ABSTRACT

STEPHENS, AMANDA MAE. Reduction of Cardiovascular Disease Risk Factors and Atherosclerosis in Male Syrian Golden Hamsters by Peanuts, Peanut Oil and Fat Free Peanut Flour. (Under the direction of Timothy H. Sanders.)

Studies have demonstrated the cardiovascular protective properties of peanuts and peanut oil through the reduction of low density lipoprotein cholesterol (LDL-C) while maintaining healthy levels of high density lipoprotein cholesterol (HDL-C). The cardiovascular protective effects of fat free peanut flour have never been evaluated despite the fact flour contains arginine, flavonoids, folates and other compounds beneficial to cardiovascular health. In addition, there is a growing body of evidence suggesting unsaturated fatty acids and arginine may promote bone strength. The objective of this study was to evaluate the effects of fat free peanut flour and other peanut components on plasma cholesterol risk factors for cardiovascular disease, atherosclerosis and bone strength in male Syrian golden hamsters. Seventy-six hamsters were randomly divided into four groups and each group was fed a different isocaloric diet for twenty-four weeks. Each experimental diet was a modification of the high fat and high cholesterol control diet. Modifications to the control diet were made by substituting fat free peanut flour (<0.5% oil), peanut oil or whole peanuts for similar metabolic components. Randomly selected hamsters from each diet group were euthanized at 0, 12, 18 and 24 weeks at which point blood plasma, aortas and femurs were collected. Plasma was analyzed for total plasma cholesterol (TPC) and lipoprotein distribution by high-performance gel chromatography. Aortic total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE), a metabolic atherosclerosis indicator, were determined as mg/g protein. Hamsters consuming diets containing fat free peanut flour, peanut oil or whole peanuts had significantly lower TPC and LDL-C than the control group.

There were no significant differences in HDL-C among any of the diet groups. Aortic TC, FC and CE were significantly lower in the fat free flour, peanut oil and peanut diet group hamsters compared to those in the control group. Results indicated that peanuts, peanut oil and fat free peanut flour in diets reduced blood chemistry risk factors and slowed the development of atherosclerosis, as indicated by low levels of CE, in male Syrian golden hamsters fed a high fat and high cholesterol diet. The results for bone strength were variable and overall the effects of fat free peanut flour, peanut oil and whole peanuts on bone strength were inconclusive.

Reduction of Cardiovascular Disease Risk Factors and Atherosclerosis in Male Syrian Golden Hamsters by Peanuts, Peanut Oil and Fat Free Peanut Flour

by
Amanda Mae Stephens

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APPROVED BY:

Dr. Jack Odle	Dr. Jonathan C. Allen Co-Chair of Advisory Committee

Dr. Timothy H. Sanders
Co-Chair of Advisory Committee

DEDICATION

I dedicate this work to my parents for their loving support.

BIOGRAPHY

Amanda Mae Stephens was born October 14, 1983 in Aurora, Colorado to Bob and Ann Stephens. She has one younger brother, Ben Stephens. Amanda moved to Houston, Texas in 1993. She spent the majority of her high school life as a goalkeeper on the soccer field and coaching youth soccer.

She graduated from Humble High School in May 2002 and began her Bachelor of Science at Jacksonville State University in Jacksonville, Alabama. Amanda was a four year scholarship athlete on the Jacksonville State Women's Soccer Team. She was the 2002 All-Freshman Team goalkeeper in the Atlantic Sun Conference and 2004 First Team All Conference goalkeeper and 2004 Defensive Player of the Year in the Ohio Valley Conference. At Jacksonville State, she also worked in the Academic Center of Excellence (ACE) tutoring chemistry, biology and mathematics. Amanda graduated with a B.S. in Chemistry and a minor in Nutrition and Foods in April 2006.

Amanda moved to Raleigh, North Carolina to pursue a Master of Science at North Carolina State University. She is currently pursuing a double major in Food Science and Nutrition. Outside of school and research she is a FIFA soccer referee and enjoys traveling. After graduation Amanda will hold a six month co-op position in Research and Development at Campbell's Beverage Department in Camden, New Jersey. She plans to return to North Carolina State University in January 2009 to pursue her PhD in Food Science under the direction of Dr. Timothy H. Sanders.

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LITERATURE REVIEW

Introduction

According to the American Heart Association (AHA) - 2008 update (1), cardiovascular disease (CVD) is still the leading cause of death in the United States. Over half of all CVD deaths are due to coronary heart disease (CHD). Epidemiological studies have indicated that peanut consumption decreases CHD by reducing low density lipoprotein cholesterol (LDL-C) without reducing high density lipoprotein cholesterol (HDL-C) (2). Peanuts, *Arachis hypogaea* L, are legumes that are commonly associated with nuts and mainly used for human consumption. They are a rich source of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), proteins, vitamins, minerals and bioactive materials (3). The weight of peanuts consumed per year in the United States is greater than all other nuts combined (4).

The Peanut

Peanuts are an important crop throughout numerous parts of the world. India, China and the United States are the world's premier peanut producing countries (5). In 2007, shelled peanut production in the United States was about 3.20 billion pounds (6). In the United States 99% of peanuts are grown in seven states: Georgia (41%), Texas (24%), Alabama (10%), North Carolina (9%), Florida (6%), Virginia (5%) and Oklahoma (5%) (7).

Peanut composition is dependent on cultivar, maturity, production environment and year (8). There are four types of peanuts based mainly on seed size. The different peanut types are runner, virgina, spanish and valencia. Runner-type peanuts have a medium seed size and the most prevalent, accounting for about 80% of peanuts produced in the United States, and frequently used in peanut butter. Virginia-type peanuts have the largest seed size

and account for about 15% of production. Spanish-type peanuts have small, round seeds and high oil content. They account for about 4% of production and are mostly used in peanut candy, as salted nuts and in peanut butter. Valencia-type peanuts have an intermediate seed size and are often used for boiling. They only account for about 1% of production (7).

Peanut oil

Peanut seed lipid content ranges from 35 – 54% for various varieties (9) with a mean of about 50% (10). Triacylglycerols (TAG) account for about 95% of peanut oil composition (11) with most of the fatty acids being MUFA (8, 12). Fatty acids commonly found in peanut oil are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidic (20:0), eicosenoic (20:1), behenic (22:0), lignoceric (24:0) and nervonic (24:1). Unsaponifiable material (phytosterols) is also found in peanut oil (8, 9). The total phytosterol content in peanuts is about 137 mg per 100 g of peanuts, comprised mainly of beta-sitosterol (13).

The ratio of the fatty acids is important in the stability of peanut oil. A higher ratio of oleic acid to linoleic acid leads to improved stability in peanuts (14, 15). High oleic lines of peanuts may contain up to 80% oleic acid and much less linoleic acid resulting in a large difference in the oleic:linoleic ratio compared to peanut lines with normal fatty acid profiles. Isleib et al. (16) compared peanuts from several cultivars grown variously in FL, GA, NC, and TX along with a high oleic peanut line that was also grown at all locations and determined there is a higher ($p<0.01$) amount of beta-tocopherol and lower ($p<0.01$) amount of alpha-tocopherol, total tocopherols, sucrose, raffinose, stachyose and total sugars found in the high oleic peanuts compared to regular peanuts. Overall, conventional genetic selection for high oleic concentration in peanuts has not result in undesired changes in peanut chemical composition (17).

Peanut protein

Peanut protein is found in flour (69% protein concentration), concentrates (70 - 85% protein concentration), or isolates (86 - 100% protein concentration). Peanut flour is primarily produced by using a hydraulic press, screw press or solvent extraction (18).

Peanuts contain 7.6 g of protein per one ounce serving which amounts to 15% of the Reference Daily Intake (RDI) (19). Raw peanuts contain 22 - 30% protein which is composed of globulins; albumins, arachin and conarachin (9) with about 90% of the protein globulins being arachin and conarachin (18, 20). While 10 amino acids are found in significant quantities in peanuts, their concentrations vary among cultivars (18). The average amino acid content and ammonia concentration of peanuts are reported in Table 1.0 (21).

Table 1.1: Amino Acid Compositions of Peanuts (21)

% (g/100g of protein)					
Amino acid	**Total[#]**	**Amino acid**	**Total[#]**	**Amino acid**	**Total[#]**
lysine*	3.49	glutamic acid	18.79	isoleucine*	3.18
histidine*	2.46	proline	5.16	leucine*	6.38
arginine*	12.39	glycine	6.00	phenylalanine*	5.19
aspartic acid	11.98	alanine	4.62	ammonia[+]	2.37
threonine*	4.33	cysteine	Trace	glutamine	na^
serine	4.81	valine*	4.21	asparagine	na^
methionine*	0.74	tyrosine	3.90	tryptophan	na^

[#] Ranges are reported as g / 100 g protein
* Essential amino acid
[+] Amino compound not an amino acid
^ Mean was not determined

While peanut protein is commonly used for fortification of foods (22), the nutritional quality of peanut protein is limited due to the low amount of essential amino acids: lysine (3.5%), tryptophan (1.0%), threonine (2.6%) and methionine (1.2%). Hoffpauir (9) reported that peanuts are an overall fair source of essential amino acids, and peanut protein is

generally considered to be a better sources of protein compared to other plant sources (22). Furthermore, the digestibility of peanut flour was suggested to be better than soy protein and comparable to animal protein by Hussein et al. (23).

Carbohydrate

The carbohydrate content in peanuts is comprised of starch (4%), cellulose (2%), pectin material (4%) and sugars (2-5%) (9). Starch content increases during maturation, peaks during mid maturity and then remains constant (24). Sucrose is the most prevalent sugar followed by glucose, fructose, arabinose and galactose. Sucrose increases while glucose decreases with maturity (8). Sucrose content can range from 2.86 - 6.35% in peanuts across different cultivars (25). A one ounce serving of peanuts provides about 2.4 g of dietary fiber (19).

Vitamins/Minerals

Peanuts are high in vitamins E and contain measurable amounts of vitamins A and D. The vitamin E content in one ounce of peanuts is equivalent to 29% of the RDI (19). The most active form of vitamin E found in peanuts is α-tocopherol. Grimm et al. (26) reported tocopherol levels to be as high as 565 ppm in Florunner peanut oil. Sanders et al. (27) reported tocopherol values to range from 419 - 644 ppm in peanut oil across six U.S. cultivars. While small amounts of vitamins K and C may be present in peanuts (9), Ahmed and Young (24) reported no evidence of vitamin K and only 5.8 mg per 100 g of vitamin C available in peanuts. Vitamin B is also present in the forms of folate, thiamine, pyridoxine and riboflavin (8). Thiamine is the main form and it is present at about 1 mg per 100 g in the seed and about 4 mg per 100 g in the skin (28).

The mineral content of manganese, copper, iron, calcium, potassium, magnesium, zinc and sodium was determined in peanut flour, peanut isolate and raw peanuts from Florunner, Starr and Florigiant varieties of peanuts grown variously in GA, VA, OK and TX. Peanut flour was determined to be overall higher in Ca, Fe, K, Mn, Cu and Zn than peanut protein isolate and raw peanuts. Mg and Na are lower in peanut protein isolate compared to peanut flour (29). Copper concentration can range from 23 - 323 ppm in raw peanuts (30). The amount of folate in one ounce of roasted peanuts is 10% of the RDI (19). Riboflavin and niacin are also present in peanuts with a concentration of about 3.1% and 15% respectively. Galvao, Lopez and Williams (31) reported 10 of 13 essential minerals in raw peanuts and peanut butter from the same lot of virginia-type peanuts (Table 1.2). The peanut butter contained 91.8% roasted peanuts, 5% sugar, 1.2% salt and 2% stabilizers. Data demonstrated that calcium content in a one ounce serving of raw peanuts or peanut butter was 1.7% of the Recommended Daily Allowance (RDA). A one ounce serving of peanuts supplies 15.0%, 12.7%, 6,2%, 4.9%, 4.0%, and 2.0% of Cu, Mg, Fe, P, Zn, and Ca, respectively.

Table 1.2: Mineral Content in Raw Peanuts and Peanut Butter (31)

Mineral	Raw Peanut*	Peanut Butter*
Ca	48	46.7
Cl	10.1	581
K	687	682
Mg	157	148
Na	8.39	388
P	137	188
Co	<0.022	0.11
Cr	0.16	0.26
Cu	0.74	0.67
Fe	1.99	1.62
Mn	1.7	1.41
Zn	3.29	2.91

*values reported as mg/100g

Skins and Hulls

The testa or skin on the outside of the peanut seed makes up about 3% of the total seed weight (9). The testa can be consumed but is generally removed before human consumption because they are bitter. Nutritionally, testae are high in fiber and contain bioactive constituents such as resveratrol, flavonoids, phytosterols, tocotrienols and saponins. Sanders, McMichael and Hendrix (32) reported a range of 0.02 -1.79 ug resveratrol per g of peanut across 15 cultivars and 3 peanut market types. The seed coats of runner- and virginia-types peanuts contained the highest resveratrol content, approximately 0.65 ug per g of seed coat. Raw testae contain large amounts of heat-labile thiamine (8). Peanut shells potentially inhibit lipoprotein lipase and pancreatic lipase (33).

Atherosclerosis

Atherosclerosis is degeneration, hardening, and loss of elasticity, in the coronary arteries. Atherosclerosis is one of the principle causes of CHD. According to the AHA – 2008 statistical update, CHD is responsible for over half of deaths caused by CVD which is the number one cause of death of people in Western societies (1). Several different factors may increase the risk for CVD. A person's heredity, gender and age (34) are considered to be uncontrollable risk factors. Tangier disease is an example of a hereditary disease which drastically reduces the level of circulating HDL-C (35). Other hereditary diseases may affect the way an individual produces, carries or stores cholesterol resulting in hypercholesterolemia (36). Premenopausal women have a much lower incidence of CHD than men and postmenopausal women. This is thought to be due to estrogen levels which potentially increase HDL-C thus providing protection against CHD (37, 38). Several CVD

risk factors, know as lifestyle factors, are controllable by the individual. Lifestyle risk factors include; obesity (39), hypertension, diets high in saturated fat and cholesterol, cigarette smoking and diabetes mellitus.

