ Biohazard ___

Signature of ACUC Representative Lynda Haworth

Date Received by Safety/Radiation/Biohazard Committee: _____

Review Board Action: Date: _____
Approved ___ Approved Contingent ___ Disapproved ___ Returned for Revision ___
Remarks:

Signature of Safety/Radiation/Biohazard Representative _____

# APPENDIX B

# DESCRIPTIONS OF CASEIN AND SOY PROTEIN ISOLATE

**BIOSERVE**
One 8th Street, Suite I • Frenchtown, NJ 08825
Phone: 908-996-2155 • Web Address: www.bio-serv.com • Fax: 908-996-4123

## PRODUCT #1100
## Casein

### Typical Analysis

| | |
|---|---|
| Protein (N x 6.38) | 88.00 % |
| Fat | <0.50 % |
| Dietary Fiber | <0.50 % |
| Ash | 2.00 % |
| Moisture | <10.00 % |
| Carbohydrate | <0.50 % |

| MINERALS AND VITAMINS | | AMINO ACID PROFILE | % |
|---|---|---|---|
| Calcium | 0.04 % | Lysine | 7.1 |
| Sodium | 0.03 % | Tryptophan | 1.5 |
| Phosphorus | 1.00 % | Phenylalanine | 4.4 |
| Potassium | 0.10 % | Methionine | 2.4 |
| Magnesium | 28.00 ppm | Threonine | 4.3 |
| Manganese | 1.00 ppm | Leucine | 8.0 |
| Iron | 15.00 ppm | Isoleucine | 5.3 |
| Zinc | 40.00 ppm | Valine | 6.3 |
| Selenium | 0.10 ppm | Arginine | 3.6 |
| Riboflavin | 0.60 mg/lb | Histidine | 2.7 |
| Vitamin A | <75.00 IU/lb | Tyrosine | 5.5 |
| Thiamine | <0.005 mg/100 gm | Cystine | 0.3 |
| Niacin | 0.050 mg/100 gm | Serine | 5.5 |
| Pyridoxine | 0.005 mg/100 gm | Glutamic Acid | 19.5 |
| Vitamin B12 | 5.260 mcg/100 gm | Aspartic Acid | 6.2 |
| | | Glycine | 2.4 |
| | | Alanine | 2.6 |
| | | Proline | 9.8 |

### MICROBIAL ANALYSIS

Standard Plate <5000/gm
Coliform <10/gm
Thermophiles <1000/gm
Yeast and Mold <100/gm
Salmonella Neg/25 gm

## PRODUCT #1510
## Soy Protein Isolate

**Description:** Soy Protein Isolate is a specially processed soy protein which has a protein value of 90+ %. It has a bland flavor. It can be used for its functional properties (dispersibility, solubility, emulsion capacity) and/or as a nutritional supplement.

### Typical Analysis

**CHEMICAL CHARACTERISTICS**

| | |
|---|---|
| Protein (N x 6.38) | 93.00 % |
| Fat | 0.30 % |
| Ash | 3.90 % |
| Moisture | <5.00 % |
| Fiber (crude) | 0.25 % |
| pH | 6.25 - 6.95 |
| Solubility | <50 % St. N Mthd. |
| Suspension | good, 1-2 hours |

**MINERALS AND VITAMINS**

| | |
|---|---|
| Calcium | 1.6 gm/kg |
| Phosphorus | 8.0 gm/kg |
| Potassium | <2.0 gm/kg |
| Sodium | <12.0 gm/kg |
| Choline | 1.36 gm/kg |

**MICROBIOLOGICAL DATA**

| | |
|---|---|
| SPC Maximum | 10,000 /gm |
| Yeast/Molds | <100 /gm |
| E. coli | <3 /gm |
| Salmonella | Negative |

**AMINO ACID PROFILE** **%**

| | |
|---|---|
| Lysine | 5.5 |
| Tryptophane | 1.1 |
| Phenylalanine | 4.6 |
| Methionine | 1.2 |
| Threonine | 3.3 |
| Leucine | 7.2 |
| Isoleucine | 4.3 |
| Valine | 4.4 |
| Arginine | 6.7 |
| Histidine | 2.3 |
| Tyrosine | 3.3 |
| Cystine | 1.1 |
| Serine | 4.6 |
| Glutamic Acid | 16.8 |
| Aspartic Acid | 10.2 |
| Glycine | 3.7 |
| Alanine | 3.8 |
| Proline | 4.5 |

