ABSTRACT OF THE THESIS

Anti-Obesity Effects of Resveratrol, Black Tea Extract, and Caffeine in Mice

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Obesity continues to be a major public health issue in the United States. The aims of this study were to investigate whether the dietary resveratrol, black tea extract, caffeine and their combination could show anti-obesity effects in CD-1 mice fed with a high-fat diet. Five groups of CD-1 mice, with 10 mice in each group, were fed with 0.06 % resveratrol (RTL), 0.2 % black tea extract (BTE) and 0.05 % caffeine (CF), as well as their combination containing 0.03 % RTL, 0.1 % BTE and 0.025 % CF for 16 weeks. Mice on the CF diet significantly ($p<0.01$) reduced body weight gain by 46 %, while those on the RTL, BTE, CF and the combination diets showed reductions by 12 %, 14 % and 28 %, respectively. There were significant inhibitions in parametrial (77 %), retroperitoneal (77 %) fat pads and brown adipose tissue (57 %) for mice on CF group.

Mice on the BTE diet showed significant ($p<0.05$) inhibition of leptin levels in plasma by 59 %, while those on the CF and combination diets demonstrated significant ($p<0.01$) inhibition by 88 % and 79 %, respectively. These results suggest that diets with RTL, BTE, and CF reduced body weight gain, fat tissue depositions and leptin levels in female CD-mice during the 16-week animal studies.

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Chapter 1.

Introduction

Obesity is prevalent and continues to increase in developed, developing and even some underdeveloped nations. It has become pandemic worldwide as a result of changes in lifestyles, especially in eating habits. Obesity is not only associated with a number of serious medical complications but also raises the risk of diseases such as hypertension, type II diabetes, coronary heart disease and certain cancers (Rudelle, Ferruzzi, Cristiani, Moulin, Mace, Acheson et al., 2007). Therefore, obesity prevention and treatment are essential to the promotion of public health (Murosaki, Lee, Muroyama, Shin, Cho, Yamamoto et al., 2007).

Treatment of obesity is beneficial because the weight loss reduces the risk of mortality and morbidity. However, the only two FDA approved drugs, Orlistat and Sibutramine, can both induce serious side effects, such as palpitation and hypertension (Rudelle, et al., 2007). Orlistat (Xenical) is an intestinal lipase inhibitor that decreases the digestibility of dietary fat and increases fat excretion, while Sibutramine is a serotonin and noradrenaline uptake inhibitor that reduces appetite and increases energy expenditure (Bray, 2000). To avoid the undesirable side effects of these drugs, dietary concepts look to play an important role in weight control programs. Therefore, a rapidly expanding field in therapeutics is in the use of natural supplements (Kovacs & Mela, 2006).

The objective of this research is to investigate the anti-obesity effects of resveratrol, black tea extract, caffeine and their combination in CD-1 mice. The results will not only contribute to obesity research in general but also benefit the development of drugs for obesity treatment. Moreover, the potential applications for these compounds are unlimited. They can be incorporated into dietary supplements in pursuit of safe and effective weight loss products. The preliminary work of soy lecithin-based emulsion systems is conducted to reach the goal of increasing the bioavailability of oil soluble compounds and leading to greater efficacy in the use of these compounds for the prevention of obesity.

1.1. Obesity Background

For a good introduction to the subject of obesity, please consult the textbooks Clinical Obesity in Adults and Children (Kopelman, Caterson & Dietz, 2005) and Obesity: Etiology, Assessment, Treatment, and Prevention (Anderson, 2003), as they contain distinctive and comprehensive information about this subject. These textbooks include information about the causes of obesity, its complications and also novel methods for obesity prevention and treatment.

The severity of the obesity issue is seen in clarity by looking at the percentage of obese individuals within the total population, which is defined as those aged 15 and above. The definition of obesity will be described in the following section. Figure 1.1. is compiled from the fact book reported by the Organization for Economic Co-operation and Development (OECD) in 2008. There is an obese population of 32.2 %, 23.0 %,

12.0 %, 9.5 %, 3.2 % and 3.0 % in the United States, the United Kingdom, Turkey, France, Korea and Japan, respectively in 2005. Countries with greater obese populations not only face serious public health problems, but also must deal with many non-medical consequences of obesity on their societies. The most serious impact is the increased expense to public and private entities which may further influence the economy as a whole (Wolf & Colditz, 1998).

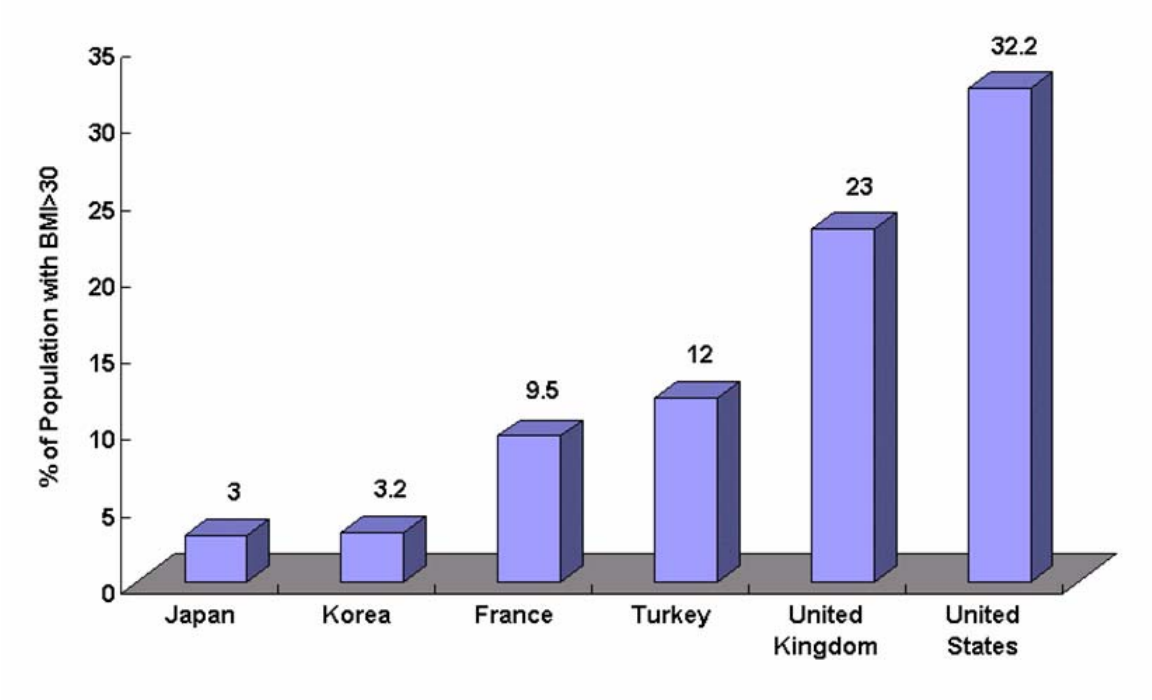


Figure 1.1. Obese population in selected countries in 2005 [(OECD), 2008)].

1.1.1. Obesity and Human Health

Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired (Wolf & Colditz, 1998). Obesity

is commonly characterized by measuring the Body Mass Index (BMI). BMI is the most widely used method for body fat estimation. The equation is listed as follows:

$$BMI=kg/m^2,$$

where kg refers to the weight in kilograms of the subject and m refers to the height in meters of the subject (Sweeting, 2007).

According to the definition from the World Health Organization (WHO) in 2000, there are three major categories for BMI values. The value under 18.5 refers to underweight, while the range from 18.5 to 24.99 refers to a normal condition, and the range from 25 to 29.99 refers to overweight. BMI greater than 29.99 refers to obese. With the rising BMI value, the risk of comorbidity becomes more severe. People with an obesity problem easily develop a large number of medical conditions, through either an increase of fat mass or an increase in the number of fat cells. Overall, people with higher BMI values tend to have higher risks of coronary heart disease (CHD), atherosclerotic diseases, and type II diabetes (Haslam & James, 2005).

1.1.2. Adipose Tissues

In addition to the BMI approach to define obesity, there are other indications to verify obesity levels, such as adipose tissues and lipid profiles. Adipose tissue is loose connective tissue which is composed of adipocytes. The general function of adipose tissue is that it is the major storage site for fat in the form of triglyceride. It also serves as

an important endocrine organ responsible for the secretion of leptin and resistin (Albright & Stern, 1998). There are two different kinds of adipose tissues: one is white adipose tissue (WAT) and the other is brown adipose tissue (BAT) (Kershaw & Flier, 2004).

White adipose tissue serves several functions, providing a source of energy, heat insulation, and a cushion for organs. White adipocytes are cells with larger fat droplets than found in brown adipose tissue (Albright & Stern, 1998), as is illustrated in Figure 1.2. White adipose tissue is found directly below the skin which contributes mainly to heat insulation. The size of white adipose cells ranges from 25 to 200 microns whereas brown adipose cells are roughly 60 microns (Lowell & Flier, 1997).

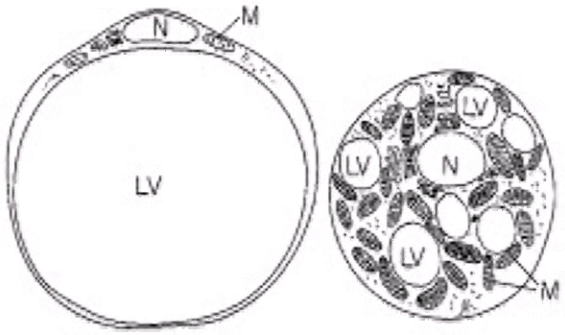


Figure 1.2. Cross Section Images of White (left) and Brown Adipose Cell (right) (Albright & Stern, 1998).

LV: lipid vacuole; M: mitochondria; N: nucleus.

Brown adipose tissue is named for its color and is present in newborns and in a specialized form in hibernating mammals. Brown adipose tissue is rich with vascularization and densely packed with mitochondria and appears to be single lipid

droplets, as shown in Figure 1.2. Brown adipose tissue is generally located in deep cervical regions such as the interscapular and paravertebral regions. In order to function as a heat generation site, a series of reactions take place in the mitochondria in these cells and result in the breakdown of fatty acids. This metabolic degradation process is also called beta-oxidation. If fatty acids undergo beta-oxidation for ATP production, they move from adipocytes into the blood and are carried to the tissues as an energy source (Albright & Stern, 1998). The thermogenic process is extremely important to newborns exposed to cold environments (Himms-Hagen, 1990).

Chapter 2.

Literature Review

2.1. Resveratrol (RTL)

Resveratrol (3,5,4'-trihydroxystilbene) was first isolated from the roots of white hellebore (Veratrum grandiflorum O. Loes) in 1940 (Baur & Sinclair, 2006) and later also purified from the roots of the oriental medicinal plant Polygonum Capsidatum in 1963 (Holme & Pervaiz, 2007). Resveratrol is not abundant in typical diets but relatively rich in the skin of grapes (Gu, Creasy & Kester, 1999). The concentration of resveratrol in red wine ranges from trace amounts to 14 mg/L with a mean of 1.9 ± 1.7 mg (Stervbo, Vang & Bonnesen, 2006) depending on the year of production and the wine aging process. Resveratrol is also found in less significant concentrations in peanuts, at about 0.02-1.8 mg/L (Sanders, McMichael & Hendrix, 2000). The bioavailability of resveratrol is very low. Once it is absorbed, it is immediately metabolized to glucuronide and sulfate derivatives (Espin, Garcia-Conesa & Tomas-Barberan, 2007) and the levels of metabolites are dose dependent (Marier, Vachon, Gritsas, Zhang, Moreau & Ducharme, 2002). Resveratrol is a fat soluble compound. The structure of trans-resveratrol and its major derivative, piceid (resveratrol-3-O-β-D -glucoside) are illustrated in Figure 2.1.

Resveratrol is characterized as a polyphenolic phytoalexin and most noticeable for its anti-thrombogenic, anti-inflammatory, cardio-protective (Bradamante, Barenghi & Villa, 2004), neuro-protective, and cancer preventive and therapeutic activities (Jang, Cai,

Udeani, Slowing, Thomas, Beecher et al., 1997). Additionally, anti-aging effects are observed in yeast (Howitz, Bitterman, Cohen, Lamming, Lavu, Wood et al., 2003; Valenzano, Terzibasi, Genade, Cattaneo, Domenici & Cellerino, 2006) as well as in vertebrates (Valenzano, Terzibasi, Genade, Cattaneo, Domenici & Cellerino, 2006).

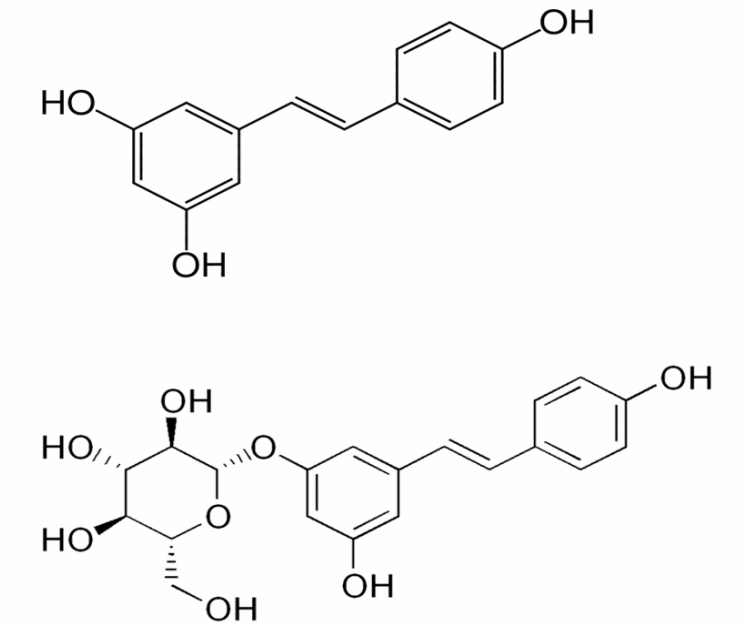


Figure 2.1. Structures of Trans-Resveratrol (upper) and Piceid (lower) (Baur & Sinclair, 2006).

2.1.1. Resveratrol and Obesity

Resveratrol can be related to the obesity issue through different aspects. Resveratrol has been considered to demonstrate caloric restriction actions in lower organisms (Wood, Rogina, Lavu, Howitz, Helfand, Tatar et al., 2004). Caloric restriction refers to reduction of energy intake (typically 30-40 % in rodents) and is widely considered to be the most productive way to promote health and further extend longevity through the activation of sirtuin proteins (Barger, Walford & Weindruch, 2003).

From the lipid profile point of view, it was reported by Arichi et al. in 1982 that resveratrol could inhibit the cholesterol and triglycerides depositions in livers of rats and decrease the rate of triglyceride synthesis (Arichi, Kimura, Okuda, Baba, Kozawa & Arichi, 1982). Also, in the same paper, it was found that piceid (resveratrol β-glucoside or polydatin) is an effective regulator of serum lipid concentrations (Arichi, Kimura, Okuda, Baba, Kozawa & Arichi, 1982). However, in recent in vivo studies, there were no significant effects of resveratrol on cholesterol and triglyceride concentrations (Turrens, Lariccia & Nair, 1997). Despite this, resveratrol has been shown to reduce the formation of atherosclerotic plaques in rabbits fed a high-fat diet (Wang, Zou, Cao, Hsieh, Huang & Wu, 2005).

2.2. Black Tea Extract (BTE)

Teas are derived from the leaves of Camellia sinensis. Different teas result from different processing methods. Black tea, for example, is produced in a four-step process: withering, rolling, oxidation and drying (Winsome, 2008). The objective of the withering step is to reduce moisture content by up to 70 %. The rolling process breaks the tea leaves to aid in the oxidation process. Fresh tea leaves are rich in catechins and also contain polyphenol oxidase in separate cell compartments from catechins. The most significant difference between black and green tea is that black tea undergoes an oxidation process, while green tea does not. When tea leaves are broken, contact between catechins and polyphenol oxidase causes the catechins to form dimmers and polymers known as theaflavins and thearubigins, whose structures are shown in Figure 2.2.

The process of making black tea maximizes the interaction between catechins and polyphenol oxidase. Therefore, black tea is rich in theaflavins and thearubigins, but relatively low in catechins, such as EGCG (Lakenbrink, Lapczynski, Maiwald & Engelhardt, 2000). Black tea contains about 200 mg flavonoids per 235 ml cup (Wiseman, Balentine & Frei, 1997). Table 2.1. shows the main components found in black tea beverages (Dufresne & Farnworth, 2001).