Atherosclerosis is a long-term process that starts in young individuals, but is usually only discovered when it becomes more severe later in life. The disease starts to develop when the lining of the tunica intima is stripped away or damaged; however, little is known about why or how the initial damage occurs. After the damage occurs, leukocytes start to attack and adhere to the damaged areas. Plaque, a combination of lipids, calcium and fibrous connective tissue, then accumulates on the proliferating area. Macrophage cells up-regulate receptors that take up modified LDL-C and release a variety of cytokines and growth factors. Macrophages accumulate cholesteryl ester and can be converted to foam cells (40, 41). At this stage, the diseased artery is said to have developed an atheroma or fatty streak (42). Fatty streaks are the earliest visible identifiable lesions of atherosclerosis (43). Plaque continues to build up, eventually progressing to blockage and hardening of the arteries which can result in hypertension. Soft plaque can suddenly rupture creating a thrombus stopping blood flow through the artery. Myocardial infarctions occur when a thrombus stops blood flow (42, 44).

Fatty streaks and lesions are commonly quantified using either a lipid stain (45) or unstained and then measured as percent area. Image analysis software or pixel determination is commonly used to determine percent area (46). The development of atherosclerosis can be chemically determined by measuring alterations in the metabolism of the arterial wall. Cholesterol can be esterified in the arterial tissue by lecithin-cholesterol-fatty acyl transferase (LCAT), cholesteryl ester synthetase, or fatty acyl-CoA-cholesterol acyl transferase (ACAT)

(47). Cholesteryl esters in arterial tissue are the end result of the esterification of cholesterol. Cholesteryl esters are one of the first metabolic precursors of atherosclerosis (48). In an atherosclerotic artery cholesteryl ester increases more than any other single component (49). Phospholipids and triacylglycerols also increase in atherosclerotic arteries (50), but that increase is not a prerequisite for the increase in cholesteryl ester (51). In an atherosclerotic artery, cholesteryl ester concentration may increase up to fifty times higher than in a non-atherosclerotic artery before any visible lesions are seen, while at the same time phospholipids and triacylglycerols increase only slightly (48, 52). Chobanian and Manzur (53) concluded that cholesterol esterification in experimentally induced lesions occurs in the same manner that it does in human atherosclerotic lesions. If a regression of atherosclerosis occurs, a reduction of cholesteryl ester concentration also occurs (54).

Atherosclerosis is an inflammatory disease (55). Antioxidants are thought to retard atherosclerosis development by reducing LDL-C oxidation and overall inflammation (56). Oxidized LDL-C is up taken more readily by macrophages and induces foam cell formation in the artery (57) and has been linked to increased CVD (58).

Diets high in saturated fat are more atherogenic than diets high in cholesterol because the saturated fats increase aortic cholesterol accumulation and increase the inflammatory marker IL-1β in the arterial wall (59). Some studies have indicated that cholesterol can act as an antioxidant (60, 61) but several studies contradict this hypothesis (58, 62-64).

Homocysteine has been indicated to increase the risk of CVD (65). Homocysteine is a metabolic byproduct of methionine and is usually converted to cystathionine by the co-enzyme, B_6. The development of atherosclerosis from homocysteine usually occurs when an individual is deficient in vitamin B_6 (66). In addition, folate may have an inverse relationship

on plasma homocysteine concentration (67). Not only do peanuts contain about four times the amount of folate as other nuts making up 60% of the RDA for folate in healthy, non-pregnant individuals, but they also have a B_6 to methionine ratio of 1.2 (42).

Blood Chemistry

The AHA states that hypertension and hyperlipidemia are two of the primary risk factors for atherosclerosis (1). Hyperlipidemia accelerates plaque formation increasing the risk of developing CHD (68). Growing evidence has indicated that the combination of high TAG and hyperlipidemia accelerate the development of CHD (69). According to the AHA, individuals in the general population who have total cholesterol levels above 240 mg / dL are at more than double the risk of developing CHD. However, a decrease in total plasma cholesterol (TPC) and LDL-C and simultaneous increase in HDL-C can reduce the risk of CHD (70). The AHA also reports that individuals who have HDL-C levels below 40 mg / dL also suffer from an increased risk of developing CHD (1). Therefore, a decrease in HDL-C is considered just as severe of a risk factor of CHD as an increase in LDL-C (35, 71, 72). The main stimulation of cholesterol esterification in the arterial wall seems to be the interaction with plasma LDL-C (73).

Diets high in MUFA and PUFA are associated with healthy cholesterol distribution profiles (74) commonly associated with protection against CVD (75, 76). Studies have indicated that diets high in MUFA protect against LDC-C oxidation better than diets high in PUFA (77-79). Oleic and linoleic acids are both effective at lowering TPC and LDL-C but oleic acid may be more protective against a reduction in HDL-C than linoleic acid (80).

Saturated fat intake is positively associated with an increase in very low density lipoprotein cholesterol (VLDL-C) and LDL-C concentrations.

A review article by Kubow (81) suggests the digestibility and rate of clearance of TAG is dependent of the arrangement of saturated fatty acids (SFA) in the molecule and that the delay of chylomicron remnants clearance is associated with atherosclerosis. Meals high in SFA cause a longer postprandial rise in TAG than meals high in PUFA (82). TAG metabolic enzymes are specific for sn-1 and sn-3 positions. TAG with SFA at the sn-2 position decreases the metabolism of chylomicrons and remnants compared to SFA at the sn-1 or sn-3 positions (81). Peanut oil only has 4 - 7% long chain SFA and almost all are present on the sn-3 position (83).

HMG-CoA reductase is the rate-limiting step in animals' cholesterol biosynthetic pathway and also plays a large role in cholesterol biogenesis in animals. Lalitha, George, and Ramasarma (84) demonstrated that in raw peanuts the rate limiting step of isoprenoid biosyntheses is the Mevalonate-5-pyrophosphate (MVAPP) decarboxylase. MVAPP decarboxylase is inhibited by phenolic acids but not by light or abscisic acid.

Health Effects of Peanut Oil

Linoleic acid, a highly prevalent n-6 fatty acid in peanuts, has demonstrated to have cardiovascular protective effects (85, 86), reduce platelet aggregation and vasoconstriction (87), reduce atrium fibrillation (88), retard atherogenic plaque formation (89, 90), reduce LDL-C oxidation (91) and reduce overall blood glucose (92). Peanut oil was shown to promote atherosclerosis by the high retention of TAG in one study in which very high concentrations (15%) of peanut oil were used (93). These results suggested that arachidic

and behenic acids and TAG structure in peanut oil was responsible for the atherosclerotic development in rabbits.

Vitamin E in peanut oil has been demonstrated to benefit heart health. Rein et al. (94) examined the effects of vitamin E on atherosclerosis in hamsters. In the study a hypercholesterolemic diet containing 2 g cholesterol and 90 g butterfat per kg with either 3 or 30 international unit (IU) of vitamin E was used. While the 30 IU vitamin E diet significantly decreased the amount of aortic fatty streaks a reduction of plasma lipid values was not observed.

Sun et al. (95) examined the effects of intravenous injection of β-carotene (25 mg/kg twice weekly), dietary supplement of α-tocopherol at 0.5%, and α-tocopherol plus β-carotene to the normally atherogenic diets fed to rabbits. Alpha-tocopherol may reduce oxidation of LDL-C by acting as a peroxyl radical scavenger (91). Beta-carotene decreased TPC and LDL-C concentrations with no effect on LDL-C oxidation (95). While α-tocopherol reduced the susceptibility of LDL-C to oxidation, there were no significant changes to TPC levels. The combined use of α-tocopherol and β-carotene decreased TPC, LDL-C, and susceptibility of LDL-C oxidation. All three experimental groups had decreased atherosclerotic lesion area and intimal thickness. While peanuts contain high levels of α-tocopherol, the concentrations of carotenoids are variable. Pattee and Purcell (96) reported that β-carotene is present in immature peanuts at about 60 ug and decreases with maturity.

Stampfer et al. (97) and Rimm et al. (98) examined supplemental α-tocopherol consumed by groups of women and men. Both studies indicated that an increase in α-tocopherol intake lowered the risk of CVD. Rimm et al. (98) reported men who took at least 100 IU of vitamin E per day for two years reduced risk of CHD by 27% compared to those

who did not take supplemental vitamin E. In addition, men who consumed 60 IU vitamin E reduced the risk of CHD by 24% compared to men who consumed minimal, inconsistent amounts of vitamin E. Stampfer et al. (97) reported that women who consumed vitamin E supplements for more than two years reduced the risk of CVD by 41%.

Many studies have examined the different fatty acid concentrations in oils. MUFA and PUFA have cardiovascular protective effects when substituted for SFA in the diet (80, 99, 100). Rudel, Parks and Sawyer (101) replaced PUFA with MUFA in the diet designed to promote coronary artery atherosclerosis (CAA) in monkeys. The five year study directly compared MUFA, n-6 PUFA and SFA. At the end of the study, monkeys fed MUFA diets had the lowest LDL-C / HDL-C ratio and highest CAA, at a level comparable to the SFA group. At the same time, the group fed n-6 PUFA had the lowest amount of CAA at the end of five years. The authors suggest that PUFAs prevent LDL-C composition changes in the artery, whereas MUFAs do not. In this study, the MUFA diet contained greater than 70% oleic acid whereas the PUFA contained greater than 70% linoleic acid. This study agrees with Mattson and Grudy's (80) finding that oleic acid is more protective against a reduction of HDL-C than linoleic acid.

In contrast, some studies have concluded that a dietary intake of MUFA instead of PUFA decreases atherosclerosis (102). Nicolosi et al. (103) observed that hamsters fed MUFA enriched sunflower oil had less development of early aortic atherosclerosis than those fed PUFA enriched sunflower oil. In an additional study, Raven et al. (77) compared specific fatty acids on plasma lipoproteins, oxidation of LDL-C and the potential of each fatty acid to cause atherosclerosis. Results indicated that MUFA reduces the risk of CVD by protecting against LDL-C oxidation. MUFA appears to prevent the lowering of HDL-C better than

PUFA. However, PUFA may decrease the incidences of LDL-C oxidation (104). Hargove et al. (105) demonstrated that diets high in MUFA sources with varying ratios of MUFA to PUFA do not increase the susceptibility of LDL-C oxidation. The best course seems to be a dietary ratio of MUFA and PUFA and low amounts of SFA to maintain healthy blood chemistries (76, 106)

Peanut Protein and Bioactive Fractions

A diet high in protein may provide a protective effect against CVD (107, 108). High dietary protein intake has been inversely associated with blood pressure in experimental animals (109) and humans (110). In addition, the exchange of protein for carbohydrates significantly reduced LDL-C and TAG without reducing HDL-C in humans (107, 111). It has been suggested that hyperinsulinemia increases pro-oxidants in the blood and may promote atherosclerosis (111). The source of protein affects the health benefits that protein use provides. Diets low in SFA and cholesterol with primarily plant protein have potential health benefits. Protein diets high in SFA and cholesterol and low in fiber have been significantly associated with CVD (108, 112).

Neucere et al. (113) investigated the effects that heating peanut protein had on nutritional value determined by weight gain in Sprague Dawley rats. Each of the ten treatments; wet heated 110°C-155°C or dry heated 110°C-155°C, were compared to a commercial casein diet. The peanuts were heated as intact seeds and then the fat was extracted with hexane. Heating did increase a trypsin inhibitor, but changes in essential amino acids were seen which had a larger impact on growth than the increase in trypsin inhibitor.

Hexane extracted peanut flour from various sources and other oil seeds were examined for the protein quality by evaluating the effect of diet on growth in albino rats and the microorganism *Tetrahymena pyriformis*. Peanut flour was determined to be deficient in methionine, isoleucine and lysine as well as low in threonine and tryptophan, based on values determined by the Food and Agriculture Organization (FAO) (114). Even though peanut flour has limited amounts of certain amino acids, the rats grew 43% better than the group fed a casein diet. The *T. pyriformis* peanut flour group grew 26% better than the casein and there was a 3-fold increase in growth of the microorganisms when the peanut flour diet was supplemented with methionine, isoleucine and lysine. Peanut flour diets supplemented with methionine, isoleucine and lysine did not improve overall growth in rats suggesting that methionine, isoleucine and lysine supplementation does not improve the protein nutrient content responsible for growth. In the same study, the addition of lysine to sunflower and sesame flours and the addition of methionine, isoleucine and valine to soybean flour increased growth performance in rats (115).

Arginine is an abundant amino acid in peanuts. Cooke et al. (116) studied the effects of arginine supplements on atherosclerosis in high cholesterol diets for male rabbits. The experimental group received a six-fold increase in daily arginine intake. The control group on a standard commercial diet exhibited no intimal lipid accumulation after staining with Oil Red O. The cholesterol group, 1% cholesterol and 0.9% arginine, had about 40% intimal lipid accumulation and the arginine group, 1% cholesterol and 3.15% arginine, had less than 10% intimal lipid accumulation indicating the cardio protective effect of arginine. The CVD protection of arginine was not evident in a long term human evaluation (117).

Peanuts contain significant amounts of phytosterols, mainly in the form of β-sitosterol (118). Flavonoid intake has been associated with a lower CVD risk (119). Defatted peanut meal had antioxidant activity at 180°C roasting for 60 minutes however; the hydrolysis of the peanut meal by proteases greatly increased its antioxidant activity (120).

Human Experimental Studies

Alper and Mattes (121) conducted a 30 wk crossover study with 15 adults (7 females, 8 males) with healthy blood lipid values to determine the effects of peanut consumption on serum lipids, magnesium and homocysteine. Energy intake did not significantly differ between the baseline and treatment periods. There was no significant change in mean TPC or total plasma homocysteine concentrations in the three treatments. Results also concluded there was a significant increase in dietary magnesium intake across treatments and the changes were significantly negatively correlated with reductions of TPC, TAG, and HDL-C in the free-feeding group. O'Byrne, Knauft and Shireman (122) performed a similar study with peanuts as the base for a low-fat, MUFA rich diet compared to a normal and low fat diet in hypercholesterolemic post menopausal women. The group that consumed peanuts had significant reductions in TPC and LDL-C. The study did report a slight decrease of HDL-C in the peanut diet group and speculated that it was because of the low amount of MUFA consumed overall in the daily diet. Supporting studies by Kris-Etherton et al. (74, 123) concluded that peanuts, a high MUFA food, lowered TPC by 10% and LDL-C by 14% without decreasing HDL-C concentrations in individuals. When trying to lower cholesterol, diets rich in MUFA foods may be preferable to diets low in fat because MUFA diets lead to more desirable blood chemistries (106).