# APPENDIX C

## PROXIMATE ANALYSIS OF CASEIN AND SOY PROTEIN ISOLATE DIETS

# POPE TESTING LABORATORIES, INC.

CONSULTING ANALYTICAL CHEMISTS
AND TESTING ENGINEERS

FOODS, FEEDS, DAIRY PROD.
WATER, MISCL. ANALYSES
COTTON SEED PRODUCTS
PACKING HOUSE PRODUCTS
FERTILIZERS

P. O. BOX 903
DALLAS, TEXAS
75221
(214) 742-8491

OFFICIAL CHEMISTS
WEIGHERS AND INSPECTORS
NATL. COTTONSEED PRODUCTS ASS'N.
REFEREE CHEMISTS
AMERICAN OIL CHEMISTS SOCIETY

**TO** Texas Woman's University
P0105162-Nfs Omb 307
Denton, TX

**Date Rec'd** 5-15-98

**Report of Tests on** Animal Diet

**Received from**

**Identification Marks** Sample # 1 Casein

| | |
|---|---|
| Moisture | 3.7 % |
| Protein | 19.7 |
| Fat | 9.0 |
| Fiber | 2.7 |
| Ash | 3.0 |
| Nitrogen Free Extract | 61.9 |

**LAB NO.** 32641

**POPE TESTING LABORATORIES, INC.**

**By** Leon Hunter

F-80—40M

# POPE TESTING LABORATORIES, INC.

CONSULTING ANALYTICAL CHEMISTS
AND TESTING ENGINEERS

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AMERICAN OIL CHEMISTS SOCIETY

**TO** Texas Woman's University
PO105162-Nfs Omb 307
Denton, TX

**Date Rec'd** 5-15-98

**Report of Tests on** Animal Diet

**Received from**

**Identification Marks** Sample # 2 30Y

| | |
|---|---|
| Moisture | 3.0 % |
| Protein | 20.5 |
| Fat | 8.8 |
| Fiber | 1.8 |
| Ash | 2.8 |
| Nitrogen Free Extract | 63.1 |

**LAB NO.** 32642

**POPE TESTING LABORATORIES, INC.**

By Flora Hunter

F-50—40M

# APPENDIX D

# PROCEDURES FOR TUMOR TISSUE PREPARATION AND IMPLANT

## Tumor Implantation Procedure

1. A rat of 220-230 g body weight will use 0.15 mL ketamine (100 mg/mL) and 0.05 mL xylazine (20 mg/mL)
2. Clean off top of rodent cocktail covering with alcohol swab, and measure cocktail using a #25 guage needle and syringe.
3. Roll the rat in a towel or restrain rat in appropriate manner.
4. Inject the mixture of ketamine and xylazine (i.m.) on the right hind leg.
5. When the rat is in deep sleep, make a small (1/4 - 3/8") incision in the left hind flank.
6. Tunnel underneath opening with a micro hemostat as far as possible.
7. Insert tumor cube in opening as far as possible.
8. Apply wound clip to close opening.

## Tumor Tissue Preparation

1. Pour Tissue culture media into a petri dish.
2. Asphixiate tumor-bearing rat using a suitable substance (i.e. $CO_2$).
3. Cut along the base of the tumor.
4. Use a scalpel to separate the tumor (encapsulated) from the skin.
5. Remove the encapsulated tissue and make an incision.
6. Live tumor tissue (white /pinkish) is on the outside of the tumor while the necrotic tissue is on the inside.
7. Make another incision parallel to the first cut (2 mm from the original cut).
8. Cut crosswise at 2 mm distance to make 2 mm squares.
9. Cut the top layer of tumor tissue off to get 2 $mm^3$ pieces (avoid blood vessels and necrotic tissue).
10. Place the tumor cubes into the tissue media and cut away any blood vessels and necrotic tissue if present.