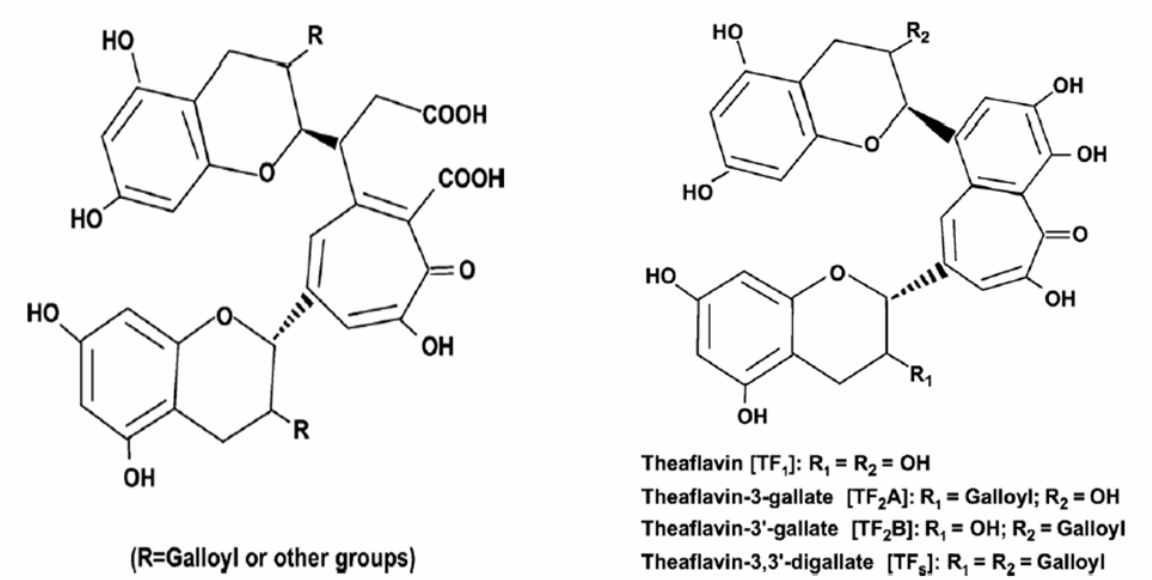


Figure 2.2. Structure of the Thearubigins (left) and Theaflavins (right) (Lambert & Yang, 2003).

Catechins	3-10	Methylxanthins	8-11
Theaflavins	3-6	Carbohydrates	15
Thearubigins	12-18	Protein	1
Flavonols	6-8	Mineral matter	10
Phenolic acids and depsides	10-12	Volatiles	<0.1
Amino acids	13-15		

Table 2.1. Main Components of Black Tea Beverages (Dufresne & Farnworth, 2001).

Components measured in wt% of extract solids.

2.2.1. Black Tea and Obesity

It is a common belief in Asian countries that tea can help control and prevent obesity. Obese people are seldom found among long-term tea-drinking individuals (Dufresne & Farnworth, 2001). This general concept applies to all kinds of teas. Based on biochemical and pharmacological studies of tea catechins, the mechanism in tea for obesity prevention in mice may be through stimulating hepatic lipid metabolism (Murase, Nagasawa, Suzuki, Hase & Tokimitsu, 2002), inhibiting gastric and pancreatic lipases (Han, Takaku, Li, Kimura & Okuda, 1999), stimulating thermogenesis (Han, Takaku, Li, Kimura & Okuda, 1999), modulating appetite, and demonstrating synergism with caffeine (Zheng, Sayama, Okubo, Juneja & Oguni, 2004). Tea catechins are also suggested to suppress fatty acid synthase (FAS) in breast cancer cells (Yeh, Chen, Chiang, Lin-Shiau & Lin, 2003).

Black tea consumption has been shown to reduce total and low-density lipoprotein (LDL) cholesterol by 11.1 % in mildly hypercholesterolemic adults (Davies, Judd, Baer, Clevidence, Paul, Edwards et al., 2003). In diet-induced obese rats, a Keemun black tea extract reduced food intake, body weight and plasma triglyceride levels via oral administration in rats. In the same study, it was also found that black tea extract inhibited fatty acid synthase (FAS), although this positive effect was reduced when the black tea extract was prepared with boiling water (Du, Wang, Wu & Tian, 2005). A powder formulation of African black tea extract can provide steady glucose-lowering effects both in short- and long-term treatments in mice (Shoji & Nakashima, 2006).

2.3. Caffeine (CF)

Caffeine belongs to a class of compounds called methylxanthines, and the structure of caffeine and its metabolites are illustrated in Figure 2.3. (Fredholm, 1995). Caffeine can be found in a large number of plants, including coffee, tea, cola nuts, cacao beans, and guarana (Kovacs & Mela, 2006). Brewed coffee contains about 563 mg/L of caffeine, while green tea contains about 63 mg/L and soft drinks about 96 mg/L (Interest, 1996).

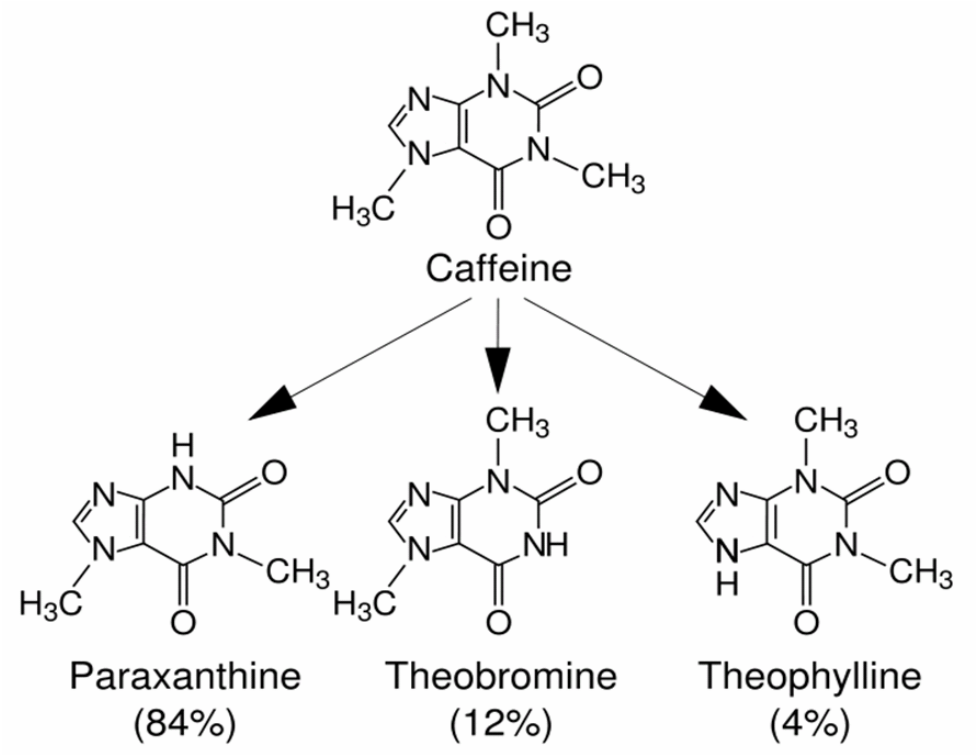


Figure 2.3. Caffeine and its Metabolites (Fredholm, 1995).

2.3.1. Caffeine and Obesity

Caffeine acts through inhibition of phosphodiesterase which is an enzyme that degrades intracellular cyclic AMP (cAMP) and results in increased cyclic AMP

concentration in the cell and prolonged noradrenalin release. It also acts through adenosine antagonism (Dulloo, Seydoux & Girardier, 1992). The pathway is illustrated in Figure 2.4. Some animal studies have shown that methylxanthines are effective compounds for energy expenditure and weight control. There is also report which found the administration of caffeine and ephedrine combined to be an effective weight control method through increased energy expenditure or reduced food intake in rodents and monkeys (Dulloo & Miller, 1987).

For human studies, short term thermogenic effects of caffeine in the range of 100-600 mg have been reported in lean and obese individuals (Koot & Deurenberg, 1995). The thermogenic impact of methylxanthines may be due to the stimulation of substrates in the Cori cycle (the conversion of glycogen and glucose to lactate) or the FFA-triglyceride cycle (Astrup & Toubro, 1993). Also, there is evidence showing that caffeine stimulates lipolysis and fat oxidation. However, caffeine lacks any long term effect on thermogenesis. One possible explanation may be the compensatory effect on appetite and energy intake which would have an inverse effect on energy expenditure (Kovacs & Mela, 2006).

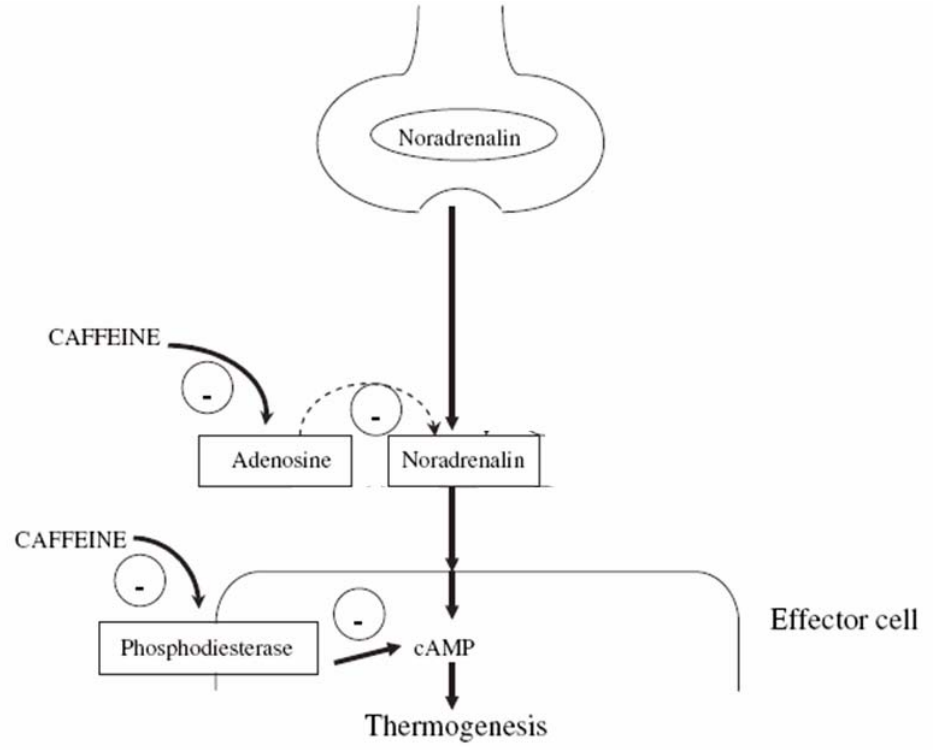


Figure 2.4. The Pathway of Caffeine's Influence on Thermogenesis (Kovacs & Mela, 2006).

Chapter 3.

Materials and Methods

3.1. Animal and Diets

Female CD-1 mice, 5 weeks old, were purchased from Charles River Laboratories (Kingston, NY) and the animals were kept in the animal facility for at least one week before use. CD-1 mice were randomly assigned to a control group and four test groups ([*]n = 10). The initial average body weight in each group was kept at 24.7 grams. All mice were housed in cages under controlled environmental conditions with temperatures maintained between 70 to 74 degree Fahrenheit, humidity at 45 to 55 % and a 12 hour light- dark cycle (lights on from 6 A.M. to 6 P.M.). The bedding type used was the Beta-chip (heat-treated laboratory bedding), which was changed weekly. All mice were given free access to tap water and diets.

Control animals were fed with a high-fat diet consisting of 20 % corn oil in AIN-76A diet which is a specified rodent formula recognized by the American Institute of Nutrition (AIN). Test groups were fed with selected kinds of natural compounds added in high-fat diets. Resveratrol and caffeine were purchased from Sigma-Aldrich Inc. and black tea was a kind gift from Dr. Chi-Tung Ho, Rutgers University, New Jersey. The diets for all groups had equal calorie distribution. Groups include:

[*] The symbol “n” indicates the number of mice in each group.

(1) High-fat diet (20 % corn oil in AIN-76A).

(2) 0.06 % resveratrol in high-fat diet.

(3) 0.2 % black tea extract in high-fat diet.

(4) 0.05 % caffeine in high-fat diet.

(5) 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet.

All diets were purchased from Research Diets Inc. (New Brunswick, NJ) and the diet compositions are depicted in Table I.2. All animals in the study were maintained on their respective diets for 16 weeks before sacrifice.

3.2. Food Intake and Water Consumption

Food and water intake were measured three times a week on a per-cage basis. The average food and water intakes were calculated every week. The unit for food intake is gram/day/mouse; for water consumption it is ml/day/mouse.

3.3. Body Weight Measurement

The body weight of each mouse in each group was measured once a week on a per-cage basis. The average body weight was calculated every week.

3.4. Sample Collections

After 16 weeks on the special diets, all mice were sacrificed. Parametrial fat pads, retroperitoneal fat pads, brown adipose tissues, spleens and livers were removed and

weighed immediately. The anatomy of the abdominal region in female mice is illustrated in Figure 3.1. (Cook, 1965). After weighing, all tissues were frozen and kept at -80 degree Fahrenheit. Blood samples were collected from the jugular veins into test tubes with sodium heparin and then underwent certain lipid profile determination methods including the blood glucose level method and the high density lipoprotein cholesterol (HDL-C) level method. Some blood samples were then processed in the centrifuge at 12500 rpm for 1 hour at 4 degree Celsius. The supernatant fractions formed the plasma used to determine the total cholesterol, total triglyceride and leptin levels. The plasma samples were kept at -80 degree Celsius. This project was approved by the Animal Care and Facilities Committee at Rutgers University under the protocol numbers of 08-046 and 99-015. All project members were certified to conduct animal experiments. The procedures for sample collection were performed under consultation (Huang, 2007).

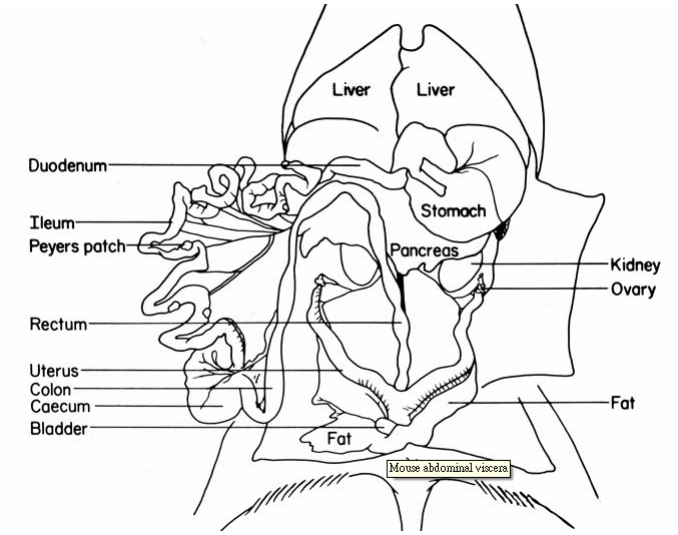


Figure 3.1. The Anatomy of Abdominal Viscera Displayed in Laboratory Female Mice (Cook, 1965).

3.5. Blood Glucose Assay

Blood glucose was measured by the PTS PANELS glucose test strips for use with CardioChek Brand Analyzers manufactured by Polymer Technology Systems, Inc. (Indianapolis, IN). The following procedure was adopted from the product information sheet. The principle of the test is based on the analyzer's reading of differences in light reflection. Each strip contains glucose oxidase (Aspergillus niger), peroxidase (horseradish), 4-aminoantipyrine and N-N-disubstitute aniline as the active ingredients. The analyzer reads light reflected off a test strip that changes color after the blood has been placed on it. The darker the color is, the higher the glucose level. The concentration limitation ranges from 20 to 600 mg/ml.

3.6. High Density Lipoprotein Cholesterol (HDL-C) Assay

Blood HDL levels were measured by the PTS PANELS Lipid Panel Test Strips for use with CardioChek P.A. Analyzer manufactured by Polymers Technology Systems (PTS), Inc. (Indianapolis, IN). The following procedure was adopted from the product information sheet. The principle of the test is based on the concept described below. When blood is applied to a test strip, the blood has enzymatic reactions with chemicals in the strip to produce a color which is read by the analyzer using reflectance photometry built inside the analyzer. The intensity of the color produced is proportional to the concentration. The level limitation ranges from 15 to 100 mg/dl. The schematic principle is shown in Figure 3.2.