An epidemiological study conducted by Fraser et al. (124) demonstrated that frequent nut consumption (> 4 servings / wk) resulted in a 52% reduction in fatal CHD events with a confidence interval of 36 – 76% and also had 49% fewer non-fatal myocardial infarctions with a confidence interval of 28 – 89% compared to subjects who consumed nuts rarely (< 1 serving / wk). The fatty acid profile in nuts may reduce the risk of CHD by not only having a favorable effect on CVD risk blood chemistries but also by providing antioxidant protection. Jiang et al. (125) studied the effect nuts had on inflammatory markers such as C-reactive protein (CRP), interleukin-6 and fibrinogen in people between 45 – 84 years old. The conclusions suggest frequent nut consumption is associated with lower inflammation indicated by a decrease in CRP, interleukin-6 and fibrinogen. Hypercholesterolemic men who took slow-release vitamin C and vitamin E with a meal had reduced atherosclerotic progression by about 26-33% in the six year Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (126). Copper may also have antioxidant properties, but a 1997 study (127) concluded copper supplements (2 mg/d for 4 wks) had no effect on CVD risk factors; plasma lipoprotein(a), VLDL-C and LDL-C oxidation, in adult hypercholesterolemic men.

The intake of soybean oil on CVD risk factors in humans was investigated by Lichtenstein (128). Thirty subjects (16 women and 14 men) over 50 years old with high LDL-C (> 130 mg / dL) consumed five different soybean oil diets to determine the effects on CVD risk factors. The diets (30% fat) contained soybean oil, low saturated fatty acid soybean oil, high oleic acid soybean oil, low α-linolenic acid soybean oil, or partially hydrogenated soybean oil. Not all fat came from a single variety of soybean. The high oleic acid soybean oil diet had four times more oleic acid than native soybean oil, which

represented about 85% of the total fatty acid makeup. Blood analyses concluded that the high oleic soybean oil diet had about a 40% higher concentration of MUFA than the other diets. HDL-C concentrations were significantly greater in individuals who consumed the high oleic soybean diet compared to individuals who consumed the soybean diet. Not including the partially hydrogenated soybean oil diet, no significant difference was found between groups in VLDL-C, TAG, lipoprotein(a), or CRP concentrations in all subjects.

Peanut oil may decrease CVD risk factors in men and women because of the potential ability to reduce appetite and body composition in healthy adults. Coelho et al. (129) conducted a feeding study in lean and overweight adults with peanut oil to examine effect on energy intake and expenditure, body mass index (BMI), body composition, appetite, and plasma lipids. The results concluded no changes in saturated fatty acid composition and an increase in MUFA and PUFA. By the end of the eight week study, plasma vitamin E concentrations had significantly increased in both groups. Median body weight significantly increased by about 5 lbs in the overweight group, but in the lean group body weight did not significantly increase throughout the study. Resting energy expenditure significantly increased by about 5% in the overweight group but not in the lean group. HDL-C increased significantly and LDL-C decreased significantly in overweight individuals at four weeks but not at eight weeks. Other studies (74, 121, 124) have also found that the increase in caloric intake by consumption of nuts does not parallel weigh gain in humans probably because peanuts have a high satiety value.

Experimental Rodent Feeding

Rodent studies provide extensive knowledge and quicker results than human studies due to the inability to visually examine internal systems in health studies. The length of

human lifespan and inability of tight control over daily factors is another reason to use experimental rodents. The need for digestion, absorption and metabolism of the dietary nutrients precludes the use of in vitro systems.

CVD has been studied in several animals including pigeons (130), golden Syrian hamsters (131, 132), monkeys (50), rabbits (133), rats (134), and mice (135), but all experimental models are comparable to the formation of atherosclerosis in humans. Hamsters closely resemble humans with respect to rates of hepatic cholesterol synthesis (136, 137) and lipoprotein distribution in that they carry the majority of circulating cholesterol as LDL-C (94, 138, 139). They also respond to high saturated fat and high cholesterol diets much like humans (131, 140, 141). Rats and mice do not naturally have cholesteryl ester transfer protein (CETP) activity and their major plasma lipoprotein is HDL-C (142).

German et al. (143) examined cholesterol distribution in four groups of golden Syrian hamsters fed diets differing in dietary fiber and saturated fat. Blood was drawn by cardiac puncture and plasma analyzed by ultracentrifugation and HPLC. A superpose 6HR FPLC column was used for parallel plasma lipid analyses. Two groups of hamsters were fed α-cellulose with either olive oil or fish oil. The fish oil fed group had significantly higher total cholesterol than hamsters fed olive oil. The other two groups of hamsters were fed barley fiber, a hypocholesterolemic fiber, with either olive oil or fish oil. Unlike in the α-cellulose group, fish oil did not affect the total cholesterol compared to olive oil; however, although total cholesterol concentrations were lower, the HDL-C to LDL-C ratios were not favorable in this study. Hamsters fed diets that contained fish oil had higher TAG concentration compared to groups fed olive oil. The amount of total cholesterol in the diet affects lipoprotein remodeling in hamsters, and dietary fiber intake may help slow lipoprotein

remodeling when an individual has a change in total cholesterol. Dietary fiber did not appear to affect lipoprotein remodeling when the HDL-C / LDL-C ratio changed.

Golden Syrian hamsters are good animal models for atherosclerosis (131, 144). Using golden Syrian hamsters, Nicolosi et al. (43) demonstrated that LDL-C oxidation is inversely associated with aortic total and esterified cholesterol concentration. The research further indicated no significant associations between lipoprotein cholesterol values in hamsters fed high oleic, mid oleic, or high linoleic oil. Fatty acid profile seems to play a large role in oxidation of LDL-C and PUFA oxidize faster than MUFA. Hamsters fed higher MUFA diets had lower early aortic atherosclerotic lesions.

Angiotensin (Ang) II and cardiac chymase concentrations are up-regulated after myocardial infraction. Ang II promotes cardiac remodeling via angiotensin converting enzyme (ACE). Chymase also plays a role in cardiac remodeling but is not regulated by ACE inhibitors and can cause detrimental effects if not controlled. Chymase is an Ang II converting enzyme in humans, hamsters, monkeys and dogs but in rats and rabbits it acts as an Ang I degrading enzyme (145).

Differing amounts of cholesterol and cholate were added to a modified semipurified AIN-76A rodent chow to examine atherosclerotic lesion development and hyperlipidemia in mice (64). The use of a semipurified diet allowed precise control of nutritional factors and maltose dextrin was added to prevent high lipid concentrations from "settling out" in the pellet rodent chow. Data were collected on four groups of rats after twelve weeks of diet consumption. Diet 1 was the control (10% lipid Calories); diet 2 was high fat (40% lipid Calories) and moderate cholesterol (0.5% by weight); diet 3 was high fat and high cholesterol (1.25% by weight); diet 4 was high fat, high cholesterol and 0.5% sodium cholate. Lipid

plasma was collected at zero, six and twelve weeks and plasma analyses were performed with a Technician RA1000. Atherosclerotic lesions were analyzed by image analysis software (NIH Image) on the aortas stained with oil red O (ORO) staining. The procedure for ORO staining was modeled after a method reported by Nunnari et al. (45) which was used with hypercholesterolemic rats. The data demonstrated that the ORO stain at certain concentrations can be used to collect reliable quantitative atherosclerotic lesions measurements in small rodents. Several different studies have also reported the successfully use of ORO to quantify atherosclerosis (45, 64, 90, 94). Nunnari et al. (45) also evaluated TPC and TAG concentrations. Plasma measurements at six weeks indicated an increase in both TPC and TAG concentrations in all three groups compared to the control. There was a slight difference between diet 2 (high fat, moderate cholesterol) and diet 3 (high fat, high cholesterol), in that diet 2 had greater TAG levels and lower cholesterol levels. There was a significant difference in diets 2 and 3 versus diet 4 (high fat, high cholesterol and cholate). The final TPC and TAG concentrations were taken at twelve weeks. Results indicated diet 1 had significantly lower total cholesterol than diets 2, 3, and 4. VLDL levels increased in the three experimental groups with HDL decreasing proportionally per diet. Diet 4 produced the largest change in cholesterol levels but triglyceride levels did not change significantly. There was a significant difference in cholesterol levels between diets 2 and 3 at twelve weeks. Fast protein liquid chromatography-gel-filtration was used to determine cholesterol in each fraction. Atherosclerotic lesions were examined on the aortic arch, the descending thoracic aorta, the abdominal aorta and the total aorta. Serum liver enzyme levels and liver derived products were measured at twelve weeks. The tests were comparable between all groups indicating the liver parenchyma and biliary systems were not seriously damaged by any of

the diets. Fatty degeneration of the liver was found in groups 3 and 4. The greatest fatty changes were observed in group 4. No histological evidence of hepatocyte necrosis, fibrosis, cirrhosis or apoptosis damage was found in any group. Gallstones were present only in group 4, which contained cholate.

A thirteen week rat feeding study (146) concluded that rubber seed oil does not have toxic potential and may be used for edible purposes. This study used peanut oil as the control. Ten percent of each oil was used as the sole source of fat in the assigned diets. There were no significant differences in body weight, food consumption, food efficiency of rats, organ weights, hemoglobin and cell counts between the two groups. No abnormal histopathological lesions were found in either group. There was a significant difference in fecal fat content between the two groups. The absorption of fat in the group fed peanut oil was 94.3%; this value agrees with other published literature (147) for rat efficiency of fat absorption.

Diestschy, Turley and Spady (148) compared the role of the liver in the cholesterol maintenance in several different animal species, including humans. Mice, golden Syrian hamsters, guinea pigs, dogs and several species of monkeys produce approximately 10 mg / day per kg less cholesterol for each 10-fold reduction in body weight compared to humans. Sprague-Dawley rats and New Zealand white rabbits produce greater amounts of cholesterol than the "main stream" whole body cholesterol synthesis described in this review article. Cholesterol is needed during growth and maturity of all animals. The older spraque-dawley rat and golden Syrian hamster synthesize about 60% less cholesterol per kg of body weight than when they were younger and sexually mature (134, 148).

Cintra (149) examined the effects of different high fat diets on body weight, liver cholesterol deposits and fatty acid profiles in male Wistar rats. The study consisted of a control (AIN-93) diet and 5 high fat diets which contained 10% of soybean, trout, flaxseed, chicken skin or peanut. The high fat diets include 1% cholesterol, 5% lard. Each diet was kept isocaloric by adjusting the amount of corn starch and protein (fat source). The peanut-fed group had significantly lower body weight than the other groups. Overall, the peanut-fed rats had the lowest food efficiency ratio.

Bone Health

Storing bone at -20°C for up to 100 days does not affect the mechanical properties (150-152). Osteoblast-like cells are thought to synthesize and secrete an insulin growth like factor 1 (IGF-I), which is a regulator in bone metabolism (153, 154). Pharmacological doses of arginine increase IGF-I at the mRNA and protein level in mouse osteoblast-like MC3T3-E1 cells which may directly or indirectly stimulate bone growth (154). Reich and Gefen (150) used trabecular (spongy) bone in an *in vitro* study to test the loss of critical mass and the effects on whole bone. They concluded that a loss of 10% of trabecular bone results in an overall significantly weaker total bone. They tested the strength of chicken femurs after removing cartilages, tendons and marrow. Marrow was removed using water and air pressure and special care was given not to damage the epiphysis trabecular bone. Bones were stored at -20°C and later brought to room temperature on the day of testing. A pendulum-based impact testing system was used (Figure 1.1). The Charpy hammer strikes the epiphysis that is held in place by two jigs. If the bone did not fit on the jigs, it was cut transversally at the end of the diaphysis region. A strain gauge attachment was placed on the

bone to measure voltage to break the bone. The bone was subject to a 3-point bending impact at a contact speed of 1.85 meter per sec and impact energy of 0.9 Joule.

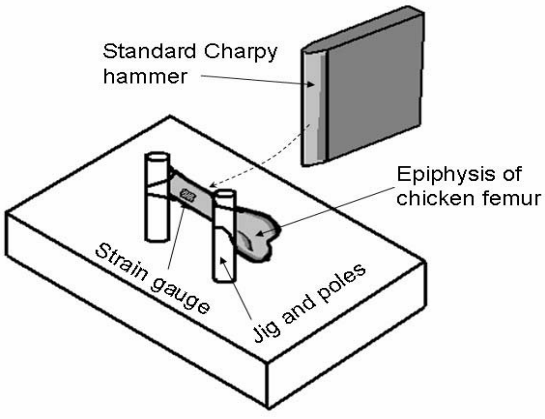

Figure 1.1: Pendulum Strength Test. The test is commonly used to replicate the natural motion of falling and stress applied to the bone (150).

Studies (155-157) have concluded that statins inhibit HMG-CoA reductase which may increase bone formation and reduce the risk of osteoporosis. A study (155) examined the effect of lovastatin, a lipid lowering drug, on HMG-CoA reductase. The results indicated a 30 - 60% and 150% increase in trabecular bone volume after 5 days and 4 weeks, respectively.

The relationship between bone and muscle systems in ten different strains of mice were evaluated by the humerus breaking strength determined with an Instron Mechanical Tester (158). The length of the probe was 4 mm and the rate was applied at 10 mm per minute. The probe was rounded to prevent shearing and cutting. The humerus was placed with the anterior surface upwards and laid horizontally so that the probe was at the mid-shaft of the humerus. Strength was recorded in Newtons at the maximum force of failure.

Another study (159) tested the point of maximum failure as a measurement of femur strength using an Instron model 4204 (Figure 1.2). The left and right femur of each mouse was radiographed to measure bone geometry and then frozen. Femurs were thawed the day of testing and length was measured with vernier calipers and mid-shaft diameter was measured with a non-contact laser micrometer. The non-embedded length was found to be about 60% of the shaft length. The femurs were mounted with periodic wetting of the sample with phosphate buffered solution onto a torsion tester. Each femur was tested with a 5 kg load cell at 0.13 rads per second.

