EFFECT OF ∂-TOCOTRIENOL AND GRAPE SEED POLYPHENOL ON LIPID PROFILE IN C57BL/6J MICE WITH A WESTERN-LIKE DIET-INDUCED NON-ALCOHOLIC STEATOHEPATITIS

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ABSTRACT

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EFFECT OF ∂-TOCOTRIENOL AND GRAPE SEED POLYPHENOL ON LIPID PROFILE IN C57BL/6J MICE WITH A WESTERN-LIKE DIET-INDUCED NON-ALCOHOLIC STEATOHEPATITIS

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Non-alcoholic steatohepatitis (NASH), the second phase in liver disease, occurs when adults consume high calorie, high fat diets. Fat accumulated in the liver causes inflammation and irreversible hardening. Grape seed polyphenol (GSP) and ∂-tocotrienol (∂-T3) may have benefits in reducing NASH. We observed the effects of dietary GSP & ∂-T3 (separated and combined: GSP - 2%; ∂-T3 - 0.05%) in C57BL/6J mice fed a high calorie high fat diet for 20 weeks. Hepatic cholesterol, triglycerides, and vitamin E; serum cholesterol, triglycerides, vitamin E, and free fatty acids were assayed. Hepatic total cholesterol was lowest in GSP diet and GSP(2%)+dT3(0.05%) diet groups. Hepatic triglycerides were lowest in the GSP(2%)+dT3(0.05%) diet group. Hepatic vitamin E levels were lowest in the GSP(2%)+dT3(0.05%) diet group. Serum cholesterol was lowest in GSP diet and GSP(2%)+dT3(0.05%) diet groups. Serum vitamin E was lowest in the GSP diet group. Serum triglycerides and FFA's were similar among the groups.

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CHAPTER I

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by an increase in fat content in the liver. According to the National Digestive Diseases Information Clearinghouse of the National Institute of Diabetes and Digestive and Kidney Diseases; as of 2006, 10 to 20% of American adults had NAFLD, while two to five percent have NASH [1]. NAFLD is associated with obesity. Basarangolu et al., reported that 95% of obese adults also have NAFLD and 3% have NASH [2]. The progression of liver disease starts with consumption of unhealthy fatty food items, leading to fat accumulation and storage in the liver; this stage is called fatty liver disease (FLD). As time passes and intake of fatty foods continues, FLD progresses to NASH where lipid peroxidation and oxidative stress cause hepatocyte injury and inflammation of the liver [2]. At this stage tissues become hardened and scarred, but it can be reversed if diet changes. The next and last stage is cirrhosis, where all tissue becomes fibrous and scarred. This stage is irreversible, and death occurs.

A two-hit theory for the progression of NASH states that excessive fat, specifically free fatty acids (FFA), is stored in the liver due to insulin resistance and oxidative stress from reactive oxygen species (ROS). In those with steatosis, ROS oxidizes hepatic fat (lipid peroxidation) causing cellular necrosis and collagen synthesis, leading to fibrosis [3]. ß-oxidation is impaired because the

increase in FFA is overwhelming and FFA is not converted to acetyl-CoA [4]. This increases lipid accumulation in the liver. The second hit occurs when ROS forces secretion of pro-inflammatory cytokines in the liver. Research shows that antioxidants can impair ROS activation, thus improving hepatic health [3].

Because of the growing percentage of obese patients with NASH, research must focus on therapeutic treatments to both prevent and treat this disorder. Patients with NASH have the opportunity to reverse the disease by changing their diets. Sibeler found that setting goals of weight management and a calorie deficit of 500-1000 kcal/day for overweight/ obese adults is an effective method to weight loss [4]. These two changes alone were found to significantly decrease weight, waist circumference, visceral fat, fasting blood glucose, insulin resistance, serum triglycerides, serum levels of liver enzymes and histological score.

The best treatment to decrease oxidative stress is the use of antioxidants. Basarangolu reported indirect evidence regarding the benefits of antioxidants, such as vitamin E in treating NAFLD [2]. Vitamin E has anti-oxidative properties that fight free radical-induced oxidative damage and protect molecules. Lavine prescribed vitamin E to pediatric patients with NASH, and found that liver function was returned to normal [5]. However, when the vitamin E supplementation was discontinued, the children were unable to sustain normal function.

Vitamin E consists of four types of tocopherols and four types of tocotrienols. Tocopherol structures have saturated tails, while tocotrienols have

unsaturated tails [6]. Vitamin E is fat-soluable and requires an intake of food items containing fat for adequate digestion and absorption. Once inside of the small intestine, the pancreas secretes pancreatic esterase and the gallbladder secretes bile for fat emulsification. This creates micelles of fat, releasing free fatty acids from triglycerides for incorporation into chylomicrons [6]. No studies have reported that absorption is different between the eight isomers of vitamin E [6].