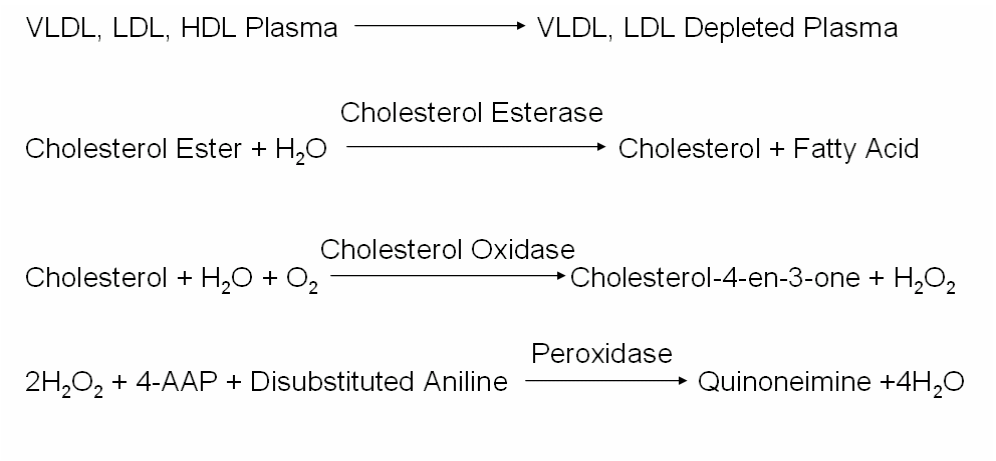


Figure 3.2. The Enzymatic Reaction of the HDL-C Assay.

(Adopted from the product information from PTS, Inc.).

3.7. Total Cholesterol Assay

The cholesterol assay was purchased from Cayman Chemical (Ann Arbor, MI). The following procedure was adopted from the product information for catalog number: 10007640. This cholesterol assay is based on a simple fluorometric method and is sensitive enough to quantify cholesterol levels in serum or plasma. The reactions first take place with cholesteryl esters in serum hydrolyzed by cholesterol esterase into cholesterol. Then the cholesterol is further oxidized by cholesterol oxidase to yield hydrogen peroxide and ketone products. Hydrogen peroxide is then detected by ADHP (10-acetyl-3,7-dihydroxyphenoxazine) which is a sensitive and stable probe for hydrogen peroxide. Then ADHP reacts with horseradish peroxide with a 1:1 stoichiometry to produce the substrate, fluorescent resorufin which can be monitored using excitation wavelengths of 565-580 nm and emission wavelengths of 585-595 nm. The substrate level is measured by the fluorescence reader, Spectra Max M5, purchased from Molecular Devices (Sunnyvale, CA). The schematic principle is shown in Figure 3.3.

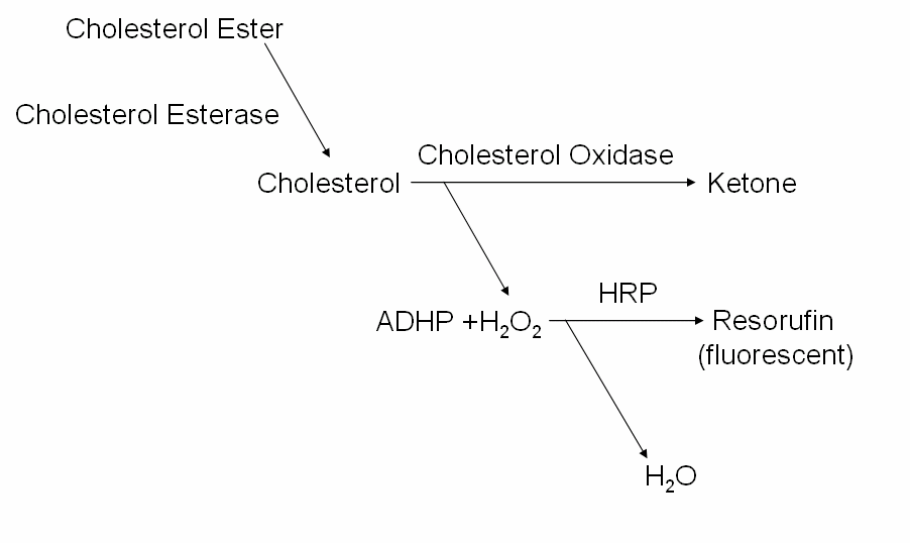


Figure 3.3. The Enzymatic Reaction of the Cholesterol Assay.

(Adopted from the product information for catalog number: 10007640).

3.8. Triglyceride Assay

The assay is conducted with Triglyceride Determination Kit by Sigma-Aldrich Inc. (St. Louis, MO). The following procedure was adopted from the product information for catalog number: TR0100. The assay is for the quantitative measurement of glycerol, true triglycerides and total triglycerides in serum or plasma. The procedure involves enzymatic hydrolysis by lipoprotein lipase to convert triglycerides to glycerol and free fatty acids. The glycerol produced is different from endogenous glycerol and is followed by coupled enzymatic reactions.

For triglycerides, the first step is the hydrolysis of triglycerides by applying lipoprotein lipase to glycerol and free fatty acids. The second step is the phosphorylation of glycerol by adenosine-5'-triphosphate (ATP) to form glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK).

G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Next, peroxidase (POD) catalyzes the coupling reaction of H_2O_2 with 4-aminoantipyrine (4-AAP) and then reacts with sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that has a maximum absorbance at 540 nm. The increase in absorbance is directly proportional to the triglyceride and glycerol concentrations of the samples. The schematic principle is shown in Figure 3.4.

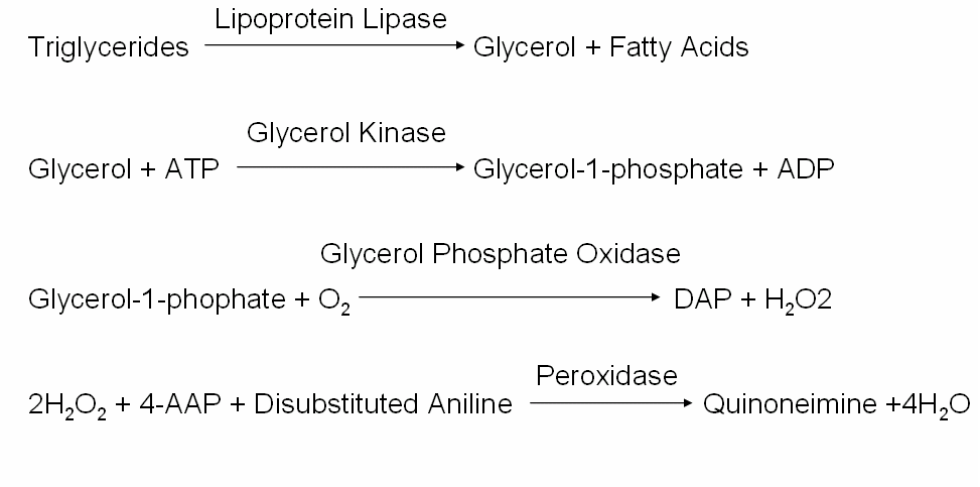


Figure 3.4. The Coupled Enzymatic Reaction of the Triglyceride Assay

(Adopted from the product information for catalog number: TR0100).

3.9. Leptin Level Determination

Leptin levels in plasma were determined by the Enzyme-Linked ImmunoSorbent Assay (ELISA). The ELISA assay kit was purchased from R&D System, Inc. (Minneapolis, MN). The following procedure was adopted from the product information for catalog number: DY498.

The procedure for the mouse leptin assay is to first coat the 96-well plastic plate with 100µl goat anti-mouse leptin (capture antibody, 2.0 µg/ml), leaving it overnight at room temperature. Each well is aspirated three times with wash buffer (0.05 % Tween 20 in PBS, phosphate buffered saline, with pH 7.2 to 7.4) and block plate with 300 µl block buffer (5 % Tween 20 in PBS with 0.05 %NaN_3) for one hour at room temperature. Repeat the wash step and apply 100µl standard mouse leptin or sample with the proper dilution ratio to each well and incubate the plate for two hours at room temperature. Repeat the wash step and apply biotinylated goat anti-mouse leptin (detection antibody, 200 ng/ml) to each well for two hours at room temperature. Again follow the wash step and add 100µl Streptavidin-HRP (streptavidin conjugated with horseradish peroxidase) to each well for 20 minutes in the dark. Repeat the wash step and apply 100µl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) as color reagents for 20 minutes in the dark. Stop the reaction by adding stop solution (2N H_2SO4) to the plate and determine the optical density immediately using a wavelength of 450 nm.

3.10. Statistical Analysis

Data were subjected to 1-way analysis of variance (ANOVA) and student's t-test using SPSS software (version 12.0 for Windows). ANOVA analysis was followed by the least significant difference (LSD) for all parameters to determine the significance of differences among groups. p values of less than 0.05 or 0.01 (as indicated) were considered to demonstrate statistical significance. All results were expressed as mean ± SD (standard deviation).

Chapter 4.

Results and Discussion

4.1. Effects of Modulated Diets on Food Intake in CD-1 Mice

Figure 4.1. shows the food intake of mice fed with modulated diets. Average food intake was not significantly different between groups. The average food intakes (mean ± SD) in the control, RTL, BTE, CF and combination groups were 3.19 ± 30.09, 3.09 ± 0.06, 3.31 ± 0.08, 3.19 ± 0.08 and 3.58 ± 0.07 g/day/mouse, respectively. Therefore, the amounts consumed were similar between the groups. This result suggests that the appetite of mice in all groups remained normal throughout the experiment.

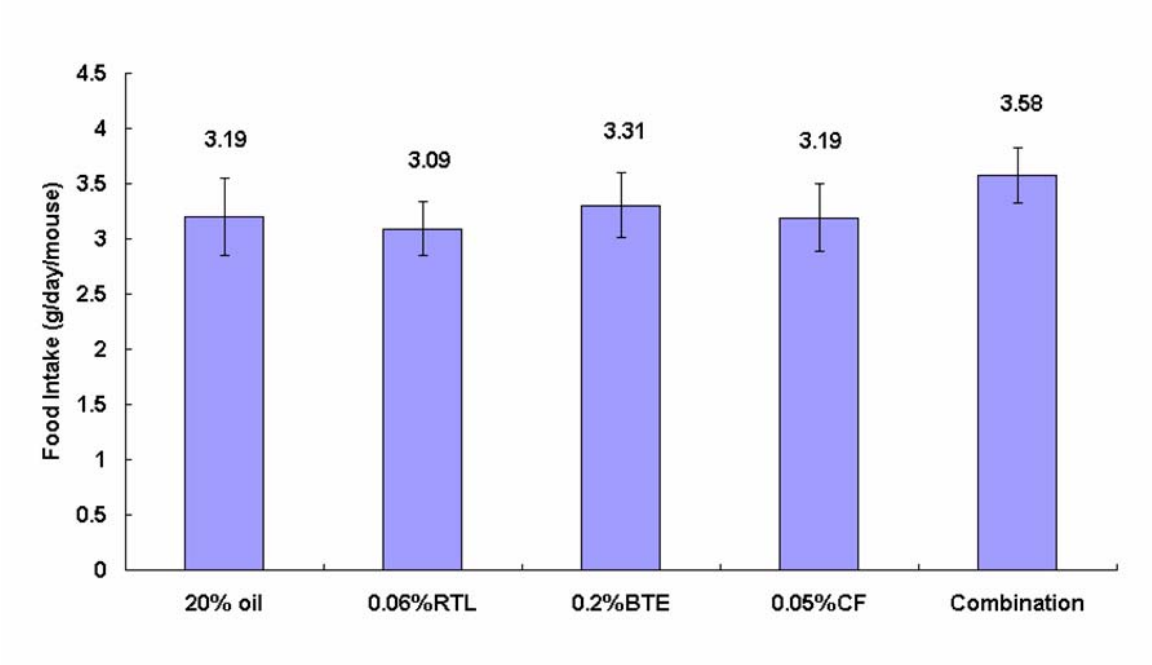


Figure 4.1. Food Intake of CD-1 Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

Food intakes were measured three times a week.

Data are expressed as mean ± SD (n = 10).

4.2. Effects of Modulated Diets on Water Consumption in CD-1 Mice

Figure 4.2. shows the water consumption of mice fed with modulated diets. Average amounts of water consumption (mean ± SD) were 5.34 ± 0.18, 4.73 ± 0.18, 5.34 ± 0.13, 7.49 ± 0.25 and 7.78 ± 0.44 g/day/mouse for the control, RTL, BTE, CF and combination groups, respectively. The water consumption increased significantly in mice on the CF and combination diets. This phenomenon may be due to the thermogenesis effect of the caffeine content in these diets (Kovacs & Mela, 2006). Thermogenesis is a

process for animals to generate body heat. To reach inner temperature equilibrium, more water is needed for animals to lower the body temperature raised by this process. The increased urine flow in humans due to caffeine consumption was reported by Neuhauser-Berthold et al. in 1997 (Neuhauser-Berthold, 1997). In order to reach the equilibrium of internal water content, mice may drink more water when consuming the diets containing caffeine.

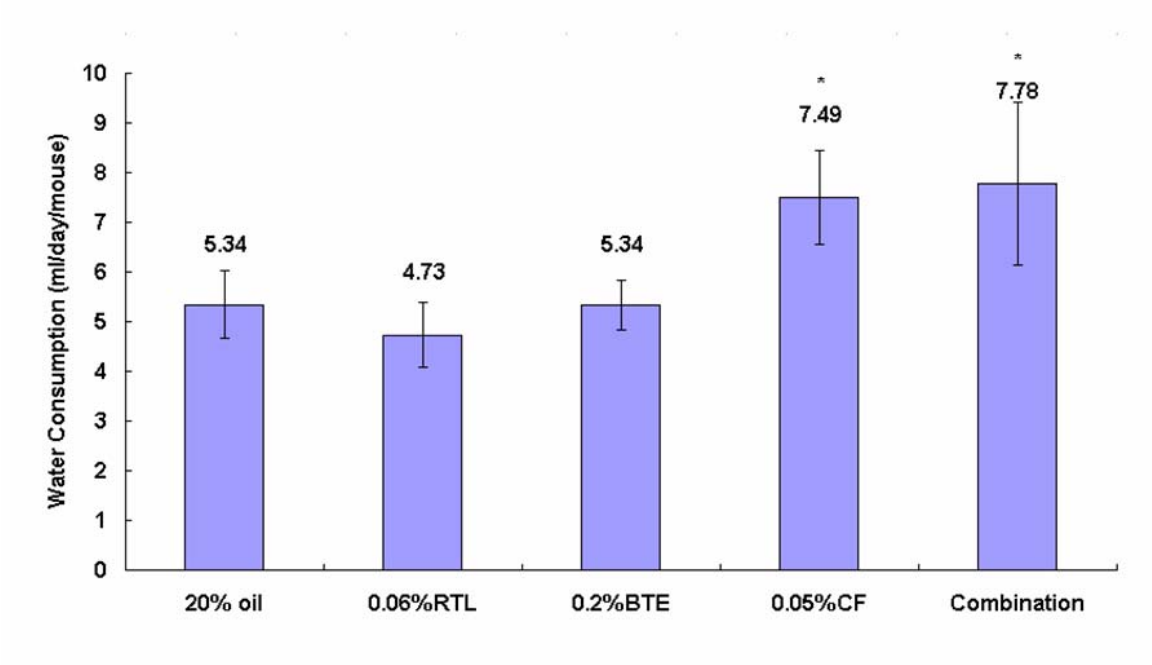


Figure 4.2. Water Consumption of CD-1 Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

Water consumption was measured three times a week.

Data are expressed as mean ± SD (n = 10).

Asterisk represents significant difference as compared to the control group ($p<0.01$).

4.3. Effects of Modulated Diets on Body Weight in CD-1 Mice

Figure 4.3. illustrates the body weight trend of CD-1 mice in all groups during the 16-week period. The average starting body weights (mean ± SD) of mice in the control, RTL, BTE, CF and combination groups were 24.70 ± 1.73, 24.05 ± 1.43, 24.68 ± 1.21 and 24.64 ± 1.43 g, respectively.

Mice in all groups started with similar body weights and steadily gained weight until the 5th week of the experiment. After the 5th week, the body weight of mice on the CF diet increased slower than all the other groups. In the 10th week, mice on CF and the combination diets started to show smaller increase in body weight compared to that of the control, RTL and BTE groups. The final body weights of the control, RTL, BTE, CF and combination groups were 42.65 ± 7.55, 40.40 ± 7.14, 34.46 ± 2.24 and 37.52 ± 5.93 g/mouse, respectively. Mice on the CF diet demonstrated the lowest end body weight at the end of the experiment, followed by mice on the combination diet.

In this experiment, the combination diet showed no synergistic activity toward weight loss in mice. The polyphenolic compounds and caffeine synergistic interaction was suggested by Zheng et al. in 2004 (Zheng, Sayama, Oguni, Juneja & Okubo, 2004). This experiment was conducted on mice fed with 2 % green tea powder, 0.3 % catechins, 0.05 % caffeine and 0.03 % theanine. The reason the combination diet in our experiment did not show expected synergism may be due to insufficient concentration of catechins content compared to that in Zheng et al.'s report.