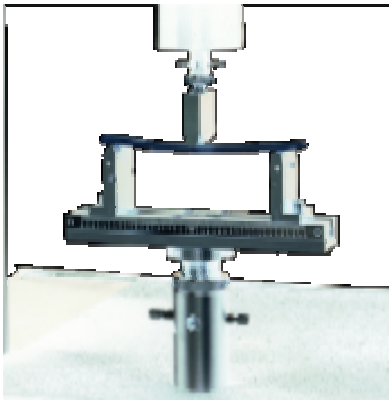

Figure 1.2: Instron 3-Point Bend. The sample is held at two points and the third point applies force until fracture. The rate that force can be applied and distance between the points can be controlled (160).

Chevalley et al. (154) demonstrated arginine stimulated IGF-I production which played a role in stimulation of bone formation through osteoblast-like cells in mice. Nitric Oxide (NO) is synthesized from L-arginine nitric oxide synthase enzymes and may have a several effects of physiological functions in the bone. NO seemed to help push bone

remodeling towards a positive balance (161-163). In addition, Turner et al. (162) demonstrated that adding L-arginine reverses the inhibitory effect of aminoguanidine on trabecular bone formation, emphasizing of the importance of the L-arginine / NO pathway on bone formation during bone remodeling.

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CHAPTER 2:

REDUCTION OF THE DEVELOPMENT OF ATHEROSCLEROSIS BY PEANUTS, PEANUT OIL AND FAT FREE PEANUT FLOUR

ABSTRACT

Studies have demonstrated the cardiovascular protective properties of peanuts and peanut oil in lowering low density lipoprotein cholesterol (LDL-C) without decreasing high density lipoprotein cholesterol (HDL-C). The cardiovascular protective effects of peanut flour have never been evaluated even though the flour contains arginine, flavonoids, folate and other compounds that have been linked to cardiovascular health. The objective of this study was to evaluate the effects of fat free peanut flour and other peanut components on cardiovascular disease (CVD) risk factors and atherosclerosis in male Syrian golden hamsters. Seventy-six hamsters were randomly divided into four groups which were fed different high cholesterol, isocaloric diets. Modifications for the experimental diets were made by substituting fat free peanut flour (<0.5% oil), peanut oil or whole peanuts for components with similar metabolizable energy in the control diet. Tissues were collected from randomly selected hamsters in each diet group at 0, 12, 18 and 24 weeks. Total plasma cholesterol (TPC) and lipoprotein distribution were determined by high-performance gel chromatography, and aortic total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE) were determined by gas liquid chromatography. Peanut component diet groups had significantly lower TPC, LDL-C and very low density lipoprotein (VLDL-C) than the control group and HDL-C was not significantly different among any of the diet groups. Peanut component diet groups had significantly lower aortic TC, FC and CE than the control diet group. Results demonstrated that peanuts, peanut oil and fat free peanut flour reduced TPC and LDL-C while maintaining HDL-C. Results also indicated that peanuts and peanut components slowed the build-up of aortic CE, thus suggesting a reduction in the development of atherosclerosis in hamsters when consuming an atherosclerosis inducing diet.

INTRODUCTION

Peanuts (*Arachis hypogaea*) are legumes but are generally considered as nuts. The weight of peanuts consumed per year in the United States is greater than all other nuts combined (1). Peanuts are comprised of about 50% lipid, 30% protein and 20% carbohydrates and are rich in vitamins, minerals and bioactive materials. They contain several heart healthy nutrients including monounsaturated and polyunsaturated fatty acids such as oleic and linoleic acid, potassium, magnesium, fiber, α-tocopherol, folates and flavonoids.

The American Heart Association (AHA) has indicated that cardiovascular disease (CVD) is still the number one cause of death of Americans in the 2008 statistical update (2). Hypercholesterolemia is one of the primary risk factors for CVD. Human clinical trials have indicated that peanuts reduce total plasma cholesterol (TPC) and low density lipoprotein cholesterol (LDL-C) while maintaining high density lipoprotein cholesterol (HDL-C) (3). The United States Food and Drug Administration (FDA) affirms the health claim that peanuts may reduce the risk of heart disease when consumed daily in small amounts (4). Peanuts and peanut oil have also been demonstrated to reduce CVD risk factors in epidemiological studies (3, 5) and human clinical trials (5-8). However, there is minimal literature on the effects of peanuts and peanut oil on atherosclerosis in rodents and there appears to be no published literature on the cardiovascular health affects of fat free peanut flour.

CVD is an inflammatory disease (9). Antioxidants have the potential to retard the early stages of atherosclerotic development by reducing the oxidation of LDL-C (10). There are several potential antioxidant compounds in peanuts. Vitamin E has been demonstrated to

reduce CHD in both men and women (6, 8). Phytosterols also directly reduce LDL-C oxidation (11). Peanuts are a source of vitamin C, magnesium, copper, arginine and niacin, folates and fiber which may be linked to cardiovascular health. Obesity has been linked to an increased risk of CVD, hypertension, hyperlipidemia and stroke (12). Peanut oil has a mild satiety value (13) and peanut consumption has either had a inverse or no affect on body weight when recommended servings are consumed, even when nut consumption increases the overall daily caloric intake (14).

The development of atherosclerosis can be chemically determined by measuring the alterations in the metabolism of the arterial wall. Cholesteryl ester (CE) is a strong indicator of the development of atherosclerosis because it is one of the first metabolic changes that occurs in diseased arteries (15). CE can increase as much as fifty times higher in atherosclerotic arteries compared to healthy arteries (15, 16). Metabolic changes that occur during the early stages of atherosclerosis development also include an increase in phospholipids and triacylglycerols (TAG), but not nearly at the same concentration as CE (17, 18). CE concentrations also decrease when there is a reduction in atherosclerosis (19).

Hamsters were used in this study because of their cardiovascular similarities to humans. Nistor et al. (20) concluded hamsters are susceptible to diet induced atherosclerosis, their major plasma cholesterol carrier is LDL-C, and therefore hamsters are a good model for experimental atherosclerosis. Hamsters also closely resemble humans with respect to rates of hepatic cholesterol synthesis (21) and response to high saturated fat, high cholesterol diets (20, 22). Another benefit of using hamsters is that they are also small, easy to handle, and relatively inexpensive. Males are generally utilized to reduce the possibility that cardiovascular protective effects were caused by estrogen and not components in the diet.

Estrogen potentially increases HDL-C providing a natural benefit to heart health (23, 24). The objective of this study was to examine the protective effects of fat free peanut flour against CVD risk factors and the effects of fat free peanut flour, peanut oil and whole peanuts on the development of atherosclerosis in golden Syrian hamsters.

MATERIALS AND METHODS

Animal Care

Eighty male Syrian golden hamsters, 6-weeks-old and weighing between 80 – 100 g, were purchased from Harlan Inc. (Indianapolis, IN). Hamsters were housed in individual cages with NEPCO (Northeastern Products Corporation) wood chip bedding (Warrensburg, NY), wire top covers and isolator top lids at the Biological Research Facility (BRF) at North Carolina State University (NCSU). Food was placed in the wire top covers for stimulation and on the bottom with the bedding for natural-type consumption.

The hamsters were maintained on a 12/12 hr light/dark cycle in an environmentally controlled room. The cages were cleaned weekly while food, water and general visible health were checked daily. Seventy-six hamsters were randomly assigned to one of four diet groups before the start of the experiment. Hamsters were weighed weekly until age 15 weeks and then bimonthly. All procedures were approved by North Carolina State University Animal Care and Use Committee (Raleigh, NC). IACUC protocol #06-104-B approved August, 2006.

Diets

The four experimental diets were fed *ad libitum* with clean water. The control diet was a modification of the AIN-76A Clinton/Cybulsky Cholesterol Series #2 semipurified diet for rodents prepared by TestDiet® (Richmond, IN). The control diet was designed to increase blood chemistry risk factors and induce atherosclerosis and its risk factors in hamsters. There were three experimental diet groups; whole peanuts, peanut oil or fat free peanut flour. All diets were adjusted to the caloric level of the control diet by substituting peanut products for cocoa butter, soybean oil, casein, corn starch, sucrose, maltodextrin, or cellulose based on metabolizable energy (fat is 9 kcal/g, carbohydrate and protein are 4 kcal/g) and not by weight.

High oleic, dark roasted peanut products [whole peanuts, peanut oil and peanut flour (12% fat)] were a gift from Golden Peanut, LLC (Alpharetta, GA). The three peanut products were collected from the same lot either before processing or after processing. During processing the oil was mechanically removed from roasted peanuts. The pressed peanut material was hexane extracted to produce a 12% oil flour. The 12% oil peanut flour was further hexane extracted by Avoca Farms, Inc. (Merry Hill, NC) to about 0.5 % oil.

The hamsters were fed their original commercial diet (Purina 5001) for one week and experimental diets were substituted for the commercial diet incrementally during the next five days. The hamsters received 25% experimental diet and 75% commercial diet on day one, a 50/50 mix on day two, and a 75/25 mix on day three. Hamsters in all diet groups were consuming 100% of their designated experimental diet by day five. The 24 week study was initiated when all hamsters were consuming 100% of the experimental diet when the animals were 8 weeks old.

Peanut Component Analysis

Fatty acid analysis was determined for the whole peanuts and peanut oil received from Golden Peanut, LLC (Alpharetta, GA). A sample of peanut oil and whole peanuts were taken from the individual containers that were sent to TestDiet® (Richmond, IN) for production of the experimental diets. Before fatty acid analysis, the whole peanuts were ground in a Braun coffee mill (Gillette Inc., Boston, MA) and then mechanically pressed to remove oil. Both samples of the oils were kept overnight in a dark area.

Fatty acids were determined in triplicate as described by Bannon et al. (25). One drop of oil (ca. 30 mg) was collected in a screw capped tube, mixed with 1 mL 0.5 M methanolic potassium hydroxide and placed in an 80°C water bath for 5 minutes. The samples were allowed to cool and then 1 mL BF_3 (14 % in methanol) (Sigma, St. Louis, MO) was added as the catalyst for the formation of the methyl esters and samples were heated in the same water bath for 10 minutes. After the samples cooled slightly, 1 mL of water and then 1 mL of hexane were added to the tube which was then vortexed. The tubes remained at room temperature until layers formed. The top layer containing the methyl esters was removed, swirled with sodium sulfate and transferred to a Gas Chromatography (GC) vial for analysis.

Analyses were conducted on a Perkin Elmer Gas Chromatograph equipped with a Flame Ionization Detector (GC-FID) and a SGE BPX70 column (30 m x 0.25 mm ID x 0.25 um dry film, Phenomenex, Torrance, California). The injection was split at a rate of 73.0 mL per min at 265°C. The carrier gas was helium at 1.82 mL per minute. The initial temperature was 60°C, held for 2 min and then ramped at 10°C per min to 180°C and then at 4°C per min until 235°C. The total run time was 27.7 minutes. Peak identifications were

made by matching retention times with authentic fatty acid methyl ester standards. The standards used were Kel-Fim FAME-5 Standard purchased from Matreya, LLC. (Pleasant Gap, PA) and GLC-21A purchased from Nu-Check Standards (Elysian, MN).

Protein content and amino acid profiles were measured in triplicate for whole peanuts and peanut flour before they were sent to TestDiet® (Richmond, IN) to manufacture the experimental diets. Protein was determined by measuring percent nitrogen on a 2400 CHN Elemental Analyzer (Perkin-Elmer Corp., Norwalk, CT). Percent nitrogen was determined based on the weight of the sample and then a conversion factor of 5.46 was used to calculate the total protein.

The amino acid analysis was conducted in duplicate for whole peanuts and fat free peanut flour using a modification of the method published by Hagen et al. (26). The whole peanuts were ground to a fine consistency in a Braun coffee mill (Gillette Inc., Boston, MA). The ground peanuts and fat free peanut flour were transferred into cellulose thimbles for Soxhlet extraction. Hexane was refluxed through the Soxhlet tubes for 6 hours. Duplicate extracted samples were hydrolyzed for amino acid analysis using a modification of the methods of Hagen et al. (26). A small amount of each sample was removed and oxidized in performic acid so that the sulfur groups of methionine and cysteine were protected and then the remaining sample was hydrolyzed using 6 N HCl containing 0.1% phenol in a heating block for 18 hr at 110°C. Methionine and cysteine samples were held an additional 30 min in an ice bath and then hydrolyzed for 18 hr at 110°C with 6 N HCl containing 0.1% phenol. An aliquot of the hydrolysate was then derivatized using AccQ·Floor™ reagent as outlined in the Waters' (Waters Corp., Milford, MA) manual (WAT052874, Rev 0). A Summit Model High Performance Liquid Chromatography (HPLC) (Dionex Corp., Sunnyvale, CA) with a

Waters Acc·QTag column (C_{18}, 4µ, 150 mm x 3.9 mm) was used in the analyses of derivatives.

In the HPLC analysis, eluant A was an aqueous phosphate buffer plus triethylamine solution from (Waters Corp., Milford, MA) diluted with deionized water. Eluant B was acetonitrile diluted with deionized water (60:40, v/v). The gradient for analysis was initially 0% B and increased to 33% in 32 min at a flow rate of 1.0 mL per min and then held for 1 minute. Post run equilibration of the column was 13 min at 0% B. The total injection volume was 20 µL, the total run time was 50 min and the column temperature was 37°C. Detection was by fluorescence with the excitation wavelength set to 250 nm and the emission wavelength set to 395 nm. The samples were spiked with an internal standard, alpha-aminobutyric acid (Sigma Chemical Corp., St. Louis, MO). A mixed standard (Pierce Biotechnology, Inc., Rockford, IL) that contained all amino acids except tryptophan was analyzed with sample aliquots to construct an analytical response curve over a range of 0.2 – 1.0 µg per mL.