Vitamin E deficiency is rare and usually only occurs when an illness or disease prevents fat absorption resulting in erythrocyte hemolysis. With prolonged deficiency, neuromuscular dysfunction of the spinal cord and retina of the eye may occur. Usually, toxicity occurs rarely, however if it were to take place, very high levels of vitamin E could disturb blood-clotting activity performed by vitamin K and/or certain medications resulting in hemorrhaging which could lead to a hemorrhagic stroke [7].

Vitamin E is found in various vegetable oils, margarines, salad dressings, seeds, and nuts. Cooking may destroy some vitamin E molecules due to heat and oxidation. Highly processed food items have little to no vitamin E; thus the best way to get daily of vitamin E is to eat it in freshly made food items [7].

The tocotrienol subfamily of vitamin E is unique from the tocopherol subfamily, in that they protect the body's neurosystem, prevent cancer, and decrease cholesterol [8]. ∂-tocotrienol (∂T3) was discovered and isolated from the other tocotrienols in 1922. After thorough research in 1980, it was found that ∂T3 lowers lipids, inflammation, and nitric oxide levels in the body system [9-10].

The current study focused on ∂T3 because, as Preedy found, it is the delta isomers in tocotrienols that reduce high cholesterol levels due to the position of methyl groups on the molecule [8]. A study done by Qureshi et al. was one of the first to find that hypercholerolemic pigs fed a tocotrienol-rich supplement resulted in a decrease in total serum cholesterol as well as in low-density lipoprotein (LDL) cholesterol, apolipoprotein B, thromboxane-B2, and platelet factor 4 [11]. Research shows that the mechanism of ∂T3 works by suppressing HMG-CoA reductase, which decreases cholesterol synthesis [8]. Later Qureshi et al., tested tocotrienols on chickens and not only did the results show a hypocholesterolemic effect, but tocotrienols also exibited significant antioxidant properties. The methyl position in tocotrienols affect overall results in reducing cholesterol, preventing cancer, and acting as an effective antioxidant [8, 10].

The current study also tests grape seed polyphenol. Polyphenols in general act as antioxidants by protecting cells against oxidative harm from free radicals. They have the ability to reduce metals by preventing iron from producing hydroxyl radicals from peroxide. Many studies have found that polyphenols lower LDL cholesterol in the body. Grape seed polyphenol is literally the extraction of oils that exist in grape seeds.

There is little research on non-alcoholic steatohepatitis in relation to grape seed polyphenol and ∂-tocotrienol. However, there is no research where both substances have been tested together on the NASH mouse model.

Purpose of the Study

The purpose of this study was to investigate the effect delta-tocotrienol, grape seed polyphenol, alone and in combination, in C57BL/6J mice fed a western-like high-fat diet to induce non-alcoholic steatohepatitis-like state. The study investigated lipid levels and vitamin E levels in the liver and sera of the mice. This investigation was part of a larger study investigating histological changes in the same condition.

CHAPTER II

REVIEW OF LITERATURE

Nonalcoholic fatty liver disease (NAFLD) is characterized by an increase in fat content in the liver. According to the National Digestive Diseases Information Clearinghouse of the National Institute of Diabetes and Digestive and Kidney Diseases; as of 2006, 10 to 20% of American adults had NAFLD, while two to five percent have NASH [1]. The progression of liver disease is as follows; humans consume unhealthy fatty food items, leading to fat accumulation and storage in the liver; this stage is called fatty liver disease (FLD). As intake of fatty foods continue, FLD progresses to NASH where lipid peroxidation and oxidative stress cause hepatocyte injury and inflammation of the liver [2]. Here, tissue becomes hardened and scarred, but it can be reversed if diet changes. The last stage is cirrhosis, where all tissue becomes fibrous and scarred. This stage is irreversible, and death occurs.

A two-hit theory for the progression of NASH states that excessive fat, specifically free fatty acids (FFA), is being stored in the liver due to insulin resistance and oxidative stress from reactive oxygen species (ROS). In those with steatosis, ROS oxidizes hepatic fat (lipid peroxidation) causing cellular necrosis and collagen synthesis, leading to fibrosis [3]. ß-oxidation is impaired because the increase in FFA is overwhelming, which disables FFA from

conversion to acetyl-CoA [4]. This increases lipid accumulation in the liver. The second hit occurs when ROS forces secretion of pro-inflammatory cytokines in the liver. Research has found that antioxidants can impair ROS activation improving hepatic health [3].

Vitamin E and NASH

A westernized diet has become a new trend in cuisine all around the world. At the same time there is an increase in non-alcoholic fatty liver disease. The western diet is very affordable, as one dollar can purchase a variety of items at fast food establishments. In addition, there is a decrease in the number of homemade meals for adults and their children. Children are not only gaining weight at a younger age, but according to the Centers for Disease Control and Prevention, this increased weight gain can increase the risk of fatty liver disease, dyslipidemia, cirrhosis, type 2 diabetes (T2DM), hypertension (HTN), metabolic syndrome (MetS), and many other life threatening illnesses at a young age [12].