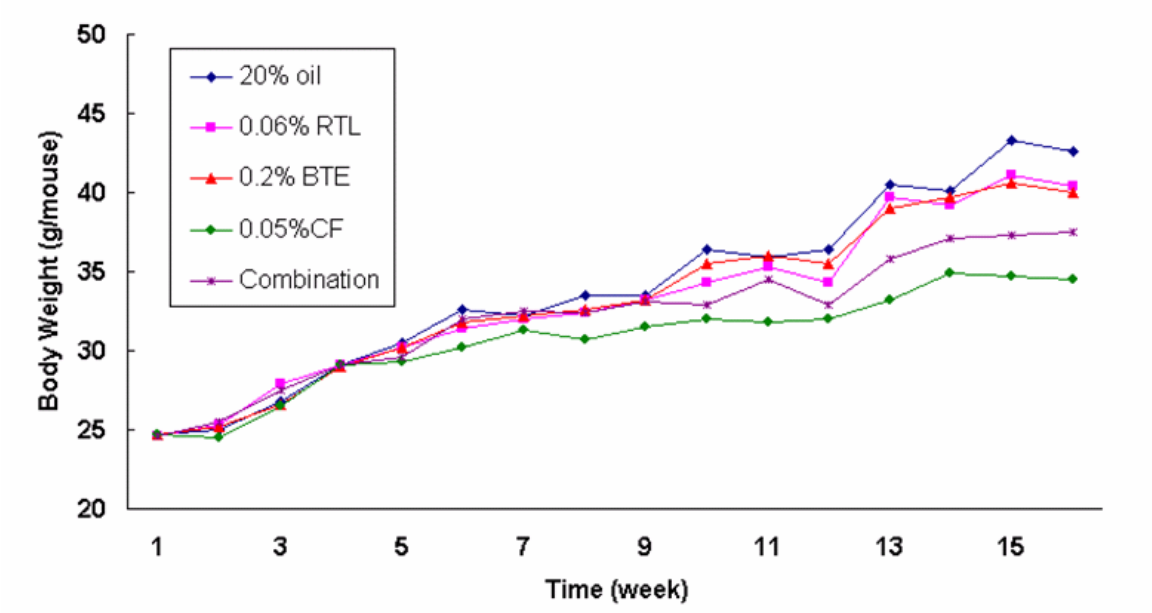


Figure 4.3. Body Weight Trend of CD-1 Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

Body weights of mice were measured weekly.

4.4. Effects of Modulated Diets on Body Weight Gain in CD-1 Mice

The average body weight gains (mean ± SD) were 17.95 ± 2.24, 15.79 ± 1.78, 15.35 ± 2.19, 9.77 ± 0.65 and 12.88 ± 1.73 g/mouse in the control, RTL, BTE, CF and combination groups, respectively. Figure 4.4. shows that the RTL, BTE and combination diets effectively reduced the body weight gain of mice for 12 %, 14 % and 28 %, respectively, as compared to the control group. Mice on the CF diet significantly reduced body weight gain by 46 %. This result suggests that CF has a significant effect on lowering the body weight of mice fed with a high-fat diet.

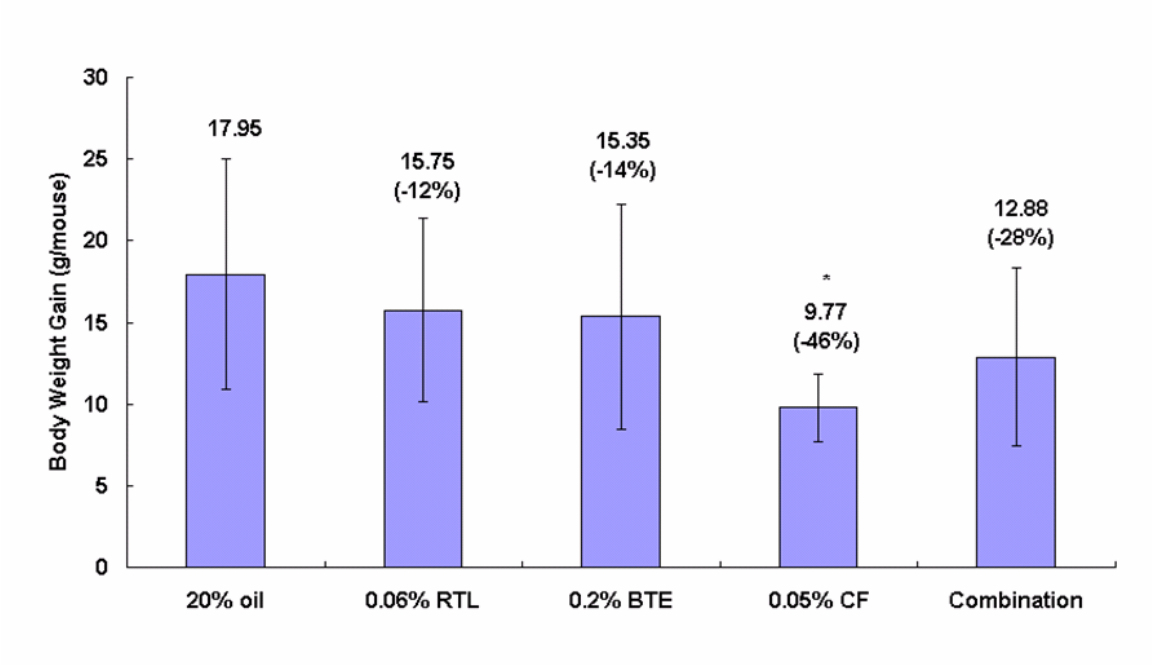


Figure 4.4. Body Weight Gain of CD-1 Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

Body weights of mice were measured weekly.

Data are expressed as mean ± SD (n = 10).

Asterisks represent significant differences compared to the control group ($p<0.01$).

The quantities in brackets "( )" represent the percentage of inhibition compared to the control group.

4.5. Effects of Modulated Diets on Parametrial Fat (P-fat) Pad in CD-1 Mice

Based on the World Heath Organization definition, obesity is defined as excessive fat tissue accumulation in the body. In order to investigate the anti-obesity effects of selected dietary compounds, P-fat pad mass was measured. The average P-fat weights in the control, RTL, BTE, CF and combination groups were 3.00 ± 0.42, 2.35 ± 0.28,

2.29 ± 0.43, 0.69 ± 0.10 and 1.87 ± 0.51 g/mouse, respectively. Figure 4.5. shows that mice on the CF diet significantly reduced their P-fat weight by 77 % as compared to the control group. The fat-lowering effect of caffeine has been demonstrated by Lu et al. in 2001, where it was shown that 0.044 % caffeine solution significantly ($p<0.01$) reduced the size of parametrial fat pad by 56 % in mice (Lu, Lou, Lin, Shih, Huang, Yang et al., 2001).

Mice on the RTL, BTE and the combination diets showed reductions in P-fat weight by 22 %, 23 % and 38 %, respectively, as compared to the control group. However, mice on the combination diet did not show the expected synergistic effect on the reduction of P-fat weight. This result reveals the similar conclusion reported by Lu et al. in 2001. Their study did not show a synergistic fat-lowering effect in mice on a EGCG-caffeine combined fluid. Therefore, more investigation into the potential for a synergistic effect of a combination diet is needed.

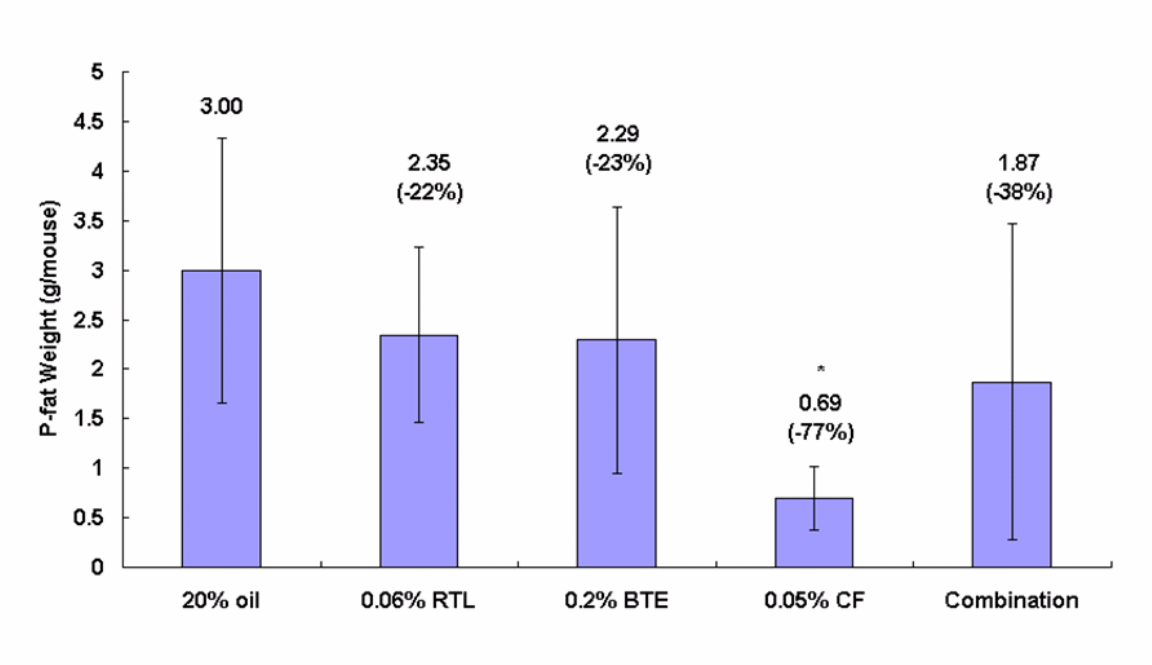


Figure 4.5. Parametrial Fat Pad Mass in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and parametrial fats were weighed.

Data are expressed as mean ± SD (n = 10).

Asterisks represent significant differences compared to the control group ($p<0.01$).

The quantities in brackets "( )" represent the percentage of inhibition compared to the control group.

4.6. Effects of Modulated Diets on Retroperitoneal Fat (R-fat) Pad in CD-1 Mice

The size of R-fat in mice is generally smaller than that of P-fat. Even though smaller in size, data showed the same pattern in all groups with R-fat. This result suggested that mice on the RTL, BTE, CF and combination diets showed consistent

reduction in white adipose tissues including P-fat and R-fat. The average R-fat weights (mean ± SD) in the control, RTL, BTE, CF and combination groups were 0.62 ± 0.14, 0.47 ± 0.07, 0.41 ± 0.07, 0.14 ± 0.03 and 0.36 ± 0.08 g/mouse, respectively. Figure 4.6. shows that mice on the CF diet significantly reduced their R-fat weight by 77 %, as compared to the control group. Even without this degree of significance, mice on the RTL, BTE and the combination diets still reduced their R-fat weights by 25 %, 34 % and 38 %, respectively, as compared to the control group.

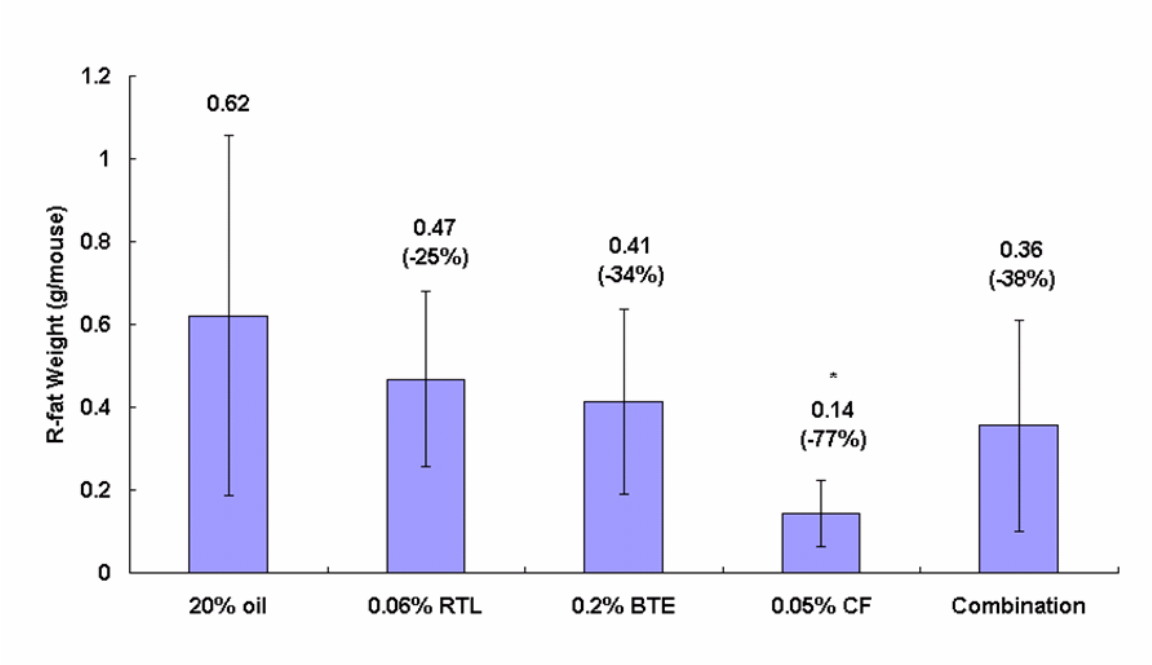


Figure 4.6. Retroperitoneal Fat Pad Mass in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and retroperitoneal fats were weighed.

Data are expressed as mean ± SD (n = 10).

Asterisks represent significant differences as compared to the control group ($p<0.01$).

The quantities in brackets "( )" represent the percentage of inhibition compared to the control group.

4.7. Effects of Modulated Diets on Brown Adipose Tissue (BAT) in CD-1 Mice

To elucidate the inhibitory effects of modulated diets on fat tissue mass, we measured the weight of brown adipose tissue. The average BAT weights in the control, RTL, BTE, CF and the combination groups were 0.51 ± 0.06, 0.50 ± 0.10, 0.49 ± 0.07,

0.22 ± 0.02 and 0.35 ± 0.07 g/mouse, respectively, as shown in Figure 4.7. Mice on CF diet showed significant reduction in BAT weight by 57 % whereas mice on the combination diet showed 32 % reduction in BAT weight without significance. However, mice on RTL, BTE diets merely reduced their BAT weight by 2 % and 5 %, as compared to the control group. This reduction rate is relatively low compared to that of white adipose tissues.

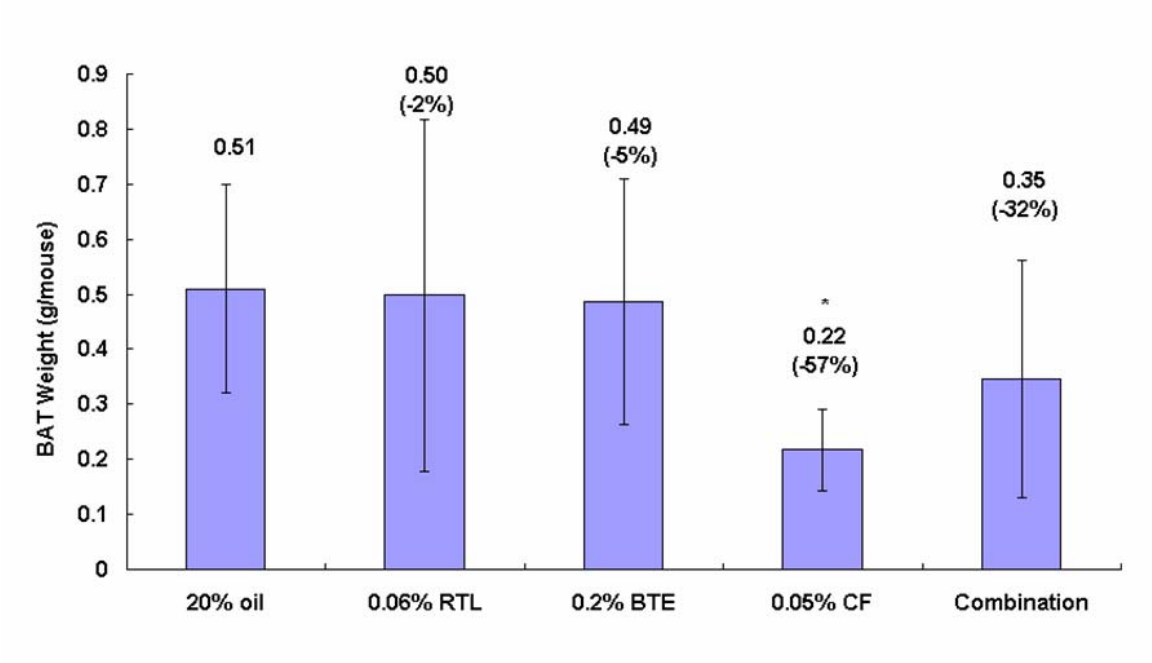


Figure 4.7. Brown Adipose Tissue Mass in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks and brown adipose tissues were weighed.