Tryptophan is acid sensitive and had to be determined from a different sample set in duplicate. The samples were digested in 4.2 M NaOH for 18 hr at 110°C and analyzed by HPLC without any additional derivatization. Tryptophan was determined by Summit HPLC (Dionex, Sunnyvale, CA) with UV detection at 254 nm and a LiChrospher 100 RP-18 column (250 mm x 4.6 mm, 5 µm, Alltech Corp., Deerfield, IL) at 30°C. The mobile phase was 90% 0.02 M phosphate buffer, pH 3.3 and 10% acetonitrile. The runs were isocratic at 1.0 mL per minute. The standard curve was determined with 1 - 100 ppm of L-Tryptophan Authentic Standard (Pierce Biotechnology Inc., Rockford, IL) in water.

Diet Analyses

Fatty acid profile of each diet was determined in triplicate by GC. Diets were ground into a powder with a Braun coffee mill (Gillette Inc., Boston, MA) and moisture was measured according to AOAC 934.01 (Official Methods of Analysis, Padmore, J.M. 1990). Exact weight of sample (ca. 2 g) was recorded and then transferred into a ceramic bowl. The samples were dried to a constant weight for 7 hr at 95°C and stored overnight in a desiccator. Fat was extracted from the diets by AOAC 954.02 animal feed method (Official Methods of Analysis, Helrich, 1990). Alcohol, 2 mL, was added to a Mojonnier tube with exact weight samples until all particles were moist to prevent clumping with the addition of acid. Ten mL of HCl were added to each sample and then the samples were shaken frequently for 30 min in an 80°C water bath. The samples were cooled to room temperature and alcohol was added until each sample volume reached the Mojonnier tube constricted portion. A rubber stopper, cleaned with alcohol before use, was used to seal the Mojonnier tubes. Twenty-five mL of ether was added and each sample was shaken vigorously for 1 minute with pressure released periodically. The samples were shaken vigorously for an additional 1 min after 25 mL redistilled petroleum ether was added. Samples were allowed to settle and then the ether-fat solution was poured off through a cotton pledget to prevent unwanted solid particles. The Mojonnier tubes were rinsed twice with ether and each time the ether-fat solution was poured off. Ether was evaporated and the samples were dried for 90 min at 100°C. The samples were placed in a desiccator and cooled to room temperature in and weighed.

The samples were analyzed on a Perkin Elmer GC with an Autosampler XL (Waltham, MA) with a Restek, RT-2560 column (100 m x 0.25 mm ID x 0.2 μm dry film, Bellefonte, PA). The initial temperature was 100°C and ramped at 3°C per min to 250°C.

The total run time was 67.0 minutes (27). A moisture correction factor was used in the calculation of percent of each fatty acid (28).

Plasma Analyses

Hamsters were euthanized by CO_2 at weeks 0, 12, 18 and 24. The hamsters were desanguinated by cardiac puncture and approximately 2 mL of each sample was anticoagulated in heparinized tubes (7% EDTA) while the remaining blood collected (ca. 1 – 3 mL) was stored in non-heparinized tubes. Tubes were placed on ice for less than 7 hrs until all samples were collected. Heparinized tubes were tempered to room temperature and then centrifuged at 6000 x g for 10 min. Plasma was decanted and 300 μL were delivered to the Department of Pathology, Lipid Science Division at the Wake Forest University School of Medicine (Winston Salem, NC) for cholesterol analyses. Total plasma cholesterol was measured and lipoprotein particle distributions were determined by high-performance gel chromatography (HPGC). A 0.9% saline solution with 0.01% EDTA and 0.01% azide at 0.4 mL per min was run onto a Superose 6 10/300 column (GE Healthcare, formerly, Pharmacia) for online cholesterol distributions. The effluent from the column was split and half was mixed with total cholesterol reagent (Cholesterol H/P, Roche Diagnostics) being pumped at 0.125 mL per minute. The area percent of each fraction (VLDL, LDL, and HDL) was determined by the chromatography software (29).

Collection of Aortas and Aortic Lesion Analysis

The heart and at least 3 mm of the aorta were removed from euthanized hamsters. A transverse cut was made at the inferior tip of the heart to expose the left ventricle. A thin

wire was fed through the left ventricle and up into the aorta. The aorta was dissected from the heart and both were frozen at -60°C. The aortas were tempered to 20 - 22°C in buffered saline solution and then removed from the metal wire. The aortas were placed on the platform of a dissecting microscope and carefully cleaned by removing all adventitia tissue and then stored in 10% neutral buffered formalin for subsequent processing.

The atherosclerotic development was quantified for each hamster in this study at week 0, 12 and 18 by aortic cholesterol ester concentrations, measured as mg per g protein. The wet weight of the aortas was recorded after being gently blotted to remove exterior formalin. The aortas were placed in 16 x 100 mm, screw-cap, round-bottomed glass tubes containing chloroform-methanol, 2:1 (v/v) containing 20.5 µg of 5-alpha-cholestane as an internal standard, and the lipids were extracted. The lipid extract was separated by filtration from the tissue dried under N_2 at 60°C and then dissolved in hexane. Analyses of free and total cholesterol were carried out with 2 injections per sample on a DB 17 (15 m x 0.53 mm ID x 1 µm) gas liquid chromatography column (J&W Scientific, Folsom, CA) at 250°C and installed in a Hewlett-Packard 5890 GC equipped with a Hewlett-Packard 7673A (Hewlett-Packard Co. LP., Houston, TX) automatic injector using online column injection and a flame ionization detector. Cholesteryl ester was calculated as the difference between free and total cholesterol, as measured before and after saponification and reextraction of the nonsaponifiable sterol into hexane. The weight of cholesteryl ester was determined by a conversion factor of 1.67. The tissue was then digested and dissolved in 1 N NaOH, and total protein was determined by the Lowry protein assay (30).

The aortas collected at week 24 were stained with Oil Red O (ORO) for aortic lesion analysis. The aortas were removed from the cadaver, stored and cleaned as described for

week 0, 12 and 18. Aortas were washed with phosphate buffered solution (PBS) and then stained for 1 hr in ORO. They were rinsed and mounted onto slides. Lesions were examined using an Olympus Vanox Upright Microscope with Adobe Photoshop & NIH Image Software (Duarte, CA). Evaluations were confirmed by Department of Pathology, Lipid Science Division at the Wake Forest School University of Medicine (Winston Salem, NC).

Statistical Methods

SAS software (Cary, NC) was used for all statistical evaluations. The plasma cholesterol and aortic cholesterol were analyzed as a two variable study accounting for time and diet effects. The plasma cholesterol results had inhomogeneity variance requiring transformation. The log transformation of the plasma cholesterol measurements was used to correct for the variance. The log transformation of aortic TC, FC and CE plus one was used for the end point of atherosclerosis because some measurements at time points were zero. Due to unpredicted factors such as spontaneous death, instrumental malfunction and sample loss there was a slight unbalance in the number of samples in each diet groups for 24 weeks. The least squared means (lsmeans) was reported to estimate the mean if the sample had been balanced. The significant differences among treatment means was assessed by Tukey's test and differences of $p < 0.05$ were considered significant (31).

RESULTS AND DISCUSSION

Hamster Weight Gain

Body weight was not significantly different among the dietary treatments (Figure 2.1). All hamsters, except for two from the peanut oil diet group, were utilized as planned.

The first animal that died was euthanized because there was a greater than 15% loss in total body weight. The second hamster that died unexpectedly tested positive for the gram negative rod *Proteus* sp. *Proteus* sp. is a water contaminant however; signs of infection were not displayed in other animals.

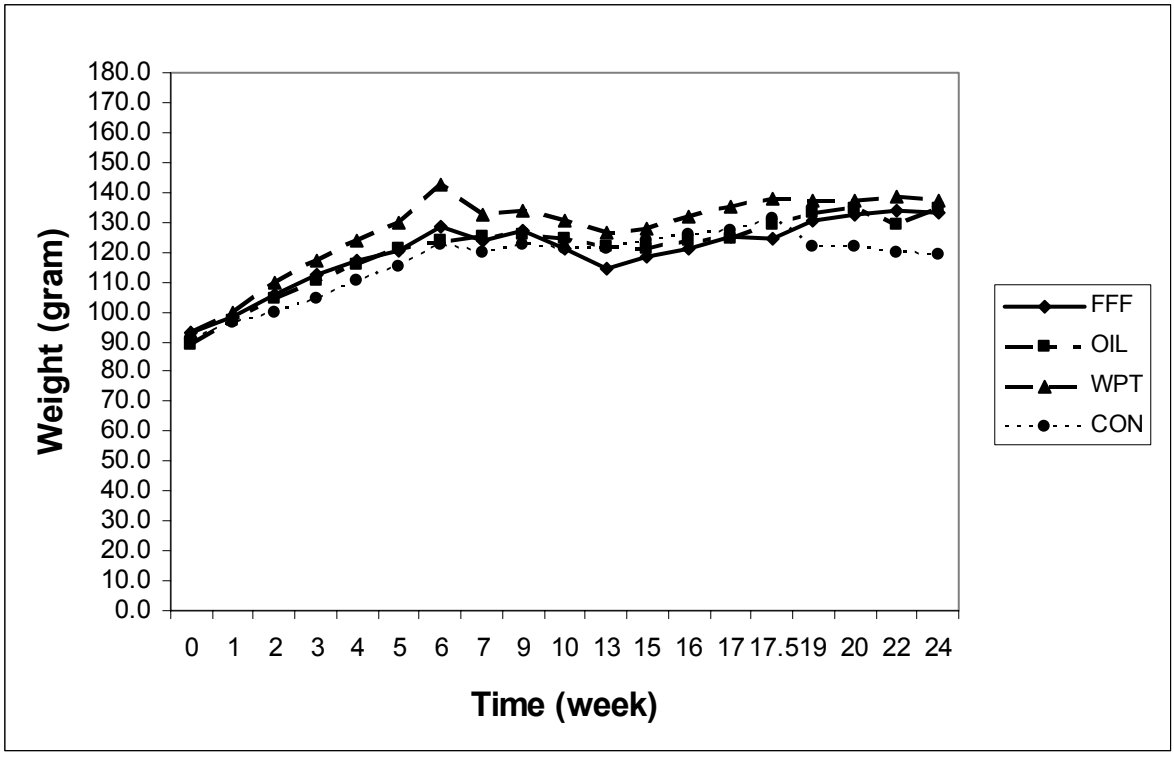

Figure 2.1 Hamster weight gain over 24 weeks

Peanut Components

Nutritional analysis was conducted on peanuts, peanut oil and peanut flour. The peanuts used in this study were high oleic peanuts. The fatty acid analysis indicated 80.5% oleic acid and 4.3% linoleic acid in the peanut oil and the whole peanuts had oleic and linoleic acid percentages of 79.1% and 3.8%, respectively. Total saturated fat concentration

was 14.3% for whole peanuts and 12.5% for peanut oil. Fatty acid content of non high oleic peanuts is about 20% and 55% of oleic and linoleic acids, respectively (32). High oleic peanuts have been demonstrated to produce the same cardiovascular beneficial affects on blood lipid profile as normal oleic peanuts (33). The total protein content of whole peanuts and fat free peanut flour was 25.6% and 49.9%, respectively. The amino acid content of the peanuts (Table 2.2) was in the range of other reported amino acid concentrations (34).

Table 2.1 Amino Acid Profile for Fat Free Peanut Flour and Whole Peanuts used in the Experimental Diet (g / 100 g)

	FFF	WP
Aspartic Acid	4.43	3.07
Serine	2.04	1.35
Glutamic Acid	7.72	5.48
Glycine	2.23	1.43
Histidine	0.71	0.58
(NH4)2SO4	7.35	4.70
Arginine	4.74	3.65
Threonine	0.79	0.77
Alanine	1.41	0.99
Proline	1.49	1.03
Tyrosine	1.41	1.18
Valine	0.78	0.94
Methionine	0.00	0.09
Lysine	1.00	0.50
Isoleucine	0.93	0.87
Leucine	2.15	1.75
Phenylalanine	1.78	1.38
Cystine	0.21	0.19

FFF = fat free peanut flour
WP = whole peanuts

Diet Analyses

Analyses were performed on all four hamster diets received from TestDiet. There was less than a 1% difference between the fatty acid analyses performed in our lab and the analytical specifications received from TestDiet (appendices 1 - 4). The experimental diets

were designed to be isocaloric to the control diet by substituting diet components of equal metabolizable energy (Tables 2.2 and 2.3). The control diet was a high fat, high cholesterol diet designed to induce atherosclerosis in rodents. All four diet groups had 0.5% cholesterol and 20 – 22% fat. The percent fat was calculated for peanut, peanut oil and fat free peanut flour at 20% of the diet and replaced soybean oil and cocoa butter in the control diet. The amount of cocoa butter replacement was limited because the saturated fat is important to induce atherosclerosis. Since fat free peanut flour had less than 0.5% fat there was not any fat substitution while peanut oil had the greatest amount of fat substitution. The differences in fat substitution produced an experimental peanut diet with a slightly higher level of saturated fat than the other experimental peanut component diets. The peanut oil and whole peanut experimental diets were higher in MUFA while the control and fat free peanut flour diets were higher in saturated fat.

Vitamin free casein was used for the protein source and substituted for the calculated protein from peanut components. A vitamin mix was added to all diets at 1.1% to insure the hamsters' basic nutritional needs for growth and health were met. Lysine was added into the whole peanut diet because of the unnaturally low amount. Average peanuts have about 2 - 3 g per 100 g of lysine and the peanuts used in this study had 0.5 g per 100 g of lysine by weight. It was determined that the addition of lysine was not needed for the fat free peanut flour diet group to ensure healthy growth.

Twenty percent of daily calorie consumption from peanuts or peanut components is not unrealistic. The hamsters were consuming about 4.5 kcal / g from their designated diet and their daily feed intake was about 14 grams. Each hamsters consumed about 12.6 – 12.7 kcal of peanuts daily. A serving of peanuts (1 oz) is about 160 kcal. Daily consumption of

peanuts by hamsters was equivalent to a human on an 1800 to 2200 kcal consuming about 2.1 to 2.6 servings of peanuts. It is not uncommon for more than one serving of peanuts to be consumed by the general population in a single sitting.