In a study performed by Lavine, 11 patients (8 boys, 3 girls) between 8 and 14 years of age, had elevated serum aminotransferase levels and were obese. Patients were given 400 IU vitamin E (α-tocopherol) capsules once a day, however if aminotransferase levels did not normalize, patients were given an additional 400 IU vitamin E capsule per day for a total of 800IU. If levels still did not normalize patients were given another capsule for a total of 1200 IU per day. The maximum intake was 1200 IU per day. Patients were followed from a minimum of four months, up to 10 months. Patients were instructed to lose

weight. Results showed no significant changes in BMI, however alanine transaminase (ALT) and aspartate aminotransferase (AST), returned to normal levels in all patients, whereas alkaline phosphatase (ALP), another indictor of liver damage, remained elevated but were close to normal levels. Five of the 11 children responded to the lower (400 IU/day) dose within one month. In the other 6 children, normalization occurred 2 to 3 months after taking supplements with 800 to 1200 IU/day. Serum α-tocopherol in all patients were within normal range before treatment, and increased with treatment [5].

Children with NAFLD are at risk of cardiovascular disease, insulin resistance and oxidative stress. In a later study performed by Lavine, et al., vitamin E (α-tocopherol) and metformin were used for the reduction in insulin resistance and oxidative stress in young human subjects for a time period of two years. 173 patients aged 8-17 years old were divided into three groups and given either 800 IU of vitamin E + metformin placebo; 1000 mg oral metformin + vitamin E placebo; or metformin placebo + vitamin E placebo for 96 weeks. Patients with diabetes, cirrhosis, pregnancy, viral hepatitis, or alcohol consumption of any amount were excluded from the study. Elevated ALT was necessary for inclusion in the study. Results showed that all of the patients had reduced ALT, AST, GGT, and ALP levels in all three groups. There was a significant decrease in ALT levels in treatment of vitamin E + placebo at week 24; however ALT levels in the full placebo group began to drop at week 72. Those who had NASH/bordering NASH, showed a resolution of NASH symptoms in the

vitamin E group. Vitamin E did not have significant effects on steatosis, inflammation or fibrosis in patients. Serum triglycerides increased, but cholesterol, LDL, HDL decreased in all groups. A possible reason patients in the full placebo group had improved ALT was a greater adherence to diet and physical activity recommendations, increased motivation in participating in the study, or the patients went through puberty [13].

In 2009, Feldstein, et al., observed children with non-alcoholic fatty liver disease (NAFLD) later in their adult lives to observe the outcome of a child with this diagnosis. NAFLD is the stage before NASH when fat collects in the arteries causing fatty streaks and foaming of fat. Typically, with increased consumption of high fat diets, NAFLD may progress to NASH. Of the 66 children; two-thirds were obese and more than half had a BMI >97th percentile with hyperlipidemia. Fifty-five children had one symptom of the metabolic syndrome (MetS) and 19 had more than three symptoms. Liver fibrosis was found in 59% of the patients. The children were instructed to consume a well balanced diet daily, encouraged to exercise, and were instructed to take either ursodeoxycholic acid or vitamin E daily for one year.

One year into the study, 49% of all children lost 10% of their baseline weight, and 86% had improved levels of aminotransferase. Ursodeoxycholic acid and vitamin E had no effect on liver enzymes. 15 years after the one year study, 76% of the patients who lost 10% of their weight, re-gained this weight. Four children, now adults developed T2DM 4, 6, 7, and 11 years after original NAFLD

diagnosis. Those with a liver biopsy showed steatosis and lobular inflammation worsened or remained the same as their baseline levels. Two patients underwent liver transplantation due to cirrhosis and had NASH post surgery, while two others died [14].

Other studies investigated the effects of vitamin E on NASH. Yakaryilmaz, et al., studied the effects of vitamin E on liver enzymes and histology in NASH. Six-teen patients (12 men, four women) had elevated aminotransferase greater than 1.5 times the upper limit of normal levels, liver histology showing NASH, serum creatinine concentration less than 1.5 mg/dL, normal serum creatine phosphokinase activity and thyroid-stimulating hormone levels. Patients were given vitamin E twice daily (800 U/day), everyday for 6 months. Results show no changes in BMI from baseline to six months, however there were improvements in liver enzymes, necroinflammation, steatosis, and serum cholesterol at the end of the study period. ALT levels returned to normal in eight patients, but remained elevated in the other 8 patients. Of the 9 patients with elevated AST at baseline, 7 returned to normal range. Overall, vitamin E improved liver function tests, steatosis, and necroinflammation, but there were no significant changes in fibrosis [3]. King et. al defined 1-aminobenzotriazole (ABT) as an antioxidant that inhibits animal cytochrome P450. Cytochrome P450 is a group of enzymes that works to synthesize and metabolize lipids and steroids. Therefore with the use of ABT there is less synthesis and metabolism of total lipids [15]. Nan, et al., observed the antioxidant effect of both vitamin E and ABT on mice given a