Data are expressed as mean ± SD (n = 10).

Asterisk represents significant difference as compared to the control group ($p<0.01$).

The quantities in brackets "( )" represent the percentage of inhibition compared to the control group.

4.8. Effects of Modulated Diets on Liver Weight in CD-1 Mice

To investigate the toxicity or any undesirable side effect of the modulated diets, we measured the live weight. The liver is generally considered to have an enzyme-induced detoxification function. Therefore, enlargement of the liver can be an indicator of possible pathological development in the body. Figure 4.8. shows the liver weights of all groups. The average liver weights (mean ± SD) of the control, RTL, BTE, CF and

combination groups were 1.56 ± 0.12, 1.61 ± 0.10, 1.50 ± 0.07, 1.53 ± 0.06 and 1.52 ± 0.07 g/mouse, respectively. There is no significant difference among all groups. Therefore, this result implies these modulated diets have no significant influence on liver weights, which were in a normal condition following experimentation period.

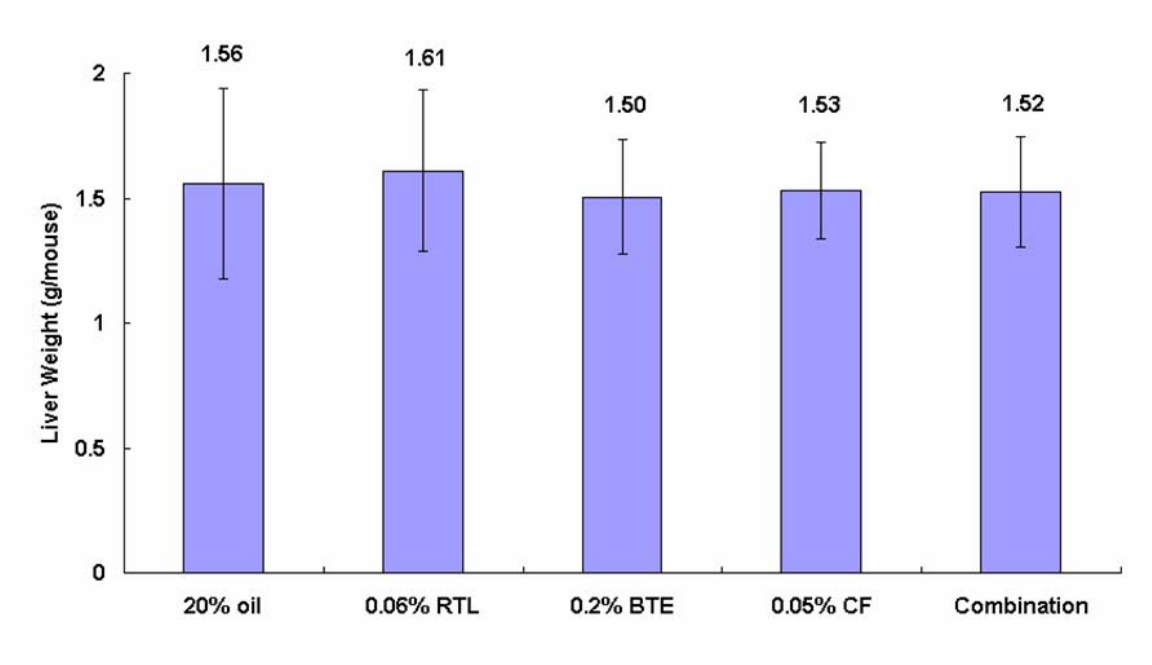


Figure 4.8. The Liver Tissue Mass in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and liver tissues were weighed.

Data are expressed as mean ± SD (n = 10).

4.9. Effects of Modulated Diets on Spleen Weight in CD-1 Mice

The spleen is regarded as one of the centers of immune system mechanisms in the body, therefore, it serves along with the liver as an indicator of toxicity. The spleen induces the immune response to foreign substances in order to protect the body. Figure 4.9. shows the spleen weights of all groups. The average spleen weights in the control, RTL, BTE, CF and combination groups were 0.15 ± 0.01, 0.13 ± 0.01, 0.14 ± 0.01, 0.14 ± 0.01 and 0.16 ± 0.01 g/mouse, respectively. There is no significant

difference in spleen weights among the groups. This result may imply the normal condition of spleens, namely that the spleens show no sign of toxicity from the diets.

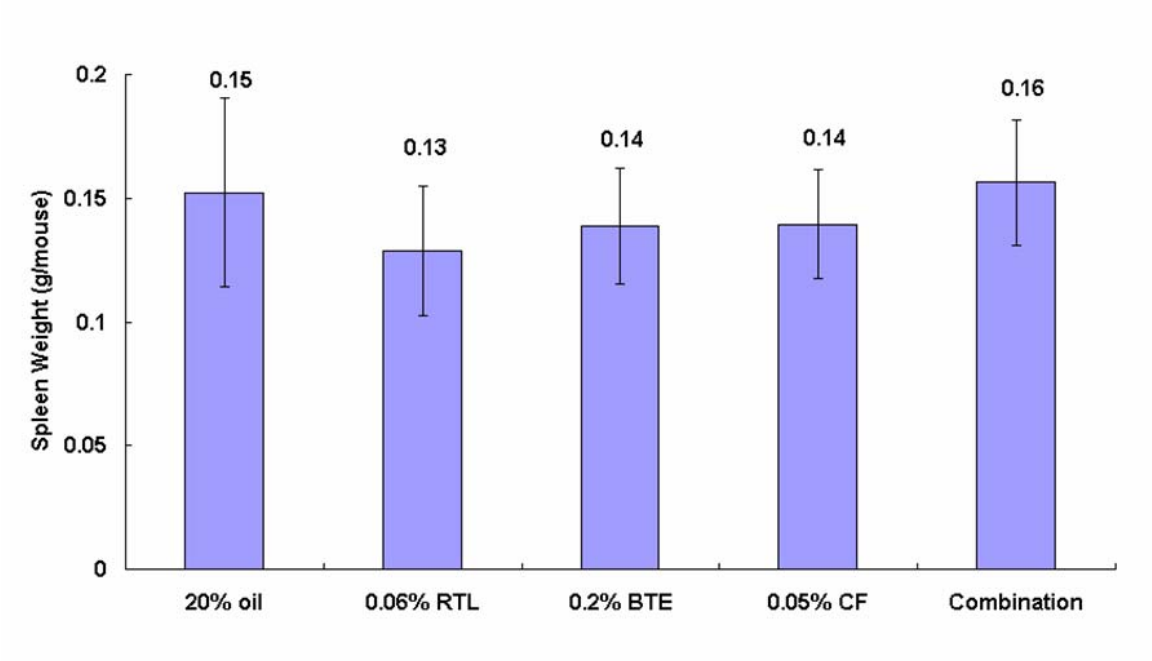


Figure 4.9. The Spleen Tissue Mass in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks and spleen tissues were weighed.

Data are expressed as mean ± SD (n = 10).

4.10. Effects of Modulated Diets on Blood Glucose in CD-1 Mice

In this experiment, the glucose-lowering effect of modulated diets in mice was investigated. The blood glucose level in mice fed with the BTE diet was suppressed by 6 % compared to that of the control group, as shown in Figure 4.10. According to results

reported by Shoji and Nakashima in 2006, African black tea extract has shown a beneficial glucose-lowering effect in diabetic mice (Shoji & Nakashima, 2006). Many papers have proposed the possible mechanisms to explain how catechins lower glucose levels. Since black tea extract is rich in theaflavin and other polyphenols, one conclusive mechanism is that these compounds suppress digestive enzyme activity such as α-amylase and α-glucosidase (Nanjo, Honda, Okushio, Matsumoto, Ishigaki, Ishigami et al., 1993). It is also proven that black tea extract can control the glucose uptake through the intestinal tract (Shimizu, Kobayashi, Suzuki, Satsu & Miyamoto, 2000) and further suppress the digestion of carbohydrates. However, mice on the RTL, CF and combination diets demonstrated no relation to a glucose-lowering effect in this study.

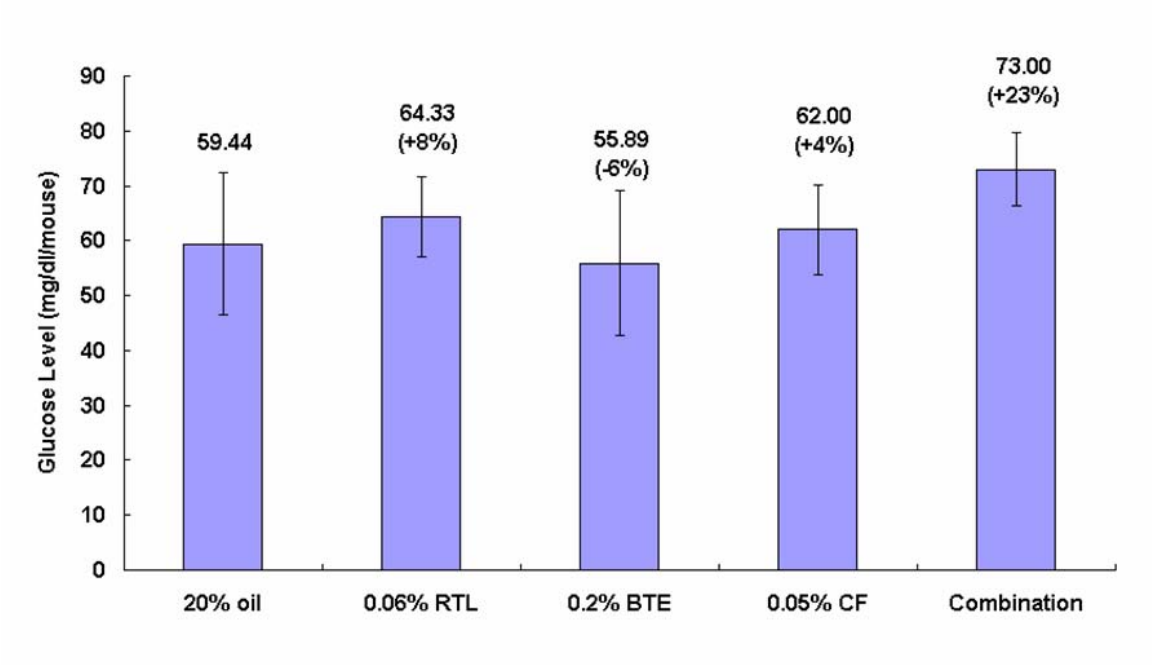


Figure 4.10. The Glucose Level in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and glucose levels were measured.

Data are expressed as mean ± SD (n = 10).

The quantities in brackets "( )" represent the percentage of inhibition or promotion compared to the control group.

4.11. Effects of Modulated Diets on Cholesterol Level in CD-1 Mice

The cholesterol-lowering effect of modulated diets in mice was investigated in this experiment. The total cholesterol level in mice fed with the BTE diet was significantly suppressed by 30 % compared to that of the control group, as shown in Figure 4.11. The catechins in black tea extract, such as theaflavins and thearubigins have

been reported to have cholesterol-lowering effect in rats fed with sucrose rich diets with total catechin content of 6.12 % (Yang, Wang & Chen, 2001).

It is interesting to note that mice on the RTL and combination diets showed significant increase in total cholesterol levels. In contrast to our results, a cholesterol-lowering effect for RTL has been reported in hamster studies. In the hamster study, RTL (0.025 g/kg diet) showed the ability to lower the total serum cholesterol and total hepatic lipid concentration levels in hamsters fed with a high-fat diet (Cho, Ahn, Kim, Choi & Ha, 2008). However, in a rabbit study, it was shown that there was no significant difference in cholesterol levels between the control group and the experimental group receiving oral RTL (Cho, Ahn, Kim, Choi & Ha, 2008). Therefore, the cholesterol-lowering effect of RTL is in need of further study.

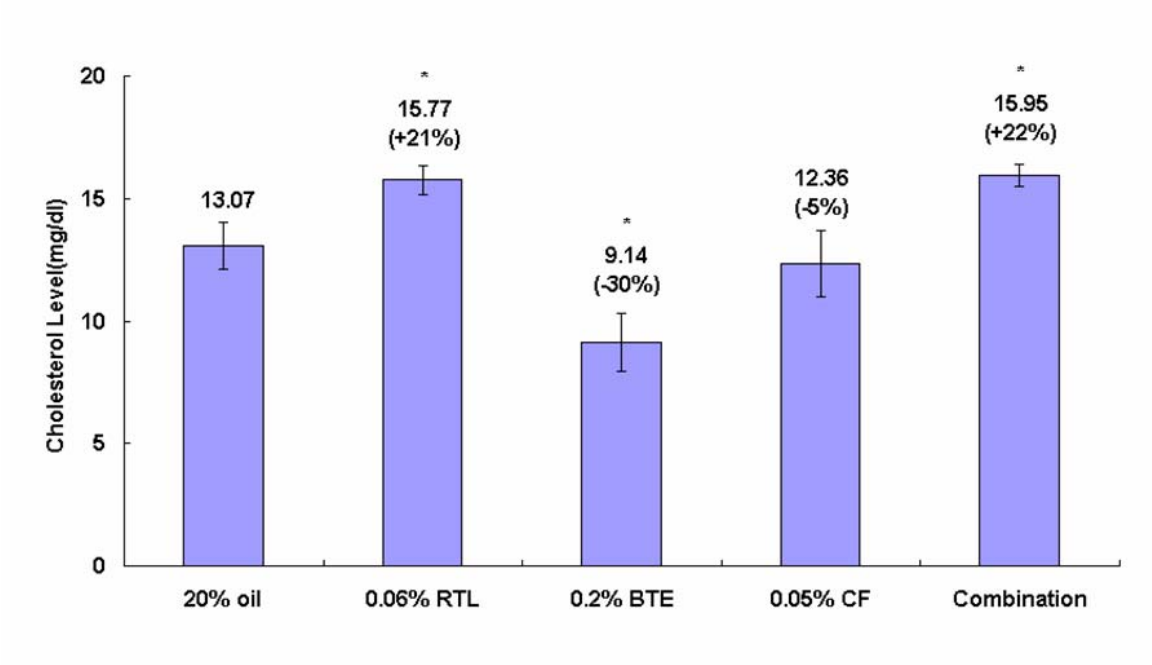


Figure 4.11. Total Cholesterol Level in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks and cholesterol levels were assayed.

Data are expressed as mean ± SD (n = 10).

Asterisks represent significant differences as compared to the control group ($p<0.01$).

The quantities in brackets "( )" represent the percentage of inhibition or promotion compared to the control group.

4.12. Effects of Modulated Diets on Triglyceride Level in CD-1 Mice

In this experiment, the total triglyceride level in mice fed with modulated diets was investigated. Mice on the BTE diet reduced their triglyceride level by 30% compared to that of the control group, as shown in Figure 4.12. Yang et al. reported that black tea with a 6.12 % catechin content can lower total triglyceride concentration in plasma (Yang,

Wang & Chen, 2001). Except for the BTE group, the RTL, CF and combination diets showed no relation to any triglyceride-lowering effect in this experiment.

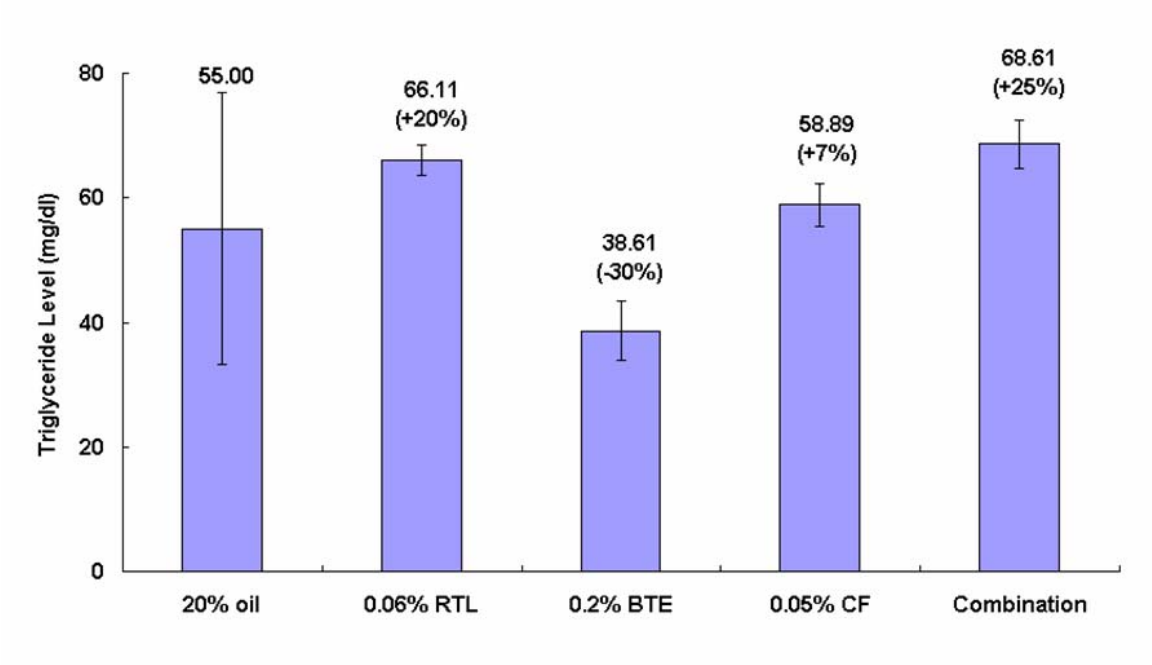


Figure 4.12. Triglyceride Level in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and serum triglyceride levels were measured.