Table 2.2 Composition of Experimental Diets*

Ingredients	Fat Free Flour	Peanut Oil	Whole Peanuts	Control
Corn Starch	21.6	27.7	23.6	23.8
Casein–Vitamin Free	10.4	22.0	16.4	22.5
Peanut Product	20.0	20.0	20.0	0.0
Cocoa Butter	16.2	0.0	9.5	17.4
Sucrose	10.4	10.1	11.5	12.7
Maltodextrin	6.5	6.0	7.0	8.0
Powdered Cellulose	4.6	5.8	3.1	5.6
Soybean Oil	2.7	1.3	1.8	2.8
AIN-76A Vitamin Mix	1.1	1.1	1.1	1.1
Dicalcium Phosphate	1.5	1.5	1.5	1.5
Clinton Salt Mix	1.1	1.1	1.1	1.1
L-Lysine	0.5	0.0	0.0	0.0
Potassium Citrate, Tribasic Monohydrate	1.8	1.8	1.8	1.9
Calcium Carbonate	0.6	0.6	0.6	0.6
Choline Bitartrate	0.2	0.2	0.2	0.2
L-Cystine	0.3	0.3	0.3	0.3
Cholesterol	0.5	0.5	0.5	0.5
Total	**100.0**	**100.0**	**100.0**	**100.0**

***** Means reported as percent by weight

Table 2.3 Proximate Percent Caloric Concentration of Experimental Diets*

	Fat Free Flour	Peanut Oil	Peanuts	Control
Protein	20.3	20.3	20.3	20.7
Carbohydrate	45.7	45.0	44.6	45.6
Fiber	6.6	5.8	4.1	5.6
Total Fat	20.4	22.0	22.8	21.4
Saturated Fat	11.7	3.6	8.3	12.3
Monounsaturated Fat	6.4	16.6	12.3	6.6
Polyunsaturated Fat	2.3	1.2	2.0	2.4

* Means reported as g / 100 g

Plasma Cholesterol Concentrations

Plasma cholesterol concentrations were significantly ($p<0.05$) lower in hamsters consuming the peanut and peanut component diets. Data in Table 2.4 indicates TPC levels remained relatively constant throughout the study for hamsters fed the whole peanut, peanut oil and fat free peanut flour diets, while TPC significantly increased with time in the control diet group. The whole peanut diet group had significantly lower TPC than all other groups at wk 12. TPC concentration for the peanut oil diet group was significantly lower than the control group but not significantly different than the fat free peanut flour or peanut oil group. The fat free peanut flour, peanut oil and whole peanut diet groups had significantly lower TPC than the control group at wk 18. In wk 24 the TPC concentration of the control group doubled to 1081.6 mg / dL while the TPC levels of the fat free peanut flour, peanut oil and whole peanut groups only increased by 96.8, 57.8 and 31.2 mg / dL, respectively. The peanut component diet groups had significantly lower TPC than the control group at the end of the study.

A blood lipid profile high in VLDL-C and LDL-C are primary risk factors of CVD (35). These results indicate that peanut components prevented an increase in TPC as well as VLDL-C and LDL-C plasma concentrations in hamsters. Data in Tables 2.5 and 2.6 indicate that all peanut component diets had a positive effect of preventing a drastic increase of VLDL-C and LDL-C concentrations compared to the control diet. The whole peanut and peanut oil groups had significantly lower VLDL-C and LDL-C than the fat free peanut flour and control groups. However, the LDL-C concentration of the control group increased with time and at 18 wk was significantly higher than the fat free peanut flour, peanut oil and whole peanut diet groups. The fat free peanut flour, peanut oil and whole peanut diet groups did not significantly differ and all had significantly lower VLDL-C and LDL-C concentrations than the control at the end of the study. The reduction of LDL-C concentration for the whole peanut and peanut oil diet groups is consistent with other published data (36). The peanut oil and whole peanut groups had diets with the highest level of MUFA which has been indicated to significantly reduce LDL-C concentrations (37). The fat free peanut flour diet group had a significantly lower amount of MUFA in the diet than the peanut oil and whole peanut groups; however at the end, there was no difference in preventing the increase in LDL-C observed in the control group. The results may be related to the difference in fiber content in the diets (Table 2.2). The fat free peanut flour group had the highest amount of fiber, about 6.6%, while the lowest amount was in the whole peanut diet with about 4.1% and the peanut oil and control diets had 5.8% and 5.6%, respectively. High levels of fiber (ca 20 - 30% of the diet) has been indicated to reduce plasma LDL-C concentrations (38, 39). The difference between diets is 1% or less. Arginine, phytosterols, flavonoids and folates have been linked to cardiovascular health and may have played a role

in the lower LDL-C concentration in the fat free peanut flour group than in the control group. Further work needs to be done to determine which non-lipid component in the peanut has the greatest cardiovascular benefit.

To reduce the risks for CVD, a higher plasma HDL-C concentration is as important as low LDL-C concentration because decrease in HDL-C is just as great of a risk factor for CVD as an increase in LDL-C (40). The control diet was a modification of the Clinton / Cybulsky cholesterol series designed to induce atherosclerosis. Our results indicate that the control diet significantly increased TPC, VLDL-C, LDL-C and HDL-C concentrations. The fat free peanut flour, peanut oil and whole peanut diets did not significantly decrease HDL-C while they prevented the increase of LDL-C and TPC. HDL-C concentrations were not significantly different from we 12 to wk 24 (Table 2.7).

Table 2.4 Total Plasma Cholesterol Concentrations for Peanut, Peanut Oil, Fat Free Peanut Flour and Control Diet Groups at week 0, 12, 18 and 24

	Time (week)			
	0	12	18	24
Diet Group	*Total Cholesterol (mg / dL) ± S.E.M.*			
Fat Free Flour	105.9 ± 8.3 a	337.1 ± 58.8 ab	348.8 ± 152.3 b	445.6 ± 168.1 b
Peanut Oil	105.9 ± 8.3 a	273.1 ± 42.4 bc	303.3 ± 55.7 b	360.7 ± 48.7 b
Peanuts	105.9 ± 8.3 a	236.9 ± 71.8 c	294.4 ± 121.8 b	325.6 ± 99.6 b
Control	105.9 ± 8.3 a	418.9 ± 161.7 a	535.2 ± 123.0 a	1081.6 ± 340.5 a

* Numbers in columns followed by the same letter are not significantly different ($p<0.05$).

Table 2.5 Very Low Density Lipoprotein Cholesterol Concentrations for Peanuts, Peanut Oil, Fat Free Peanut Flour and Control Diet Groups at week 0, 12, 18 and 24

	Time (week)			
	0	12	18	24
Diet Group	*VLDL Cholesterol (mg/dL) ± S.E.M.*			
Fat Free Flour	33.4 ± 4.4 a	89.4 ± 30.0 a	90.7 ± 61.8 ab	183.0 ± 124.3 b
Peanut Oil	33.4 ± 4.4 a	36.7 ± 12.1 b	50.8 ± 17.3 b	66.0 ± 36.1 b
Peanuts	33.4 ± 4.4 a	44.2 ± 20.1 b	90.4 ± 63.6 ab	122.3 ± 69.8 b
Control	33.4 ± 4.4 a	96.5 ± 59.9 a	154.2 ± 62.3 a	553.7 ± 338.6 a

* Numbers in columns followed by the same letter are not significantly different ($p<0.05$).

Table 2.6 Low Density Lipoprotein Cholesterol Concentrations for Peanuts, Peanut Oil, Fat Free Peanut Flour and Control Diet Groups at week 0, 12, 18 and 24

	Time (week)			
	0	12	18	24
Diet Group	*LDL Cholesterol (mg/dL) ± S.E.M.*			
Fat Free Flour	15.4 ± 4.4 a	114.1 ± 40.2 a	110.3 ± 71.6 b	141.4 ± 101.6 b
Peanut Oil	15.4 ± 4.4 a	21.8 ± 20.3 b	20.3 ± 7.9 c	60.2 ± 41.3 b
Peanuts	15.4 ± 4.4 a	9.2 ± 5.9 b	27.8 ± 28.3 b	47.5 ± 37.6 b
Control	15.4 ± 4.4 a	143.9 ± 133.2 a	224.5 ± 88.1 a	354.3 ± 91.8 a

* Numbers in columns followed by the same letter are not significantly different ($p<0.05$).

Table 2.7 High Density Lipoprotein Cholesterol Concentrations in Peanuts, Peanut Oil, Fat Free Peanut Flour and Control Diet groups at week 0, 12, 18 and 24

	Time (week)			
	0	12	18	24
Diet Group	*HDL Cholesterol (mg/dL) ± S.E.M.*			
Fat Free Flour	57.0 ± 7.9a	173.6 ± 17.2a	147.8 ± 31.5b	150.0 ± 81.0a
Peanut Oil	57.0 ± 7.9a	217.5 ± 28.4a	232.1 ± 38.6a	225.2 ± 61.1a
Peanuts	57.0 ± 7.9a	183.5 ± 52.2a	176.2 ± 36.9b	155.8 ± 62.9a
Control	57.0 ± 7.9a	178.5 ± 35.1a	156.5 ± 18.5b	163.6 ± 125.3a

* Numbers in columns followed by the same letter are not significantly different ($p<0.05$).

Aortic Lesions

Aortas from 0 and 24 wk for all diets were stained with ORO and examined for lesions as originally planned in the study. Lesions were not observed in aortas from any hamsters consuming any diet in this study. Negative results were confirmed by the Department of Pathology, Lipid Science Division at the Wake Forest University School of Medicine (Winston Salem, NC).

Aortic Cholesterol Concentrations

Chemical analyses were performed on the aortas to evaluate the concentrations of unesterified, esterifed and total cholesterol in the tissue. The primary focus of chemical analyses was CE because it is one of the first compounds associated with the development of atherosclerosis (41). The total cholesterol (TC) and unesterified cholesterol (FC) in aortas from all treatments increased from 12 to 18 weeks but concentrations were generally

significantly higher in the control diet group (Table 2.8). The aortic TC was significantly lower in the fat free peanut flour and peanut oil diet groups than the control group at wk 12 and all peanut diet groups were significantly lower than the control at 18 wk.

The data for aortic esterified cholesterol are reported as CE (Table 2.8). At wk 12 there were no significant difference among diet groups for CE aortic concentration. The CE concentration in the control group increased from 2.8 to 19.2 mg/g PR and the highest concentration in the peanut diet groups was 7.6 mg/g PR for the peanut oil diet. CE in all peanut diets was significantly lower than the control group at wk 18. The aortas that were stained earlier in the study with ORO could not be chemically analyzed. CE analysis has been demonstrated to indicate the development of atherosclerosis in previous studies (15, 18, 42). The results of this study suggest that peanuts, peanut oil and fat free peanut flour decrease the development of atherosclerosis, indicated by CE, in male Syrian golden hamsters.

Table 2.8 Total and Free Aortic Cholesterol and Aortic Cholesteryl Ester Concentrations in Peanut, Peanut Oil, Fat Free Peanut Flour and Control Diet Groups at wk 12 and 18

	TC		FC		CE	
	Time (week)		Time (week)		Time (week)	
	12	18	12	18	12	18
Diet Group	mg/gPR ± SEM		mg/gPR ± SEM		mg/gPR ± SEM	
Fat Free Flour	304.4 ± 1.1 b	456.6 ± 1.1 b	291.1 ± 1.1 b	402.6 ± 1.1 b	1.6 ± 1.3 a	3.7 ± 1.3 b
Peanut Oil	292.5 ± 1.1 b	390.5 ± 1.1 b	279.5 ± 1.1 b	301.3 ± 1.1 b	1.7 ± 1.3 a	7.6 ± 1.4 b
Whole Peanuts	350.0 ± 1.1 a	402.5 ± 1.1 b	318.5 ± 1.1 ab	346.9 ± 1.1 b	2.4 ± 1.3 a	4.0 ± 1.3 b
Control	407.7 ± 1.1 a	849.0 ± 1.1 a	370.7 ± 1.1 a	599.0 ± 1.1 a	2.8 ± 1.3 a	19.2 ± 1.3 a

* TC and FC means in columns followed by the same letter are not significantly different ($p<0.05$)
* CE means in columns followed by the same letter are not significantly different ($p<0.0001$)
* Means expressed as mg / g protein ± S.E.M.

CONCLUSIONS

These results indicate that fat free peanut flour, as well as peanuts and peanut oil, reduced TPC and LDL-C while maintaining a healthy level of HDL-C in hamsters when added to a diet known to induce atherosclerosis. Results are also consistent with other published data in that peanuts and peanut oil provide a protective effect against CVD by reducing TPC and LDL-C concentrations without decreasing HDL-C. In addition, results indicated that peanuts, peanut oil and fat free peanut flour retard the build-up of CE, one of the primary aortic metabolic parameters associated with the development of atherosclerosis.

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CHAPTER 3:

EFFECTS OF PEANUTS, PEANUT OIL AND FAT FREE PEANUT FLOUR ON BONE STRENGTH IN SYRIAN GOLDEN HAMSTERS

ABSTRACT

There is a growing body of evidence that components in peanuts such as polyunsaturated fatty acids, monounsaturated fatty acids, phytosterols, and arginine may play a role in bone health. The objective of this study was to examine the effects of peanuts, peanut oil and fat free peanut flour on bone strength in male Syrian golden hamsters. Femurs were collected at weeks 0, 12, 18 and 24 in a 24 week study using a high cholesterol control diet which was isocalorically modified with the addition of fat free peanut flour, peanut oil and peanuts for a total of four diets. The maximum strength of bone fracture was determined by a 3-point bend on a Texture Analyzer Plus. The force required for femur fracture was significantly higher in the peanut diet groups at 12 weeks but differences were not significant at 18 weeks. At 24 weeks the force required for bone fracture in all diet groups was reduced to approximately the 0 time force; however, the forces required for bone fracture in the peanut diet groups were significantly higher than those of the control diet group. The fat free flour diet was different than the control only at 24 weeks. On two of three sample dates peanut diet groups had stronger bones but overall the data are inconclusive on the effects of peanut components on bone strength.