methionine- and choline-deficient diet (MCD) to induce NASH. Sixty 8-week-old male C57BL6/J mice were divided into 10 groups. Each group was given 1 of 5 designated diets: control (no mention of ingredients), MCD, MCD + vitamin E, MCD + ABT, or MCD + vitamin E + ABT. Half of the groups were given the diets for 10 days, and the other half were given their diets for three weeks. Results show that mice given the control diet had normal liver histology at both 10 days and 3 weeks, while mice given the MCD only diet for 10 days developed mild hepatocyte steatosis and inflammation within the cells, and by 3 weeks developed macrosteatosis and inflammation. Feeding mice an MCD diet caused decrease levels of superoxide dismutase (SOD), Bcl-2, and adiponectin. When given antioxidant, levels were reversed and brought close to normal levels. In addition, antioxidant treatment improved ALT, AST, inflammation, and steatosis. This study shows that vitamin E and ABT may reduce oxidative stress, inflammation, and liver apoptosis in mice with NASH [16].A study performed by Sanyal, et al., observed the effect of vitamin E and the insulin sensitizer Pioglitazone, alone and in combination on liver histology in NASH patients [17]. Twenty Caucasian patients were randomized into either 400 IU vitamin E group, or a combination of 400 IU vitamin E + 30 mg Pioglitazone group. Patients were encouraged to eat healthy meals and exercise daily. At the end of the study, serum ALT levels were normalized in all twenty patients. Those in the Pioglitazone + vitamin E group did not have an increase in BMI, and had a decrease in steatosis, ballooning, inflammation, and pericellular fibrosis. After a

six month treatment, vitamin E + Pioglitazone was superior at steatosis grade, ballooning, metabolic clearance of blood glucose, reducing fasting insulin, FFA, and inflammation compared to the vitamin E group alone. The vitamin E group alone, had a drop in hepatic steatosis and normalized ALT levels, however there were no significant effects on histological parameters. The study revealed that vitamin E + Pioglitazone has a higher impact on patients with NASH, than vitamin E alone.

Six years later Sanyal, et al., retested their hypothesis of Pioglitazone and vitamin E effects in NASH in 247 patients without diabetes or alcoholic steatohepatitis. Each participant must have hepatocellular ballooning. Testing took place at baseline and at 96 weeks, and followed 24 weeks after the treatment was discontinued. Participants were randomly divided into three treatment groups: 30 mg Pioglitazone + vitamin E placebo; 800 IU vitamin E + Pioglitazone placebo; Pioglitazone placebo + vitamin E placebo. All patients were advised to make lifestyle changes, including diet changes on their own. In this study, vitamin E significantly improved symptoms when treating patients with NASH. This could possibly be because the time line was 2 years compared to the prior 6 month time line; also the dose amount was 800 IU vs 400 IU. Pioglitazone combined with vitamin E significantly reduced steatosis, inflammation, hepatocellular ballooning, insulin resistance, liver-enzyme levels, and steatohepatitis. Fibrosis was not reduced in either treatment group [18].

Tocotrienol

The tocotrienol subfamily of vitamin E is unique from the tocopherol subfamily, in that they protect the body's neurosystem, prevent cancer, and decrease cholesterol [8]. ∂-tocotrienol (∂T3) was discovered and isolated from the other tocotrienols in 1922. After much research in 1980, it was found that ∂T3 lowers lipids, inflammation, and nitric oxide levels in the body [9-10]. The current study focused on ∂T3 because, as Preedy found, the delta isomer reduces high cholesterol levels due to the position of methyl groups on the molecule [8].

In a study done by Zhihong, et al., four-month old and 23-month old male mice were divided into two groups, a control and a tocotrienol diet group. Mice were fed their designated diet for six weeks. The control and treatment diets consisted of a basal oil- and vitamin E-free version of the AIN-93M diet. The treatment diet contained Tocomin, a mixture of both tocotrienol and α-tocopherol (12.2% αT3, 2% ßT3, 6.2% ∂T3, 20.1% γT3, and 10.7% α-tocopherol). Both of these diets contained the same amount of α-tocopherol, but different amounts of tocotrienol. Older mice in both the control and treatment groups had a decrease of total T cells, CD4 cells, CD8 T cells, and regulatory T cells, with an increase in memory CD4, CD8 T cells, B cells, interleukin (IL)-1ß, IL-6, IL-10 and prostaglandin E2 compared to the younger mice. Older treated mice had lower levels of lymphocyte proliferation in IL-2, IL-4, and IL-10 compared to the young mice, but no difference in tumor necrosis factor-α (TNF-α), or interferon-γ. Researchers also performed in vitro studies, to determine how tocotrienol alone

affected splenocytes on both old and young mice. They found that all tocotrienol in the order of α-, γ-, and ∂- increased lymphocyte proliferation in older mice, but not in young mice. Overall this study showed that supplementation with α-, γ-, and ∂-tocotrienol increased T cell proliferation to impact immune cell function positively [19].