Data are expressed as mean ± SD (n = 10).

The quantities in brackets "( )" represent the percentage of inhibition or promotion compared to the control group.

4.13. Effects of Modulated Diets on High Density Lipoprotein Cholesterol (HDL-C) in CD-1 Mice

In this experiment, the HDL-C level in mice fed with modulated diets was investigated. Mice on the BTE, CF and combination diets showed reductions in HDL-C levels of 1 %, 16 % and 0.3 %, respectively, as shown in Figure 4.13. In contrast, mice on the RTL diet showed an increase in HDL-C levels of 24 %. From an epidemiological

point of view, high concentrations of HDL-C have a protective value against cardiovascular diseases (Lowell & Flier, 1997). The recommended range for humans is over 60 mg/dl according to the American Heart Association. Regardless of the difference between animals and human beings, higher HDL-C levels are considered to be beneficial from any health perspective.

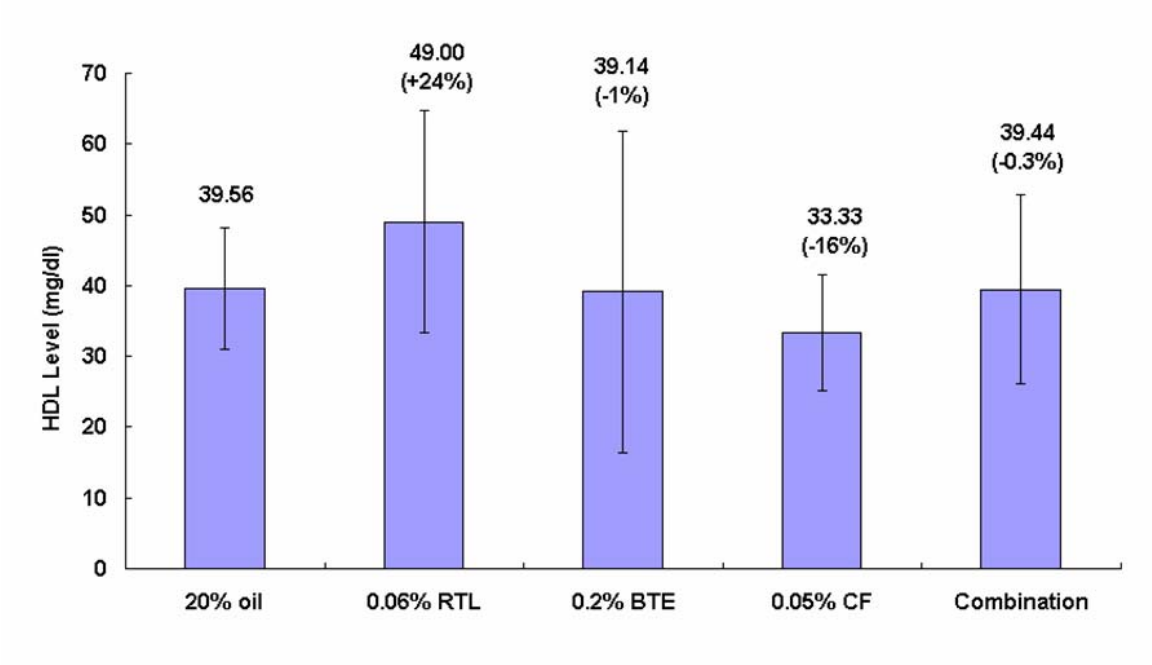


Figure 4.13. HDL-C Level in Mice Fed with Modulated Diets.

Female CD-1 mice (5 weeks old) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks and HDL-C levels were measured.

Data are expressed as mean ± SD (n = 10).

The quantities in brackets "( )" represent the percentage of inhibition or promotion compared to the control group.

4.14. Effects of Modulated Diets on Leptin Level in CD-1 Mice

To further elucidate the anti-obesity effects of RTL, BTE, CF and their combination, we analyzed plasma leptin levels as a biomarker to quantify obesity levels in mice. Leptin levels are positively correlated to the level of adipose tissue accumulated in the body. The average leptin levels (mean ± SD) in the control, RTL, BTE, CF and combination groups were 88.37 ± 14.15, 56.85 ± 18.75, 35.92 ± 10.68, 10.97 ± 1.34 and 18.25 ± 1.26 pg/mouse, respectively, as shown in Figure 4.14.

Mice on the CF and combination diets demonstrated significant reduction in leptin levels by 88 % and 79 % ($p<0.01$). Mice on the BTE diet showed significant reduction in leptin levels by 59 % ($p<0.05$) compared to that of the control. Even without significant results, mice on the RTL diet reduced their leptin levels by 36% compared to that of the control. The results revealed the same pattern with the adipose tissue. It may suggest that the BTE, CF and combination diets had a significant negative influence on leptin levels as well as on adipose tissue deposition.

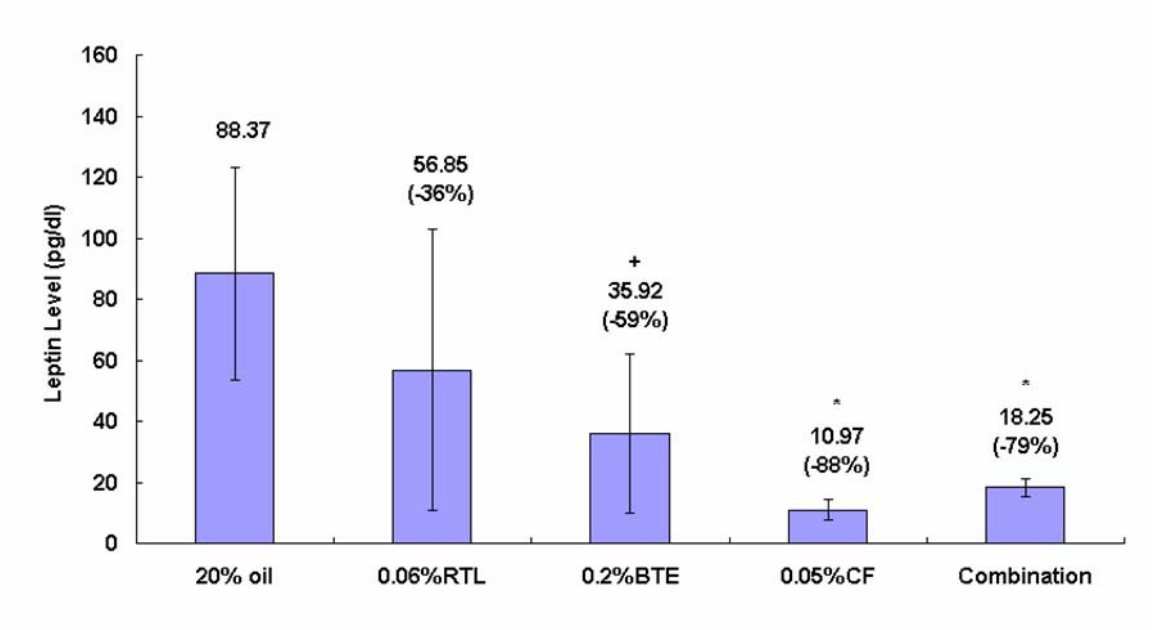


Figure 4.14. The Leptin Level in Mice Fed with Modulated Diets.

Female CD-1 mice, 5 weeks old (10 mice per group) were given a high-fat diet (AIN-76A 20 % corn oil), 0.06 % resveratrol, 0.2 % black tea extract, 0.05 % caffeine in high-fat diets individually and 0.03 % resveratrol, 0.1 % black tea extract and 0.025 % caffeine in high-fat diet in combination and water *ad libitum* for 16 weeks.

All mice were sacrificed after 16 weeks on the modulated diets and leptin levels were measured.

Data are expressed as mean ± SD (n = 10).

Asterisks represent significant differences as compared to the control group ($p<0.01$), and the symbol "+" indicates even greater difference ($p<0.05$).

The quantities in brackets "( )" represent the percentage of inhibition compared to the control group.

4.15. Summary

We demonstrated the anti-obesity effects of resveratrol, black tea extract, caffeine, and their combination in CD-1 mice. The modulated diets incorporated with specific compounds showed an effect on weight loss effect without a changed in energy input in mice. Additionally, they all reduced fat tissue accumulation in accordance to the loss in body weight. While modulated diets had no influence on animals' appetite, fluid intake,

on the other hand, was influenced by the added compounds (for the caffeine and combination groups). Generally, black tea extract had the greatest influence on most of the lipid profiles (cholesterol and triglyceride). All of the modulated diets demonstrated a leptin-lowering effect which confirmed the body weight loss and fat tissue inhibition results. The liver and spleen measurements showed no obvious toxicity in the modulated diets.

Chapter 5.

Preliminary Work

5.1. Relationship between Emulsion System and Obesity

We successfully demonstrated the anti-obesity effect of RTL, BTE, CF and their combination in mice. However, some research has shown that flavonoids show poor absorption in the body. Therefore, the ultimate goal is to incorporate the selected compounds into a stable emulsion system. Eventually, the emulsion can carry the compound as a core material and be tested on the cell or animal model. Therefore, the objective of the preliminary work is to investigate whether the emulsion system with incorporated materials is expected to show better performance compared to the compounds alone. In this preliminary section, we determine the optimum condition of a stable emulsion, a result which can be applied to weight loss products and thus serve to benefit human health.

5.2. Emulsion Background

An emulsion is a mixture of two immiscible substances. One substance (the dispersed phase) is dispersed in the other (the continuous phase). With the help of proper emulsifiers, the stability of emulsions can be enhanced. The importance of emulsion is that they control the physical properties, flavors and stability in many food systems such as butter, ice cream and salad dressing (J.M. Whittinghill, 2000). Emulsions can undergo many changes during storage that could result in breakdown and phase separation.

Therefore, it is critical to maintain the stability of the emulsion and so maintain product quality.

A stable emulsion is defined as the inevitable process of phase separation that has been slowed down to an extent that it has less influence during long term storage (J.M. Whittinghill, 2000). There are four steps for destabilization of emulsions: flocculation, coalescence, creaming and finally phase separation. The process is illustrated in Figure 5.1. The initial process is flocculation in which droplets aggregate. The second step is coalescence, where the thin film between flocculated droplets bursts and drops combine to form larger ones. The creaming process occurs due to the density of substances. Finally, phase separation takes place and results in total emulsion breakdown (Al-Bawab & Friberg, 2006).

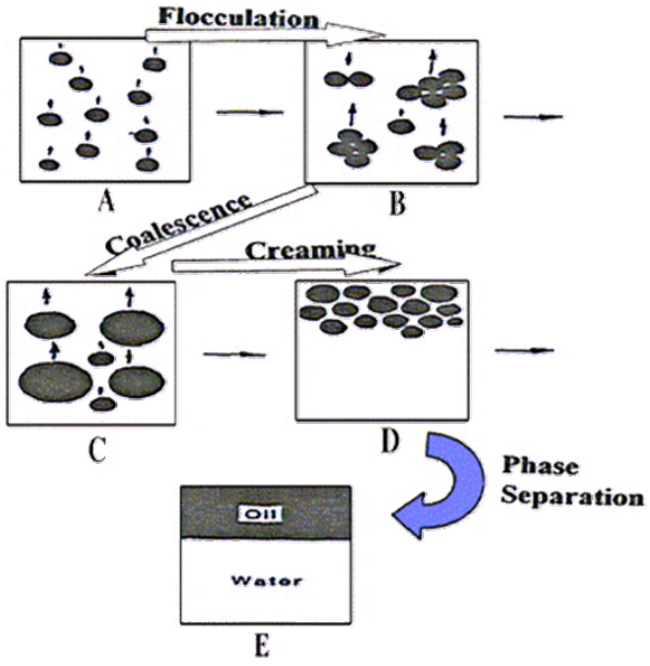


Figure 5.1. The Different Destabilization Stages of Emulsions (Al-Bawab & Friberg, 2006).

5.2.1. Soybean Lecithin

Soybean lecithin is important due to its composition of phospholipids and emulsifying properties. Phospholipids are unique natural biocompatible emulsifiers and can be used in the food industry as well as in pharmaceutical and cosmetic preparations (Jiao, Rhodes & Burgess, 2002). Soybean lecithin is obtained as a by-product in the production of oil from soybeans. The phospholipids are removed from the raw soybean oil as the precipitation components swell with water. After evaporation of the water, the precipitated mixture contains about 65 % phospholipids and 35 % raw soybean oil. This is known as commercially available soybean lecithin (Kabalnov, Tarara, Arlauskas & Weers, 1996).

Normal soybean lecithin is composed of several phosphatides dissolved in oil. The typical composition of soy lecithin is shown in Table 5.1. (Nieuwenhuyzen, 1976). It includes phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidyethanolamine (PE) and phosphatidic acids. The chemical structure of PC, PI and PE are shown in Figure 5.2. (Nieuwenhuyzen, 1976). The properties of soybean lecithin may be different depending on the concentration of phospholipids.

5.2.2. Soybean Lecithin in Emulsions

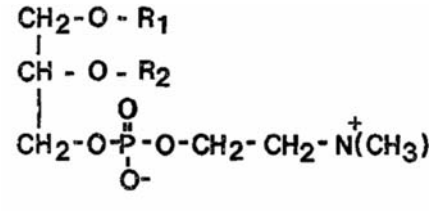
Soy lecithins are used widely in foods and beverages, cosmetics, industrial coatings and in nutrition products. They are approved by the United States Food and Drug Administration (USDA) with the status "generally recognized as safe (GRAS)". Although it has multiple uses, lecithin is most commonly used as an emulsifier either in o/w or w/o systems (John D. Weete, 1994).

Stable o/w emulsions can be prepared when the phopholipid composition is such that a lamellar liquid crystalline phase is in equilibrium with the oil and water phases (Rydhag & Wilton, 1981). Liquid crystals (LC) refer to a condition where the molecules in the liquid are oriented in a crystal-like way, and therefore flow like a liquid. Different LCs can be identified based on their unique optical properties (Martin, Keary, Thornton, Novotnak, Knutson & Folmer, 2006). A PC swells to a lamellar liquid crystalline phase incorporating less than 50 % water. Other phospholipids may influence the swelling property due to their chemical structures. For example, with the presence of PA and PI, the swelling of the lamellar phase of PC increases (Rydhag & Wilton, 1981).

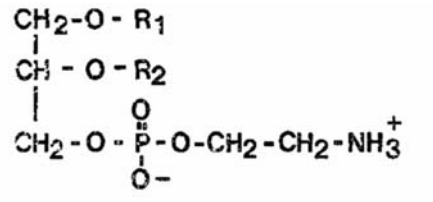
Therefore, the stability of emulsions is determined as a function of the o/w ratio and the amount of emulsifier in the system.

Table 5.1. Typical Composition (%) of Soy Lecithin (Nieuwenhuyzen, 1976).

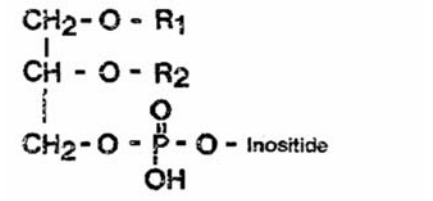
Phosphatidylcholine (PC)	20
Phosphatidylethanolamine (PE)	15
Phosphatidylinositide (PI)	20
Phosphatidic acids (PA), other phophatides	5
Carbohydrate, sterols	5
Triglyceirdes	35



(a) PC



(b) PE



(c) PI

Figure 5.2. Chemical Structure of the Three Typical Phosphatides (Nieuwenhuyzen, 1976).