INTRODUCTION

Osteoporosis affects nearly 44 million Americans or about 55% of Americans aged 50 and older (1). In 2005, health care costs associated with osteoporosis were $19 billion and that number is expected to rise to approximately $25 billion in 2025 (1). Peanuts (*Arachis hypogae*) are in the legume family but are generally considered as nuts. Peanuts are a nutrient dense food containing high levels of unsaturated fat and other compounds such as flavonoids, arginine, potassium, calcium, magnesium that have been linked to bone health, according to a review article by Nieves (2). A one ounce serving of peanuts contains about 2.0%, 4.0% and 12.7% of the Recommended Daily Allowance (RDA) for calcium, potassium and magnesium, respectively (3). In the United States the consumption of peanuts by weight is greater than all other nuts combined (4).

Peanuts are comprised of about 25% protein which is high in arginine content. Chevalley et al. (5) demonstrated that a large dose of arginine stimulates insulin-like growth factor 1 (IGF-1) and IGF-1 is known to increase bone mass and bone strength (6). Peanuts are also a good source of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). An epidemiological study concluded that people over the age of 65 who consumed high levels of PUFA had an increase risk for bone fracture but those who consumed a higher ratio of MUFA to PUFA had a reduced risk of fractures (7).

Soybean protein (8) and flavonoids (9) are reported to protect against bone loss by reducing bone turnover in ovariectomized rats. An increased intake of flavonoids resulted in higher bone mineral density in adult rats (10). The objective of this study was to examine the effects of peanuts, peanut oil and fat free peanut flour on bone fracture strength in male Syrian golden hamsters.

MATERIALS AND METHODS

Animal Care

Eighty male Syrian golden hamsters, 6-weeks-old and weighing between 80 – 100 g were purchased from Harlan, Inc. (Indianapolis, IN). Hamsters were housed in individual cages with NEPCO (Northeastern Products Corporation) wood chip bedding (Warrensburg, NY), wire top covers and isolator top lids at the Biological Research Facility (BRF) at North Carolina State University (NCSU). Food was placed in the wire top covers for stimulation and on the bottom with the bedding for natural-type consumption.

The hamsters were maintained on a 12/12 hr light/dark cycle in an environmentally controlled room. The cages were cleaned weekly while food, water and general visible health were checked daily. Seventy-six hamsters were randomly assigned to one of four diet groups before the start of the experiment. Hamsters were weighed weekly until age 15 weeks and then bimonthly. All procedures were approved by North Carolina State University Animal Care and Use Committee (Raleigh, NC). IACUC (Institutional Animal Care and Use Committee) protocol # 06-104-B approved August, 2006.

Diets

The four experimental diets were fed *ad libitum* with clean water. The control diet was a modification of the AIN-76A semipurified diet for rodents prepared by TestDiet® (Richmond, IN). The control diet was designed to increase blood chemistry risk factors and induce atherosclerosis in rodents. There were three experimental diet groups; whole peanuts, peanut oil and fat free peanut flour. All diets were adjusted to the caloric level of the control diet by substituting peanut products for cocoa butter, soybean oil, casein, corn starch,

sucrose, maltodextrin, or cellulose based on metabolizable energy (fat is 9 kcal/g, carbohydrate and protein are 4 kcal/g) and not by weight.

High oleic, dark roasted peanut products [whole peanuts, peanut oil and peanut flour (12% fat)] were a gift from Golden Peanut, LLC (Alpharetta, GA). The three peanut products were collected from the same lot either before or after processing. During processing oil was mechanically removed from roasted peanuts. The pressed peanut material was hexane extracted to produce a 12% oil flour. The 12% oil flour was further hexane extracted by Avoca Farms, Inc. (Merry Hill, NC) to about 0.5% oil.

The hamsters were fed their original commercial diet (Purina 5001) for one week and experimental diets were substituted for the commercial diet incrementally during the next five days. Experimental diets were substituted incrementally during the next five days. The hamsters received 25 % experimental diet and 75 % commercial diet on day one, a 50/50 mix on day two, and a 75/25 mix on day three. Hamsters in all diet groups were consuming 100% of their designated experimental diet by day five. The 24 week study was initiated when all hamsters were consuming 100% of the experimental diet when the animals were 8 weeks old.

Diet Analyses

Fatty acid profile of each diet was determined in triplicate by GC. Diets were ground into a powder with a Braun coffee mill (Gillette Inc., Boston, MA) and moisture was measured according to AOAC 934.01 (Official Methods of Analysis, Padmore, J.M. 1990). Exact weight of sample (ca. 2 g) was recorded and then transferred into a ceramic bowl. The samples were dried to a constant weight for 7 hr at 95°C and stored overnight in a desiccator.

Fat was extracted from the diets by AOAC 954.02 animal feed method (Official Methods of Analysis, Helrich, 1990). Alcohol, 2 mL, was added to a Mojonnier tube with exact weight samples until all particles were moist to prevent clumping with the addition of acid. Ten mL of HCl were added to each sample and then the samples were shaken frequently for 30 min in an 80°C water bath. The samples were cooled to room temperature and alcohol was added until each sample volume reached the Mojonnier tube constricted portion. A rubber stopper, cleaned with alcohol before use, was used to seal the Mojonnier tubes. Twenty-five mL of ether was added and each sample was shaken vigorously for 1 minute with pressure released periodically. The samples were shaken vigorously for an additional 1 min after 25 mL redistilled petroleum ether was added. Samples were allowed to settle and then the ether-fat solution was poured off through a cotton pledget to prevent unwanted solid particles. The Mojonnier tubes were rinsed twice with ether and each time the ether-fat solution was poured off. Ether was evaporated and the samples were dried for 90 min at 100°C. The samples were placed in a desiccator and cooled to room temperature in and weighed.

The samples were analyzed on a Perkin Elmer GC with an Autosampler XL (Waltham, MA) with a Restek, RT-2560 column (100 m x 0.25 mm ID x 0.2 μm dry film, Bellefonte, PA). The initial temperature was 100°C and ramped at 3°C per min to 250°C. The total run time was 67.0 minutes (11). A moisture correction factor was used in the calculation of percent of each fatty acid (12).

Femur Collection and Fracture Point

The femurs of each euthanized hamster were collected at weeks 0, 12, 18, and 24. Femurs were stored at -60°C until analysis. All femurs were frozen for the same length of

time before analysis. The femurs were tempered to room temperature for about 60 min in phosphate buffered saline (PBS) before analysis. Each femur was weighed and length, width and diameter were recorded. The force required to fracture the bone was used as the measure of bone strength and determined by a 3-point bend test on a Texture Analyzer TX-TA2plus (Marietta, GA). The load cell was 50 kg and the rate of probe was 5 mm per minute.

Statistical Methods

SAS software (Cary, NC) was used for all statistical evaluations. The force applied until the first fracture point was analyzed as a two variable study accounting for time and diet effects. The statistical evaluation of the maximum force of bone fracture was unique because pooled data were not used due to insurmountable inhomgeneity of variance. Due to unpredicted factors such as spontaneous death, instrumental malfunction and sample loss there was a slight unbalance in the number of rodents in each diet group at 24 weeks. The least squared means were reported to estimate the mean had the samples had been balanced. The significant differences among treatment means was assessed by Tukey's test and differences of $p < 0.05$ were considered significant (13).

RESULTS AND DISCUSSION

Bone strength as determined by force required to fracture femurs was examined in male Syrian golden hamsters. Factors such as high dietary fat intake may be contributors to age related bone loss in men (14, 15), but there is inconsistent evidence on fat intake and bone loss. The fatty acid analysis indicated the peanut oil used in the study was processed from high oleic peanuts. The proximate fatty acid composition was about 4.3% PUFA,

83.0% MUFA and 12.5% saturated fat in peanuts and peanut oil added to the experimental diets (data not shown). The fat free peanut flour diet had high amounts of saturated fat from cocoa butter and a small amount of unsaturated fat from soybean oil. Data in Table 3.1 indicates the different levels of triacylglycerol (TAG) and fatty acids in experimental diets. The peanut oil had the highest level of MUFA but the lowest level of PUFA. The whole peanut diet had the highest level of PUFA but the lowest level of MUFA. Shen et al. (16) demonstrated PUFA reduce bone turnover rate in middle aged rats (12 – 24 mo) by increasing the net bone volume.

Table 3.1 Triacylglycerol and Fatty Acid Profile of Fat Free Peanut Flour, Peanut Oil, Whole Peanuts and Control Diets

	Triacylglycerol (%)	Saturated Fat (%)	Polyunsaturated Fat (%)	Monounsaturated Fat (%)
Fat Free Peanut Flour	20.4	11.7	2.3	6.4
Peanut Oil	22.0	3.6	1.7	16.6
Whole Peanuts	22.8	8.3	12.4	2.0
Control	21.4	12.3	2.4	6.6

There was a diet and time interaction on maximum fracture force (Table 3.2). At week 0 the force required for fracture was 35.6 ± 6.2. Bone length and maximum fracture force increased between weeks 0 to 12 in all diet groups. At week 12, the peanut oil and whole peanut diet groups had significantly higher maximum fracture force than the fat free peanut flour and control diet groups. The results for the 12 week time point are consistent with literature indicating PUFA (16, 17) and MUFA (7) play a role in increasing bone strength. Blood lipid chemistries were also evaluated in this study (chapter 2). At 12 weeks

the peanut oil and whole peanut diets had higher concentrations of high density lipoprotein cholesterol (HDL-C) than the fat free peanut flour group and significantly higher concentrations of HDL-C. The combination of the data is consistent with the published work indicating HDL-C concentrations may be inversely associated with a risk of bone fracture in rats (7). However, at week 18 the significant differences among diet groups disappear. In addition, there was a decrease in the maximum fracture force between week 12 and week 18 in the peanuts and peanut oil diet groups. At week 24 there is a drastic decrease in maximum fracture force for all the diet groups. The decrease is not believed to be a diet effect because all groups decreased at about the same magnitude. All peanut component diet groups have a significantly higher maximum fracture force than the control diet group at the end of the study. However due to the varying degrees of significance at different time points among diet groups the overall results are inconclusive.

Variation in the data were not attributable to instrument malfunction nor to freezing and then thawing since studies have indicated that freezing up to 100 days does not affect mechanical properties of bone (18, 19). Weight bearing activities of the animals did not contribute to the differences since body weight did not significantly differ among all diet groups are various time points or at the end of the study.

Table 3.2 Maximum Fracture Force of Femurs at week 0, 12, 18 and 24 in Syrian Golden Hamsters

	Time (week)			
	0	12	18	24
Diet Group	*fracture force (N) ± S.E.M.*			
Fat Free Flour	35.6±6.3a	147.5 ± 33.8bc	170.9 ± 41.6a	54.0 ± 10.0a
Peanut Oil	35.6±6.3a	192.7 ± 36.3ab	174.8 ± 52.6a	64.1 ± 6.7a
Peanuts	35.6±6.3a	236.2 ± 24.3a	215.0 ± 34.7a	63.6 ± 18.2a
Control	35.6±6.3a	129.9 ± 39.8c	171.1 ± 26.2a	38.1 ± 10.0b

* Numbers in columns followed by the same letter are not significantly different ($p<0.05$).

Increasing maturity of the hamsters may be partially responsible for decrease in fracture force at week 24. Bone loss and osteoporosis has been reported to occur with increasing age in humans (1) and rats (20). The hamsters were about 32 weeks old at the 24 week experimental time point. The average life span of a hamster is about 2 years. In comparison to published data the hamsters used in this study were still relatively young at the end of this study. A decrease in osteoclastogenesis was demonstrated in 23 month old rats along with changes in bone density and structure of trabecular bone compared to 5 month old rats. However, there were no observed changes in cortical bone geometry with age (20). Literature on hamster bone integrity was not found. In the future, investigation of the effect of increasing maturity on bone health is necessary to determine the best experimental animal model for osteoporosis studies.

CONCLUSIONS

There is a growing body of evidence that certain components such as PUFA, MUFA, and arginine in peanuts may increase bone health. The peanut samples had a significantly higher fracture force at wk 12 and 24 but overall the data were inconclusive on the effect of peanut components on bone strength.

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CHAPTER 4:

CONCLUSIONS AND FUTURE WORK

Cardiovascular disease is the number one cause of death in Western societies (1). This is often attributed to the fact that people in Western societies commonly consume diets high in saturated fat and cholesterol which can lead to hypercholestermia. Unfortunately, statins or cholesterol reducing medications can be expensive, a daily inconvenience and may even cause undesirable side effects. An alternative method to reducing cholesterol is by substituting foods high in unsaturated fat for foods high in saturated fat. Peanuts are a nutrient dense food with a high ratio of monounsaturated to polyunsaturated fatty acids. Human clinical trials and epidemiological studies have demonstrated that consuming nuts, including peanuts, benefit ones overall lipid profile and reduces the risk of CVD (2-5). Peanut oil has also been demonstrated to improve the lipid profile and reduce the risk of cardiovascular disease (6, 7). However, the effects of fat free peanut flour on cardiovascular health have not been previously studied.

In this study, results suggest whole peanuts, peanut oil and fat free peanut flour have beneficial effects on reducing blood chemistry risk factors for cardiovascular disease and the development of atherosclerosis in golden Syrian hamsters fed a diet known to induce hyperlipidemia and atherosclerosis. Compared to the control diet group, whole peanut and peanut oil diet groups had a significant reduction in total plasma cholesterol, very low density lipoprotein cholesterol and low density lipoprotein cholesterol, while a healthy level of high density lipoprotein cholesterol was maintained. These results are consistent with other published data (2, 8). Fat free peanut flour also significantly reduced total plasma cholesterol, very low density lipoprotein cholesterol, and low density lipoprotein cholesterol concentrations while simultaneously maintaining a healthy level of high density lipoprotein cholesterol in hamsters. Reducing total cholesterol and low density lipoprotein cholesterol

reduces the risk of developing cardiovascular disease. However, it is important that high density lipoprotein cholesterol not decrease with the reduction of low density lipoprotein cholesterol because the risk of developing atherosclerosis can increase with a reduction in high density lipoprotein cholesterol (9).