Chin, et. al, conducted a study using over-the-counter vitamin E supplements in adults (age 35-50+). 62 subjects were divided into two groups: 35-49 year-old, and 50+ year-old. Each of the two groups were divided in two and were assigned either a palm oil placebo supplement or a tocotrienol rich fraction (TRF) supplement. The TFR supplement had 74% tocotrienol and 26% tocopherol. Specifically, each supplement had 70.4 mg α-tocotrienol, 4.8 mg ß-tocotrienol, 57.6 mg γ-tocotrienol, and 33.6 mg ∂-tocotrienol. Results showed that HDL, antioxidant levels, and plasma tocopherol concentrations were significantly increased in both treatment groups [20].

∂-Tocotrienol

∂-tocotrienol was discovered and isolated from the other tocotrienols in 1922. In 1980, it was found to be effective in lowering lipid levels within the body. The delta isomer in tocotrienols can decrease cholesterol levels due to the position of methyl groups on the molecule [8]. Not only has it been observed to decrease cholesterol, studies have found that apart from tocopherols, tocotrienols have the ability to prevent lipid peroxidation, reduce lipid levels, and reduce inflammation [9-10].

Tocotrienols have anti-inflammatory effects. Qureshi, et al., fed female chickens mixtures of ∂-tocotrienol with Quercetin, riboflavin, (-) Corey lactone (control blood pressure), Amiloride (diuretic) or Dexamethasone (inflammation reducing corticosteroid) in reducing levels of nitric oxide (NO), TNF-α, cholesterol, LDL, and triglyceride levels. One-day-old white leghorn female chickens were fed a corn-soy meal diet for one week, then divided into 24 groups (6 chickens in each group). Three control groups, each given a conventional diet for four weeks and sacrificed at different times throughout the experiment; one group given 125 microM/kg ∂-tocotrienol; 20 experimental groups given 74 or 148 microM/kg Quercetin, 66.5 or 133 microM/kg riboflavin, 71 or 142 micro/kg (-) Corey lactone, 16.5 or 33 microM/kg Amiloride, and 1.27 or 2.55 microM/kg Dexamethasone in various combinations for four weeks [10].

Chickens in the treatment groups had decreased inflammatory markers and fat infiltration in liver samples compared to control groups. Serum TNF-α and NO decreased when fed a combination of ∂-tocotrienol and Quercetin, riboflavin, (-) Corey lactone, Amiloride, or Dexamethasone. Serum cholesterol, LDL, and triglycerides were reduced in those given ∂-tocotrienol alone. When combining ∂-tocotrienol with Quercetin, riboflavin and (-) Corey lactone, and minimal Amiloride, serum cholesterol levels were reduced further but not significantly. ∂-tocotrienol alone and Amiloride alone had no impact on HDL levels, but when given together reduced HDL. Quercetin, riboflavin, and (-) Corey lactone alone lowered HDL, but did not lower any further when given in combination with ∂-

tocotrienol. Dexamethasone alone increased HDL and in combination with ∂-tocotrienol increased further. Dexamethasone + ∂-tocotrienol decreased, LDL, cholesterol, and triglyceride levels. Overall, there was a reduction in TNF-α, NO, inflammation, and improved lipid levels when ∂-tocotrienol, Quercetin, riboflavin, (-) Corey lactone, and Amiloride alone and in combination were used [10].

Studies have found that inflammation plays a role in the development of NASH. Factors like lipopolysaccharides (LPS) and proteasomes could possibly induce pro-inflammatory cytokines. Inflammatory cytokine production alters metabolism, cardiovascular, immunological, hemostat and endocrine functions leading to septic shock. Inflammatory cytokines also contribute to atherosclerosis, cancer, stoke, diabetes and liver disease. Qureshi, et al., investigated how tocotrienols modulate proteasomal activity, impact corticosteroid synthesis, and block LPS-induced signaling pathways. Results showed that tocotrienols blocked production of pro-inflammatory cytokines induced by LPS. Inhibition of chymotrypsin-like activity of rabbit muscle proteasomes was dependent on α-, γ-, and ∂-tocotrienols, but not α-tocopherol. ∂-tocotrienol itself inhibited the chymotrypsin, trypsin, and post-glutamase activities of the proteasome in macrophages by 46% and when given in higher doses, activities were enhanced. TNF-α was reduced in an LPS-stimulated macrophage when given tocotrienols. ∂-tocotrienol was effective at reducing LPS-induction of TNF-α, IL-1ß, IL-6, and iNOS in macrophages at low concentrations. At high concentrations, ∂-tocotrienol activated chymotrypsin-like,

trypsin-like, and post-glutamase activities, and up-regulates LPS-induced TNF-α, IL-1b, IL-6 and iNOS for decreased inflammation [9].

Grape Seed Polyphenol

Polyphenols act like antioxidants that protect blood cells from oxidation with the added ability of preventing iron from producing hydroxyl radicals from peroxide. Polyphenols are plant-based and can be found in tea, coffee, grains, oregano, red wine, fruits, chocolate, and grapes [7]. Grape seed polyphenol (GSP) is the extracted oil from grape seed.