R_1, R_2: fatty acids

5.3. Materials and Characterization Methods

5.3.1. Materials

The soy lecithin (PC75) used in this research was a gift from American Lecithin Company (Oxford, CT) and contains 75 % phophatidylcholine (PC). D-Limonene was purchased from Florida Chemical Inc. (Winter Haven, FL). Distilled water (W) passed through a Milli-Q water purification system (Milipore) was used to prepare the samples.

5.3.2. Emulsion Preparation

The pseudo-ternary phase system phospholipids/oil/water was studied over the emulsifier concentration region below wt50 %. The phospholipid (PC75) was dissolved in the oil, D-limonene. Samples were prepared by gradual addition of water to the PC75/ oil binary mixtures up to the desired composition in glass sample tubes. Samples then passed through the homogenization process for 5 minutes at different shear conditions. Finally, samples were allowed to achieve equilibrium at room temperature overnight (unless otherwise indicated) before any measurements were taken. The microscopic properties of the samples (phase number, physical state, and homogeneity) were examined through visual inspection. Optical microscopy images were taken at room temperature for detailed phase characterization. All sample compositions are given as weight percent except when otherwise stated.

5.3.3. Optical Microscopy

Emulsions were gently agitated in glass tubes before analysis to ensure homogeneity. A drop of emulsion was placed on a slide glass and then covered with a cover slip. The microstructure of the emulsion was then observed using inverted optical microscopy (Nikon microscopy eclipse TE2000, Nikon Corporation, Japan). The magnification of object is specifically stated.

5.3.4. Rheological Measurement

Rheological analysis of the emulsion samples were performed using the ARES Rheometer (Rheometrics Scientific, NJ) with cone and plate geometry (diameter in 50 mm and cone angle at 4 °) at room temperature. The steady rate sweep test was carried out by applying a shear rate from 1 to 500 /s. Flow behavior measurements of selected emulsion samples with different shearing conditions were performed one day after emulsion preparation. Some of each type of emulsion sample was stored for one month to measure the aging effect upon rheological properties of the samples.

5.4. Characterization of Soy Lecithin-Based Emulsion System

5.4.1. Determination of Pseudo-Ternary Phase Diagram of Water (W)/ Soy Lecithin (PC75)/ D-Limonene System

The phase diagram was studied over the low emulsifier region, below 50% of the emulsifier. Emulsion with varying oil : water : emulsifier ratios were prepared to characterize the phase behavior in the pseudo-ternary phase diagram. Visual inspection determined the one phase, two phase and stable emulsion region based on the occurrence of phase separation. Figure 5.3. illustrates the appearances of selected samples in the diagram. Samples were well homogenized with high viscosity and those that were hard to mix were considered to be in the one phase (high viscosity) or two phase region as showed in Figure 5.3. (a) and (b). Samples having precipitation or phase separation were

considered to be in the two phase emulsion region as shown in Figure 5.3. (c). Visual inspection of stable emulsions should be clear and transparent as shown in Figure 5.3. (d).

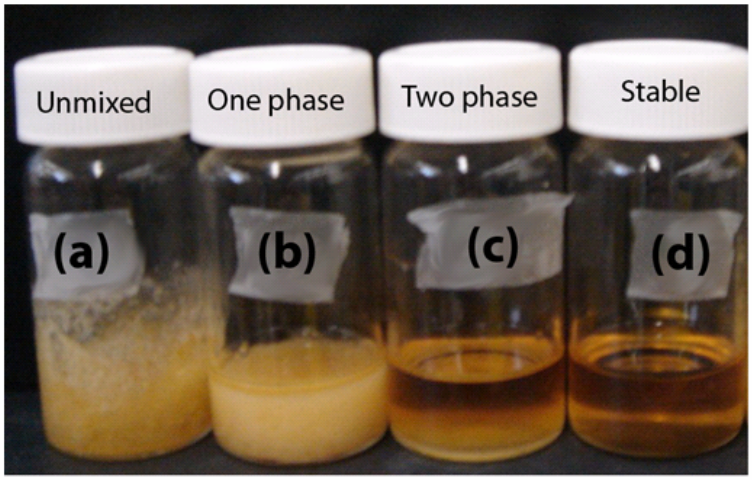


Figure 5.3. Appearance of Mixtures with Different Ratios of Water, Emulsifier and Oil.

Compositions of the four selected samples are (a) 40%:30%:30%, unmixed sample, (b) 30%:30%:40%, one phase with high viscosity sample, (c) 20%:30%:50%, two phase emulsion, and (d) 10%:30%:60%, stable emulsion.

The pseudo-ternary phase diagram could be roughly divided into these three regions: the one (with high viscosity) or two phase region, the two phase emulsion region; and the stable emulsion region as shown in Figure 5.4. The following analysis will focus on the stable emulsion region, with additional analysis of the two selected stable emulsion compositions, which are 10%:30%:60: and 10%:40%:50% in the order of water, emulsifier and oil, respectively.

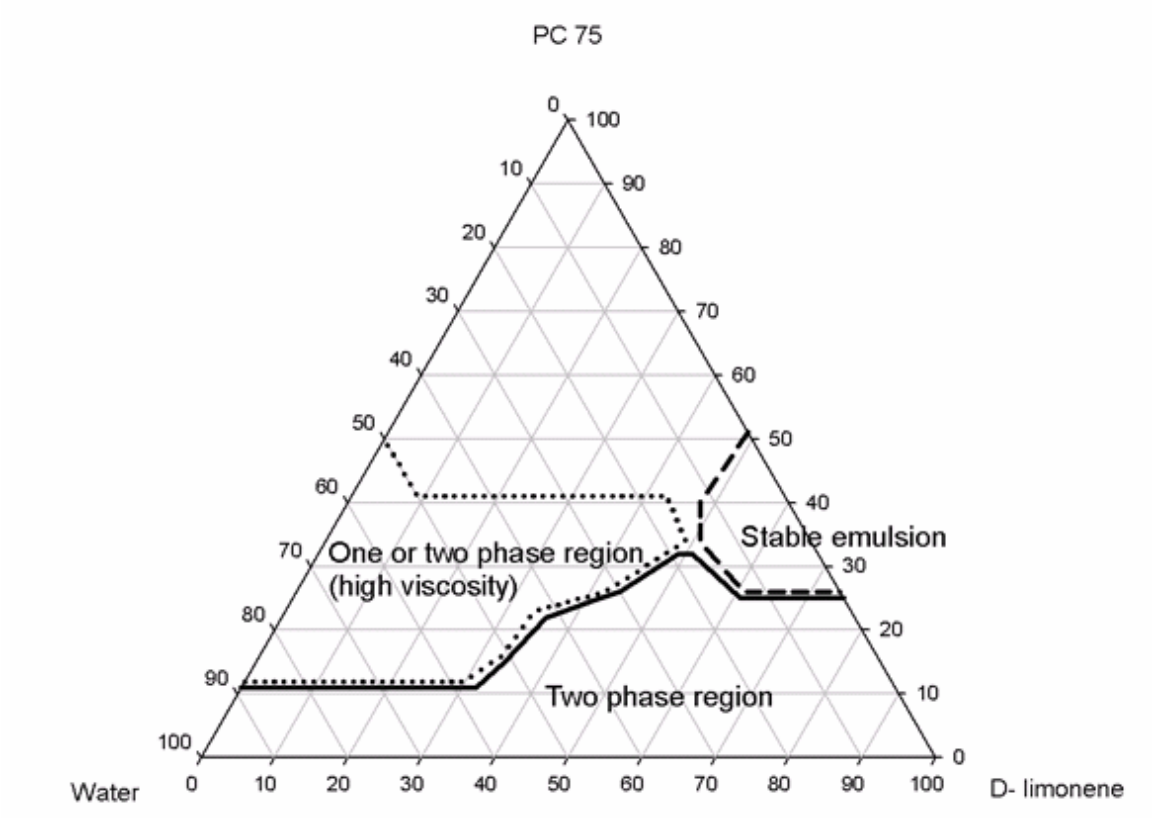


Figure 5.4. Schematic Water/PC75/D-limonene Pseudo-Ternary Phase Diagram.

Dotted boundaries delineate one phase (high viscosity) or two phase (unmixed samples) region, dashed boundaries delineate stable emulsion region and solid line boundaries delineate two phase region (phase separation). Axes units are in wt %.

5.4.2. Effect of Different Shear Conditions on Emulsion Stability

Based on the pseudo-ternary phase diagram, the stable emulsion region is our primary focus. To investigate the properties of stable emulsions prepared by PC-75, D-limonene and water, observation under optical microscopy was carried out on the two selected samples. The images were recorded using a digital camera connected to a computer. The compositions of the two selected samples are 10 % water, 30 % and 40 % PC75 and the addition of oil up to 100 %.

Figure 5.5. illustrates the emulsion images for 30 % PC75 under different shear conditions. Emulsion under a stirring condition for 30 minutes showed large oil droplets unevenly dispersed in the system at a magnification of 10 X. The corresponding size is 20 micrometers shown as a scale bar in Figure 5.5. (a). Emulsions under 6500 and 9500 rpm shear forces demonstrated evenly-spaced oil droplets and the droplets remained as individual entities as shown in Figure 5.5. (b) and (c). Oil droplets in the emulsion under the force of 13500 rpm showed homogeneity and proper array in the system as shown in Figure 5.5. (c). Under 17500 rpm (Figure 5.5. e), at the same magnification of 40 X, oil droplets in the emulsion can barely be observed. The system is more homogeneous and well dispersed than those emulsified at lower shear forces. In Figure 5.5. (b), (c), (d) and (e), the magnification is 40 X and the scale bar shown in each figure is 10 micrometers.

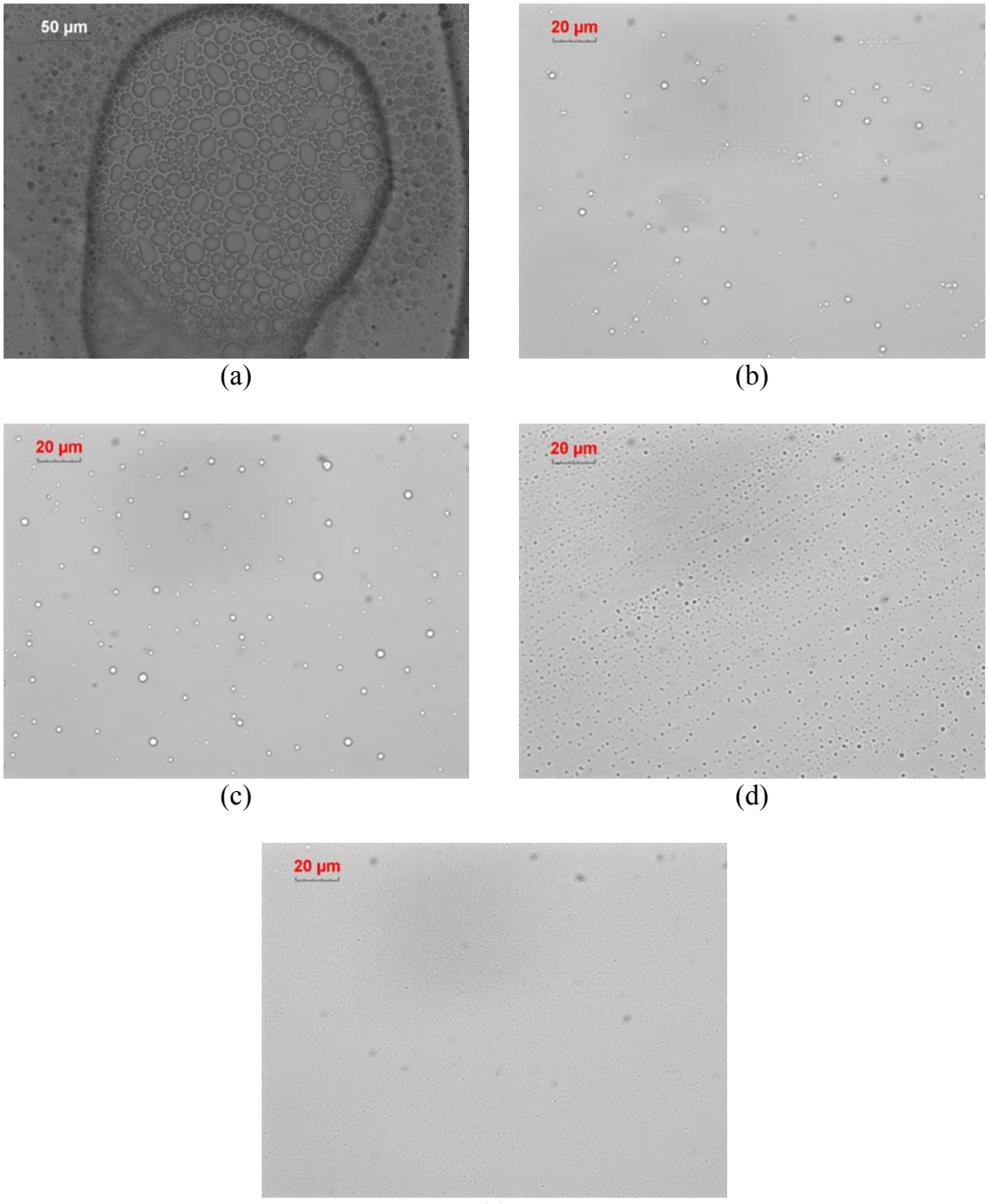


Figure 5.5. Optical Micrographs of Emulsions Containing 30 % PC75 Processed at Different Shear Conditions.

(a) stirred for 30 minutes, (b) 6500 rpm, (c) 9500 rpm, (d) 13500 rpm, (e) 17500 rpm.

Figure 5.6. shows the images of emulsion containing 40 % PC75 at different shear forces. The sample that underwent a stirring process for 30 minutes demonstrated large oil droplets unevenly spaced in the system. This was observed at 200 times magnification and the corresponding size is 20 micrometers shown as a scale bar in Figure 5.6. (a). Oil droplets in the emulsion at 9500 rpm shear force were evenly spaced in the system and not flocculated together as shown in Figure 5.6. (b). As the shear force increased from 13500 to 19500 rpm, it could be visually distinguished that the oil droplet size in the emulsions were becoming finer and remained as individual entities as shown in Figure 5.6. (c) and (d). The magnification for Figure 5.6. (b), (c) and (d) is 40 X and the corresponding size is 20 micrometers shown as a scale bar in each figure.

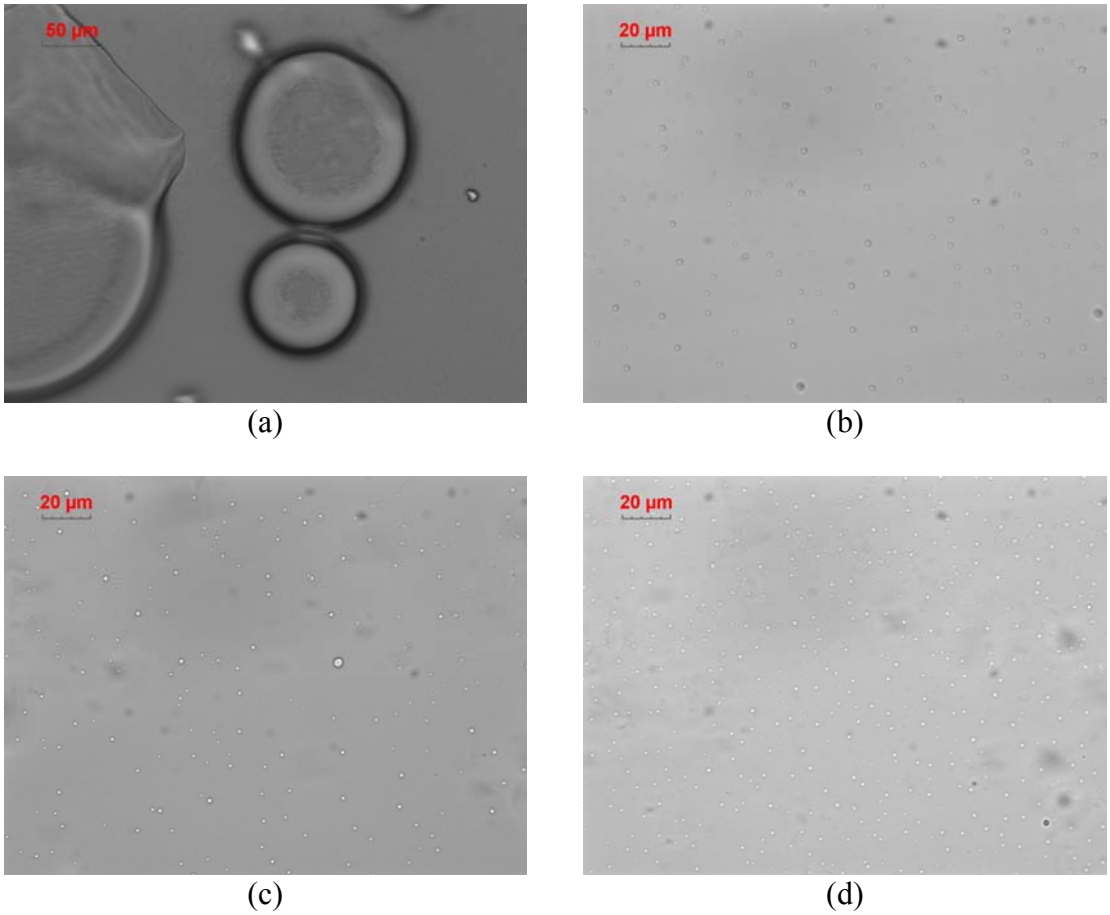


Figure 5.6. Optical Micrographs of Emulsions Containing 40 % PC75 Processed at Different Shear Conditions

(a) stir for 30 minutes, (b) 9500 rpm, (c) 13500 rpm, (d) 17500 rpm.