Even through studies have demonstrated the protective effects that nuts and peanuts have against cardiovascular disease risk factors, there is minimal research on the direct effect of peanuts on atherosclerosis. This study also examined the effects of peanuts, peanut oil and fat free peanut flour on aortic cholesteryl ester, one of the primary metabolic compounds of atherosclerosis. Results indicate that whole peanuts, peanut oil and fat free peanut flour included in the diet slow the build-up of cholesteryl ester in the aortas of hamsters fed a high cholesterol diet. The data indicate that both the lipid and non-lipid portion of the peanut are cardiovascular protective but the protective effects do not appear to be additive because the diet containing whole peanuts (lipid and non-lipid components) did not produce significantly different results. Future work should be conducted to determine which specific components in peanuts provide beneficial affects. The preponderance of bioactive compounds in whole peanuts, peanut oil, and fat free peanut flour suggest that the results obtained in this study result from the several different compounds and thus involve several different mechanisms. Additional peanut components including peanut skins should be evaluated for cardiovascular health. Peanut skins may also produce cardiovascular protective properties when consumed in a high fat diet because they are high in fiber and bioactive constituents such as resveratrol, flavonoids, phytosterols, tocotrienols and saponins.

The effects of peanuts and components on bone strength are inconclusive. The sudden decrease in strength from eighteen to twenty-four weeks can not yet be explained.

Future work on the effects of peanuts on bone development might involve studies with osteoblast cells to evaluate the contribution of several individual lipid and non-lipid components on bone development.

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APPENDICES

Appendix 1: Composition of the Control Diet

AIN-76A/Clinton-Cybulsky Cholesterol Series #2-107 58R5

DESCRIPTION

Purified Rodent Diet. Modification of TestDiet® AIN-76A, Dyed Red. Originally Manufactured as D12107, Part of the Clinton/Cybulsky Cholesterol Series.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1810020

*Other Forms Available By Request

INGREDIENTS (%)

Corn Starch	23.8042
Casein - Vitamin Free	22.4568
Cocoa Butter	17.4040
Sucrose	12.6880
Maltodextrin	7.9722
Powdered Cellulose	5.6142
Soybean Oil	2.8070
Potassium Citrate, Tribasic Monohydrate	1.8527
Dicalcium Phosphate	1.4597
Clinton Salt Mix	1.1228
AIN-76A Vitamin Mix	1.1228
Calcium Carbonate	0.6176
Cholesterol	0.5053
L-Cystine	0.3369
Choline Bitartrate	0.2246
Red Dye	0.0112

NUTRITIONAL PROFILE [1]

Protein, %	***20.7***
Arginine, %	0.79
Histidine, %	0.58
Isoleucine, %	1.07
Leucine, %	1.94
Lysine, %	1.63
Methionine, %	0.58
Cystine, %	0.42
Phenylalanine, %	1.07
Tyrosine, %	1.14
Threonine, %	0.87
Tryptophan, %	0.25
Valine, %	1.28
Alanine, %	0.62
Aspartic Acid, %	1.45
Glutamic Acid, %	4.59
Glycine, %	0.43
Proline, %	2.65
Serine, %	1.24
Taurine, %	0.00
Fat, %	***20.3***
Cholesterol, ppm	5,053
Linoleic Acid, %	1.92
Linolenic Acid, %	0.24
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.22
Total Saturated Fatty A	10.80
Total Monounsaturated Fatty Acids, %	6.32
Polyunsaturated Fatty Acids, %	2.18
Fiber (max), %	***5.6***
Carbohydrates, %	***45.6***
Energy (kcal/g) [2]	***4.52***

From:	kcal	%
Protein	0.829	18.5
Fat (ether extract)	1.823	40.7
Carbohydrates	1.823	40.7

Minerals	
Calcium, %	0.68
Phosphorus, %	0.51
Phosphorus (available), %	0.51
Potassium, %	0.67
Magnesium, %	0.06
Sodium, %	0.13
Chloride, %	0.23
Fluorine, ppm	0.0
Iron, ppm	40
Zinc, ppm	40
Manganese, ppm	66
Copper, ppm	6.8
Cobalt, ppm	0.0
Iodine, ppm	0.23
Chromium, ppm	2.3
Molybdenum, ppm	0.00
Selenium, ppm	0.00
Vitamins	
Vitamin A, IU/g	4.5
Vitamin D-3 (added), IU/g	1.1
Vitamin E, IU/kg	58.4
Vitamin K (as menadione), ppm	0.57
Thiamin Hydrochloride, ppm	6.7
Riboflavin, ppm	6.7
Niacin, ppm	34
Pantothenic Acid, ppm	17
Folic Acid, ppm	2.2
Pyridoxine, ppm	6.5
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	11
Choline Chloride, ppm	1,123
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only, NOT for human consumption.

10/26/2006

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Appendix 2: Composition of the Whole Peanut Diet

AIN Clinton Diet 58R6 w/ 20% Whole Peanuts-Dyed Red 5C5Q

DESCRIPTION

Modification of TestDiet® AIN Clinton Purified Rodent Diet, 58R6, with 20% Whole Peanuts and 0.5% Cholesterol. Dyed Red.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1812104

**Other Forms Available By Re*

INGREDIENTS (%)

Corn Starch	23.5517
TD Whole Peanuts-CA712	20.0000
Casein - Vitamin Free	16.4439
Sucrose	11.5000
Cocoa Butter	9.5000
Maltodextrin	7.0000
Powdered Cellulose	3.0602
Potassium Citrate, Tribasic Monohydrate	1.8387
Soybean Oil	1.7529
Dicalcium Phosphate	1.4487
Clinton Salt Mix	1.1144
AIN-76A Vitamin Mix	1.1144
Calcium Carbonate	0.6129
Cholesterol	0.5000
L-Cystine	0.3343
Choline Bitartrate	0.2229
Red Dye	0.0050

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only, NOT for human consumption.

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NUTRITIONAL PROFILE [1]

Protein, %	***20.3***
Arginine, %	0.57
Histidine, %	0.42
Isoleucine, %	0.79
Leucine, %	1.42
Lysine, %	1.20
Methionine, %	0.42
Cystine, %	0.39
Phenylalanine, %	0.79
Tyrosine, %	0.83
Threonine, %	0.64
Tryptophan, %	0.18
Valine, %	0.94
Alanine, %	0.45
Aspartic Acid, %	1.06
Glutamic Acid, %	3.36
Glycine, %	0.32
Proline, %	1.94
Serine, %	0.91
Taurine, %	0.00
Fat, %	***21.3***
Cholesterol, ppm	5,000
Linoleic Acid, %	1.16
Linolenic Acid, %	0.15
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.14
Total Saturated Fatty A	5.93
Total Monounsaturated Fatty Acids, %	3.49
Polyunsaturated Fatty Acids, %	1.32
Fiber (max), %	***4.1***
Carbohydrates, %	***44.6***
Energy (kcal/g) [2]	***4.56***

From:	kcal	%
Protein	0.812	18.0
Fat (ether extract)	1.917	42.5
Carbohydrates	1.784	39.5

Minerals	
Calcium, %	0.67
Phosphorus, %	0.46
Phosphorus (available), %	0.46
Potassium, %	0.66
Magnesium, %	0.06
Sodium, %	0.13
Chloride, %	0.22
Fluorine, ppm	0.0
Iron, ppm	40
Zinc, ppm	38
Manganese, ppm	65
Copper, ppm	6.7
Cobalt, ppm	0.0
Iodine, ppm	0.23
Chromium, ppm	2.2
Molybdenum, ppm	0.00
Selenium, ppm	0.00
Vitamins	
Vitamin A, IU/g	4.5
Vitamin D-3 (added), IU/g	1.1
Vitamin E, IU/kg	57.1
Vitamin K (as menadione), ppm	0.56
Thiamin Hydrochloride, ppm	6.7
Riboflavin, ppm	6.7
Niacin, ppm	33
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.2
Pyridoxine, ppm	6.4
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	11
Choline Chloride, ppm	1,115
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

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Appendix 3: Composition of the Peanut Oil Diet

AIN Clinton Diet 58R6 w/ 20% Peanut Oil-Dyed Purple 5C5S

DESCRIPTION

Modification of TestDiet® AIN Clinton Purified Rodent Diet, 58R6, with 20% Peanut Oil and 0.5% Cholesterol. Dyed Purple.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1812106

*Other Forms Available By Re

INGREDIENTS (%)

Corn Starch	27.6886
Casein - Vitamin Free	21.9455
Peanut Oil	20.0000
Sucrose	10.1000
Maltodextrin	6.0000
Powdered Cellulose	5.8000
Potassium Citrate, Tribasic Monohydrate	1.8387
Dicalcium Phosphate	1.4487
Soybean Oil	1.2746
AIN-76A Vitamin Mix	1.1144
Clinton Salt Mix	1.1144
Calcium Carbonate	0.6129
Cholesterol	0.5000
L-Cystine	0.3343
Choline Bitartrate	0.2229
Purple Dye	0.0050

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only, NOT for human consumption.

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NUTRITIONAL PROFILE [1]

Protein, %	***20.3***
Arginine, %	0.77
Histidine, %	0.57
Isoleucine, %	1.05
Leucine, %	1.90
Lysine, %	1.60
Methionine, %	0.57
Cystine, %	0.42
Phenylalanine, %	1.05
Tyrosine, %	1.11
Threonine, %	0.85
Tryptophan, %	0.24
Valine, %	1.25
Alanine, %	0.61
Aspartic Acid, %	1.41
Glutamic Acid, %	4.48
Glycine, %	0.42
Proline, %	2.59
Serine, %	1.21
Taurine, %	0.00
Fat, %	***21.3***
Cholesterol, ppm	5,000
Linoleic Acid, %	6.46
Linolenic Acid, %	0.10
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.10
Total Saturated Fatty A	3.91
Total Monounsaturated Fatty Acids, %	8.94
Polyunsaturated Fatty Acids, %	6.56
Fiber (max), %	***5.8***
Carbohydrates, %	***45.0***
Energy (kcal/g) [2]	***4.57***

From:	kcal	%
Protein	0.812	17.9
Fat (ether extract)	1.920	42.4
Carbohydrates	1.800	39.7

Minerals	
Calcium, %	0.67
Phosphorus, %	0.51
Phosphorus (available), %	0.51
Potassium, %	0.66
Magnesium, %	0.06
Sodium, %	0.13
Chloride, %	0.23
Fluorine, ppm	0.0
Iron, ppm	40
Zinc, ppm	40
Manganese, ppm	65
Copper, ppm	6.7
Cobalt, ppm	0.0
Iodine, ppm	0.23
Chromium, ppm	2.2
Molybdenum, ppm	0.00
Selenium, ppm	0.00
Vitamins	
Vitamin A, IU/g	4.5
Vitamin D-3 (added), IU/g	1.1
Vitamin E, IU/kg	56.8
Vitamin K (as menadione), ppm	0.56
Thiamin Hydrochloride, ppm	6.7
Riboflavin, ppm	6.7
Niacin, ppm	33
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.2
Pyridoxine, ppm	6.4
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	11
Choline Chloride, ppm	1,115
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

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Appendix 4: Composition of the Fat Free Peanut Flour Diet

AIN Clinton Diet 58R6 w/ 20% Peanut Flour-Dyed Blue — 5C5R

DESCRIPTION

Modification of TestDiet® AIN Clinton Purified Rodent Diet, 58R6, with 20% Low Fat Peanut Flour and 0.5% Cholesterol. Dyed Blue.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1812105

**Other Forms Available By Re*

INGREDIENTS(%)

Corn Starch	21.5967
TD Low Fat Peanut Flour-CA735	20.0000
Cocoa Butter	16.2068
Sucrose	10.4000
Casein - Vitamin Free	10.3644
Maltodextrin	6.5000
Powdered Cellulose	4.5620
Soybean Oil	2.6500
Potassium Citrate, Tribasic Monohydrate	1.8387
Dicalcium Phosphate	1.4487
AIN-76A Vitamin Mix	1.1144
Clinton Salt Mix	1.1144
Calcium Carbonate	0.6129
L-Lysine	0.5288
Cholesterol	0.5000
L-Cystine	0.3343
Choline Bitartrate	0.2229
Blue Dye #1	0.0050

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only, NOT for human consumption.

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NUTRITIONAL PROFILE [1]

Protein, %	***20.3***
Arginine, %	0.36
Histidine, %	0.27
Isoleucine, %	0.50
Leucine, %	0.90
Lysine, %	1.17
Methionine, %	0.27
Cystine, %	0.37
Phenylalanine, %	0.50
Tyrosine, %	0.52
Threonine, %	0.40
Tryptophan, %	0.11
Valine, %	0.59
Alanine, %	0.29
Aspartic Acid, %	0.67
Glutamic Acid, %	2.12
Glycine, %	0.20
Proline, %	1.22
Serine, %	0.57
Taurine, %	0.00
Fat, %	***21.3***
Cholesterol, ppm	5,000
Linoleic Acid, %	1.81
Linolenic Acid, %	0.22
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.21
Total Saturated Fatty A	10.07
Total Monounsaturated Fatty Acids, %	5.89
Polyunsaturated Fatty Acids, %	2.05
Fiber (max), %	***6.6***
Carbohydrates, %	***45.7***
Energy (kcal/g) [2]	***4.60***

From:	kcal	%
Protein	0.812	17.8
Fat (ether extract)	1.917	42.1
Carbohydrates	1.827	40.1

Minerals	
Calcium, %	0.67
Phosphorus, %	0.41
Phosphorus (available), %	0.41
Potassium, %	0.66
Magnesium, %	0.06
Sodium, %	0.13
Chloride, %	0.32
Fluorine, ppm	0.0
Iron, ppm	40
Zinc, ppm	36
Manganese, ppm	65
Copper, ppm	6.7
Cobalt, ppm	0.0
Iodine, ppm	0.23
Chromium, ppm	2.2
Molybdenum, ppm	0.00
Selenium, ppm	0.00
Vitamins	
Vitamin A, IU/g	4.5
Vitamin D-3 (added), IU/g	1.1
Vitamin E, IU/kg	57.9
Vitamin K (as menadione), ppm	0.56
Thiamin Hydrochloride, ppm	6.7
Riboflavin, ppm	6.7
Niacin, ppm	33
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.2
Pyridoxine, ppm	6.4
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	11
Choline Chloride, ppm	1,115
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

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