Many studies have found that polyphenols have benefits towards improving health. Del Rio, et al., reported that polyphenols may inhibit cancer cell growth, decrease vascularization, protect cells from oxidation, and improve vasodilation and insulin secretion [21]. Polyphenols are absorbed through the gut barrier allowing for greater antioxidant capabilities inside of the plasma. Polyphenol is converted to O-glucuronides and then transferred to the liver where metabolism continues. If polyphenols are not fully absorbed, they will travel to the colon and are metabolized by the microflora [22].

Grape seed polyphenol has resveratrol, procyanidins and proanthocyanidins, which are antioxidants that fight against free radicals and oxidation. Grape seed extracts (GSE) have been found to decrease inflammation and apoptotic cell death. To test the ability of grape seed polyphenol, Sehirli, et al., tested GSE on mice with ischemic/reperfusion-induced hepatic injury. Ischemia occurs through the constriction of blood vessels, and is so severe that

blood and oxygen cannot flow to certain body organs/ tissues. Reperfusion injury occurs after ischemia, where a lack of oxygen causes the organ and tissues to necrotize. It is important to reduce chance of this process from occurring in the liver to maintain full function. Rats with ischemic/reperfusion-induced hepatic injury, initially had decreased antioxidant glutathione (GSH) levels, while hepatic malondaldehyde (lipid peroxidation), myeloperoxide (neutrophil infiltration), AST, ALT, lactate dehydrogenase (LDH), and inflammatory cytokines levels were elevated. Once GSE treatment was given to the treatment group, each of these factors were reversed. These results suggest that GSE not only reduced ischemia and reperfusion in those with liver damage, but it also reduced lipid peroxidation and neutrophil infiltration due to its antioxidant power [23].

Grape seed extract not only reduces ischemia, reperfusion, lipid peroxidation, and neutrophil infiltration, but has been observed to decrease free-radicals in cancer, thus preventing a tumor from growing further. Khoshbaten, et al., performed a study on 30 patients with NAFLD by using a vitamin C for the control group and GSP as the treatment group for three months. Results found that the liver, spleen and portal vein size between both therapeutic techniques were not altered after treatment. AST and ALP levels did not decrease significantly, but levels of ALT decreased with the GSP therapy. AST and ALT assess liver damage. GSE decreased the level of steatosis in the treatment group, but vitamin C did not. Overall, there was improvement in liver function in

those who took grape seed extract as opposed to those in the vitamin C group [24].

Glucose accumulation may decrease AMP kinase and acetyl-CoA carboxylase phosphorylation which lead to fat accumulation in the liver. With treatment of polyphenol this process is re-activated to metabolize elevated glucose levels. In a study performed by Aoun, et al., the researchers observed the effect of red wine polyphenols on fatty acid content in rat liver on a high-fat-high-sucrose diet. A second objective was to observe the sirtuin-1-AMP kinase signaling pathway, and its role in preventing fatty liver disease. Eight-teen rats were divided into three groups: a control group; a high-fat-high-sucrose diet group (HFHS); a high-fat-high-sucrose + polyphenol diet group (HFHS+P), and were fed their respective diets for 6 weeks. Results show that supplementing a HFHS diet with polyphenols only partially prevented an accumulation of triacylglycerol (TAG) in the liver. There was no change in plasma TAG, cholesterol, or non-esterified fatty acids (NEFA) between both treatments. Polyphenol could not fully prevent steatosis from a HFHS diet, however made it less severe [25].

CHAPTER III

METHODOLOGY

The current study is part of a larger study. Briefly, 32 five-week old C57BL/6J male mice were purchased from the Jackson Laboratories in Maine, US. All mice were treated within the Texas Woman's University Animal Care and Use Committee (IACUC) guidelines. They were given food (lab chow) and water ad libitum for 2 weeks with a 12 hour light/dark cycle. Mice were then divided randomly into 4 groups, and housed 4 per cage. See figure 1 for the experimental design.