5.4.3. Viscosity and Flow Behavior of Emulsion with 40 % PC75 at Various Shear Conditions

To identify the viscosity change and flow behavior of the stable emulsion with 40 % PC75, rheological measurement was practiced. The viscosity of the emulsion increased along with the shear stress increase and as shear stress increased from 9500 to 13500 rpm, the emulsion showed typical Newtonian fluid behavior. The viscosity was independent from the shear stress. The sample processed at 17500 rpm demonstrated

shear-thinning behavior at a low shear rate and a Newtonian region at higher shear stress (Figure 5.7.).

The possible reason for the shear-thinning behavior is that with the shear stress increase, the forces become large enough to cause the oil droplets to become deformed and disrupted. At the same time, the force decreases the effective volume of the emulsion and leads to a decrease in viscosity (Chanamai, Herrmann & McClements, 1998).

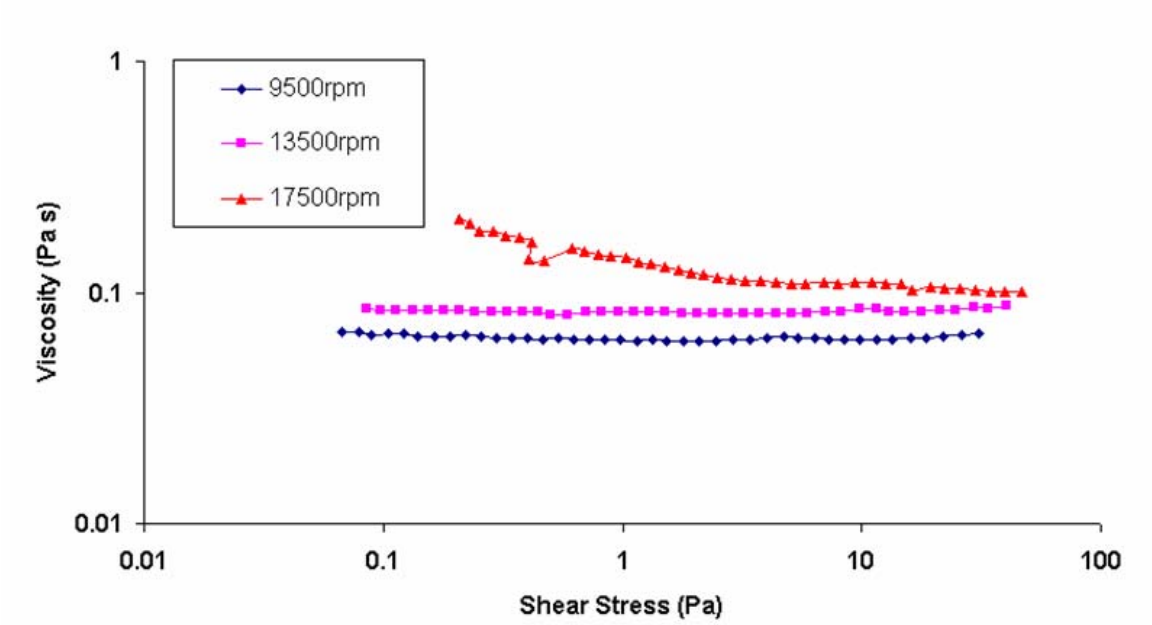


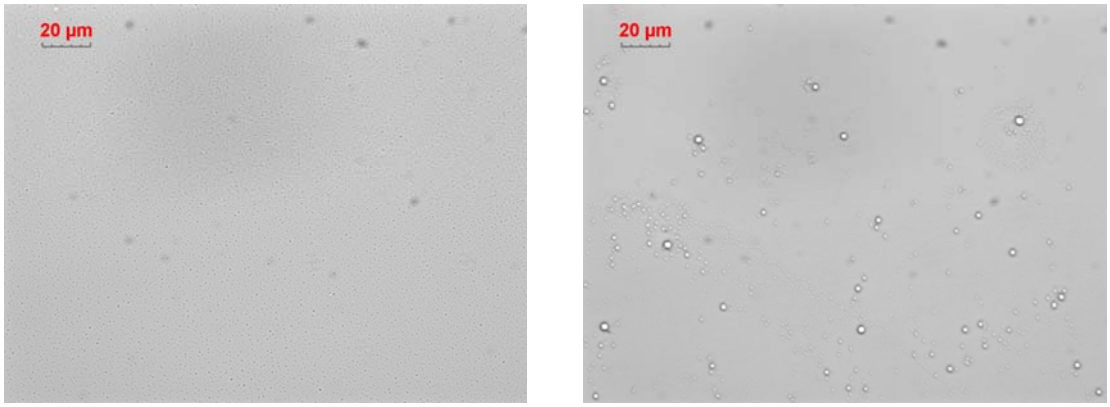
Figure 5.7. Viscosity and Flow Behavior of the 40 % PC75 Stable Emulsion at Various Shear Stress Conditions.

5.4.4. Aging Effect on the Stability of Emulsions Containing 30 % and 40 % PC75

To investigate the aging effect for selected stable emulsions, the images were taken and analyzed. The two selected emulsions were prepared. One was analyzed after

24 hours (short-term) while the other was analyzed after being stored for one month (long-term) at room temperature.

The image of the short-term storage (24h) sample with 30 % PC75 demonstrated homogeneity in the system. Oil droplets in the emulsion were spaced evenly in the system and individual entities were maintained as shown in Figure 5.8. (a). Visual inspection distinguished the particles were finer than that of the sample stored for one month. When the sample aged for a month, the flocculation phenomenon occurred. This is the initial process in the destabilization of emulsions (Al-Bawab & Friberg, 2006). Oil droplets tended to aggregate and became larger oil groups as shown in Figure 5.8. (b). However, the flocculation of the oil droplets did not result in a viscosity increase that could be illustrated through rheological analysis (Figure 5.9.). The sample with 24-hour storage presented slightly higher viscosity than that of the one month sample and this viscosity remained independent to the shear stress applied. The two stable emulsions were Newtonian fluids. They act like particles with fixed size and shape resulting in constant viscosity (Chanamai, Herrmann & McClements, 1998). All images in Figure 5.8 are at 40 X magnification and a scale bar is provided for each figure.



(a) 24 h (b) one month

Figure 5.8. Optical Micrographs of Emulsions Containing 30 % PC75 with Different Storage Periods.

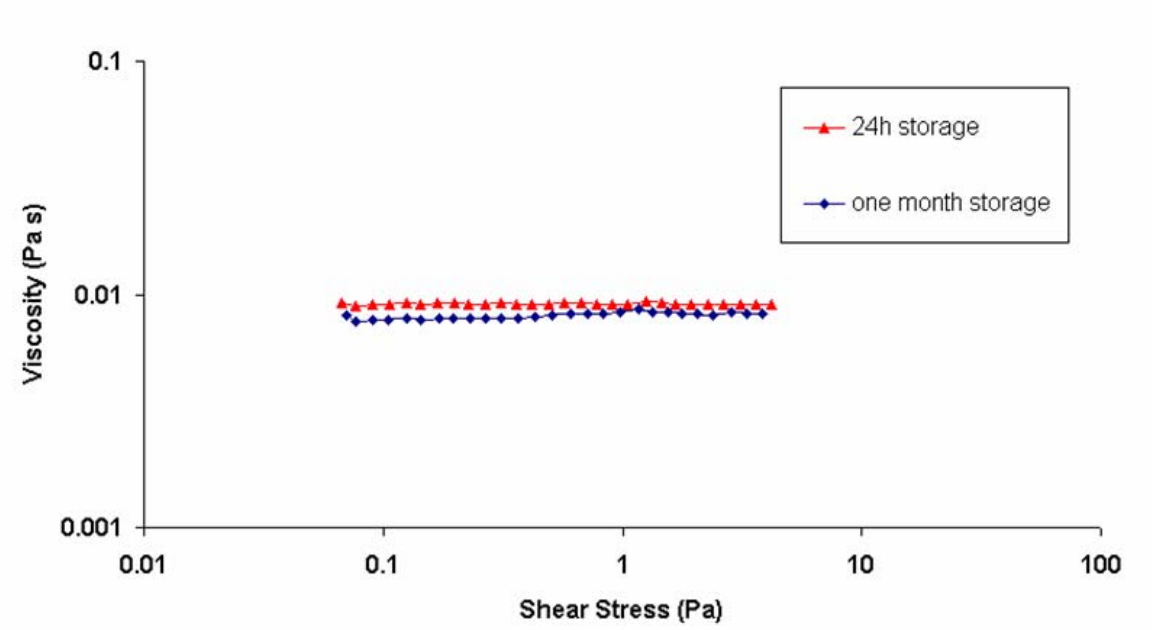
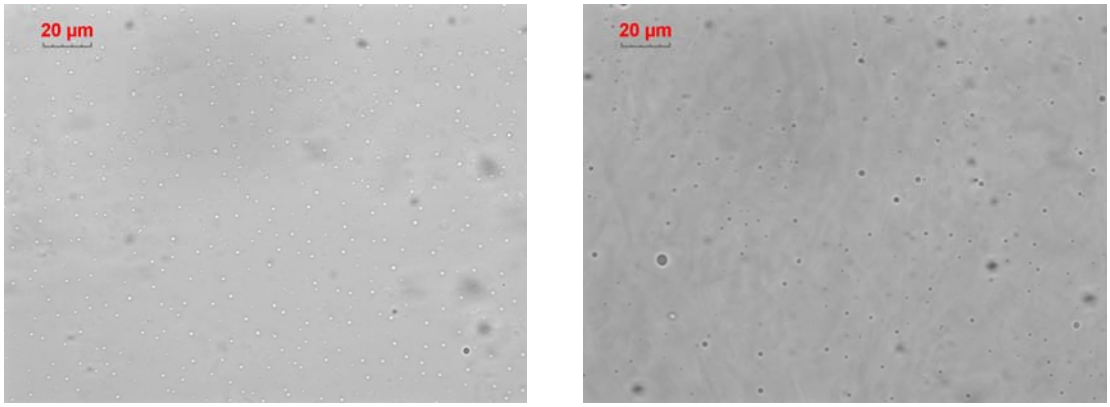


Figure 5.9. Viscosity and Flow Behavior of the Aging Effect on 30 % PC75 Emulsion Stability.

In emulsion with 40 % PC75, there was little difference between those stored for 24 hours and those stored for a month. Images show that oil droplets in the emulsion remained in a similar pattern and spaced in the system as individual entities without obvious flocculation or aggregation as shown in Figure 5.8. (c) and (d). The image results

are also confirmed by the rheological measurement. Emulsion with 40 % PC75 demonstrated shear-thinning flow behavior regardless of the storage period. Shear-thinning in flocculated emulsions results from progressive deformation and disruption of flocs as the shear stress is increased (Chanamai, Herrmann & McClements, 1998). Figure 5.11. shows the viscosity change as a function of shear rate which suggested that the viscosity of the emulsion is not influenced by the aging process.



(a) 24 h (b) one month

Figure 5.10. Optical Micrographs of Emulsions Containing 40 % PC75 in Different Storage Periods.

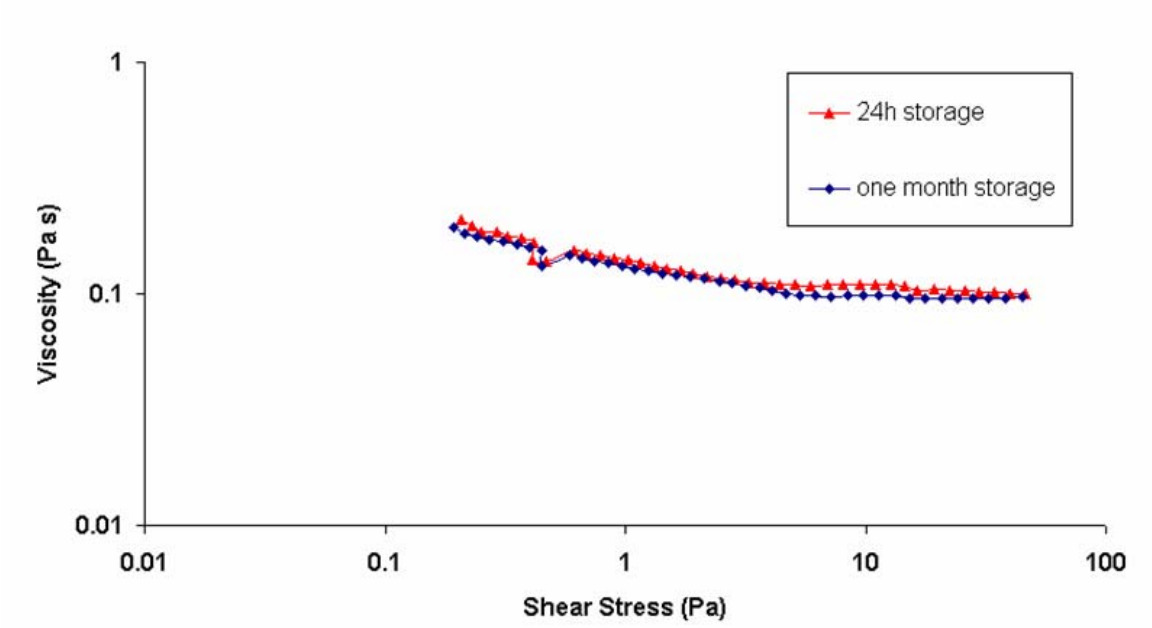


Figure 5.11. Viscosity and Flow Behavior of Aging Effect on 40 % PC75 Emulsion Stability.

Appendix

I. Diet Composition

The modulated diets including resveratrol, black tea extract and caffeine modified diets are manufactured based on the AIN-76 rodent diet with 40 kcal% corn oil which is shown in Table I.2. The protein, carbohydrate and fat compositions and calorie distribution remain the same in all diets. Total calories of all diets are 3902 kcal.

Table I.2. Composition of High-Fat, Resveratrol, Black Tea Extract, and Caffeine Modulated Diets.

Product #	**D00061203**		**D00061212**		**D00061214**		**D07100201**		**D07100202**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	24.1	*21*	24.0	*21*	24.0	*21*	24.0	*21*	24.0	*21*
Carbohydrate	44.9	*39*	44.8	*39*	44.9	*39*	44.9	*39*	44.8	*39*
Fat	20.7	*40*	20.7	*40*	20.7	*40*	20.7	*40*	20.7	*40*
Total		*100*		*100*		*100*		*100*		*100*
kcal/gm	4.62		4.61		4.62		4.62		4.62	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*
Maltodextrin	75	*300*	75	*300*	75	*300*	75	*300*	75	*300*
Corn Starch	75	*300*	75	*300*	75	*300*	75	*300*	75	*300*
Sucrose	218.8	*875*	218.8	*875.2*	218.8	*875.2*	218.8	*875.2*	218.8	*875.2*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Corn Oil	175	*1575*	175	*1575*	175	*1575*	175	*1575*	175	*1575*
Ethoxyquin	0.01	*0*	0.01	*0*	0.01	*0*	0.01	*0*	0.01	*0*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
Black Tea Extract	0	*0*	1.8	*0*	0	*0*	0	*0*	0.9	*0*
Resveratrol	0	*0*	0	*0*	0	*0*	0.5	*0*	0.25	*0*
Caffeine	0	*0*	0	*0*	0.45	*0*	0	*0*	0.225	*0*
Total	**843.81**	***3902***	**845.61**	***3902***	**844.26**	***3902***	**844.31**	***3902***	**845.19**	***3902***

Product #: D00O61203 is AIN-76A high-fat diet, D00O61212 is 0.2% black tea extract diet, D00O61214 is 0.05% caffeine diet, D07100201 is 0.06% resveratrol diet and D07100202 is the combination diet.

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