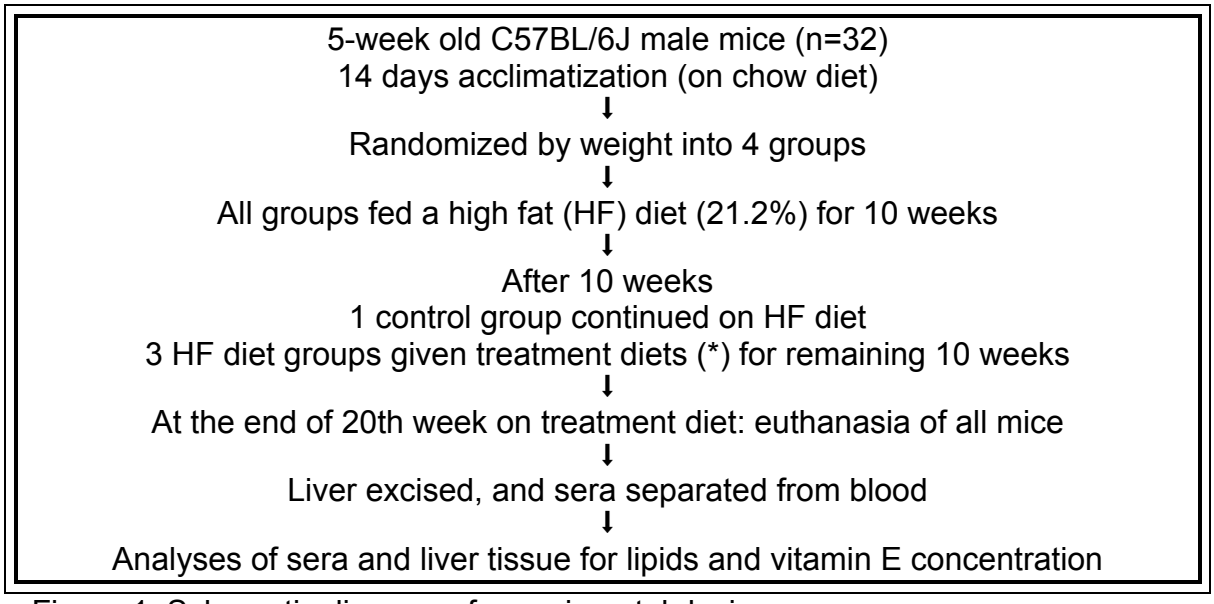

Figure 1. Schematic diagram of experimental design

*High fat diet + Grape seed polyphenol (2%); High fat diet + ∂-tocotrienol (0.05%); High fat diet + Grape seed polyphenol (2%) + ∂-tocotrienol (0.05)

Table 1. Composition of the control and treatment diets containing Grape Seed Polyphenol (GSP) and ∂-tocotrienol (∂T3).

	High fat control	GSP 2%	∂-tocotrienol 0.5%
Ingredients	g/kg	g/kg	g/kg
Casein	195.00	195.00	195.00
DL Methionine	3.00	3.00	3.00
Sucrose	341.46	341.46	341.46
Corn Starch	150.00	150.00	150.00
Maltodextrin	0	0	0
Milkfat	210.00	210.00	210.00
Soybean Oil (Vit E Stripped)	1.50	1.50	1.50
Cellulose	50.00	50.00	50.00
Mineral Mix, AIN76 (170915)	35.00	35.00	35.00
Calcium Carbonate	4.00	4.00	4.00
Vitamin Mix, Teklad (40060)	10.00	10.00	10.00
Ethocyquin, Antioxidant	0.04	0.04	0.04
GSP or ∂T3 in diet	0	20 g/kg	660 µg/kg
Total Vitamin E	123 IU/kg	123 IU/kg	123 IU/kg

For the current study, sera were assayed for total cholesterol, triglycerides, free fatty acids and vitamin E levels. Liver tissues were assayed for total cholesterol, triglycerides and vitamin E levels.

Hepatic Lipid and Vitamin E Extraction

A modified procedure of Hara and Radin was used to extract lipids from the liver [26]. The entire procedure was performed in low light to prevent oxidation of vitamin E. Briefly, thawed rat liver (250 mg) was mixed with 4.5 mL of hexane: isopropanol (1.5:1) and homogenized using an Ultra turax (speed= 270-280 rpm). The mixture was protected in foil wrap and stored at room temperature for two hours for lipid and vitamin E extraction. The mixture was then filtered with Whatman #2 filter paper and bring to 5 mL volume with hexane: isopropanol. The extract was immediately stored at -80°C until analyses. All analyses occurred within two weeks of extraction.

Determination of Hepatic Lipids

Total cholesterol was assayed on the liver extract described above using Stanbio Cholesterol Liquicolor kit (Kit #1010-225) following the manufacturer's protocol. Similarly, hepatic triglyceride was assayed using a Stanbio Triglyceride Liquicolor kit (Kit #2200-225).

Serum Lipid Profile

Total cholesterol and triglyceride were assayed following the manufacturer's protocol using Stanbio Cholesterol Liquicolor kit (Kit #1010-225) and Triglyceride Liquicolor kit (Kit #2200-225), respectively.

Determination of Serum Free Fatty Acids

This procedure was performed using BioVision Free Fatty Acid Quantification Kit (Kit #K612-100) following the manufacturer's protocol.

Determination of Serum and Hepatic Vitamin E

Vitamin E was assayed using a modified procedure of Devaraj and Jialal [27]. The entire procedure was done in low light to prevent oxidation of the sample. Briefly, 200 µL of serum or liver extract and 50 µL of internal standard (α-tocopheryl acetate [ATA] 0.3 mg/mL concentration) were pipetted into glass tubes, vortexed for 1 minute, and dried under N_2. The dried samples were reconstituted with 200 µL of ethanol, vortexed for 30 seconds, and transferred into the HPLC sample vials. HPLC wavelength was set at 292 mn, and flow rate was set at 0.8 mL/min. The mobile phase consisted of 20% dichloromethane, 60% methanol, and 20 % acetonitrile, all de-gased. After a five minute purge, an autosampler with samples was placed in the HPLC and ran for 22 minutes per sample. All samples were assayed in duplicates.

Statistical Analysis

Analysis was performed using one-way ANOVA on SPSS version 19 for Windows. $p < 0.05$ was considered significant. When significance was obtained, Tukey post-hoc test was used to determine the differences among the groups.

CHAPTER IV

RESULTS

This study is part of a larger project. The initial mean body weight was similar among the four groups of mice. The final body weight was not significantly different among the four groups of mice (Table 2).

Table 2. Body weights and liver weights of C57BL/6J male mice. Values are means±SEM. Values with different superscripts are significantly different ($p<0.05$).

Groups	Initial Mean Body wt (g)	Final Mean Body wt (g)	Mean Liver wt (g)
High Fat (Control)	25.75±1.21	49.85±0.85	3.87±0.53
GSP 2%	25.78±0.55	36.36±1.42	1.62±0.14*
∂T3 0.05%	25.52±0.59	48.20±1.41	3.75±0.38
GSP 2%+ ∂T3 0.05%	25.79±0.62	37.31±0.66	1.64±0.10*

GSP, Grape Seed Polyphenol; ∂T3, ∂-tocotrienol

Relative liver weight in both GSP diet group and GSP+∂T3 combination diet group were significantly lower than the high fat control group. There were no significant differences in relative liver weight between high fat control and ∂T3 diet groups (Figure 2).

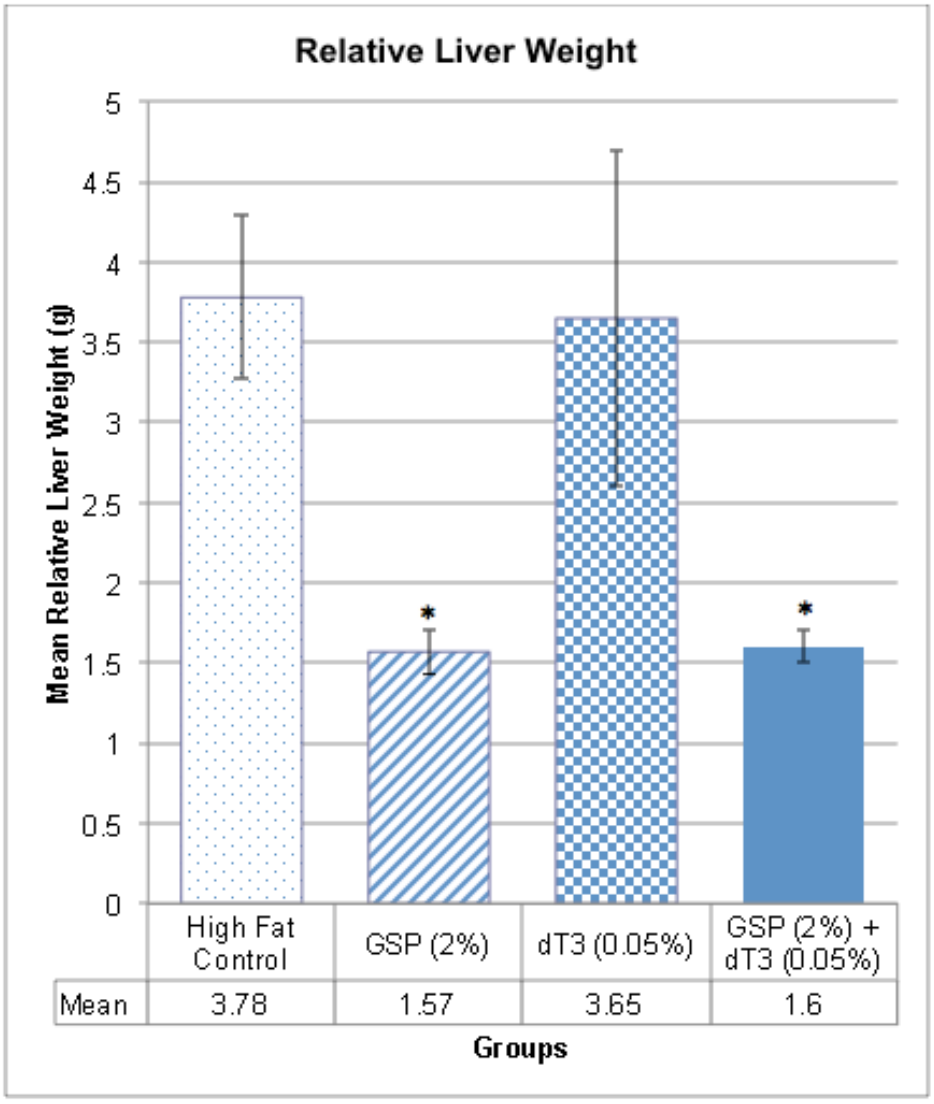

Figure 2. Mean relative liver weight of C57BL/6J male mice. Values are means±SEM.

Hepatic cholesterol was significantly lower in the GSP diet and GSP+∂T3 combination diet groups compared to both the high fat control group and ∂T3 diet group. There were no significant differences in hepatic cholesterol between the high fat control and the ∂T3 diet groups (Figure 3).

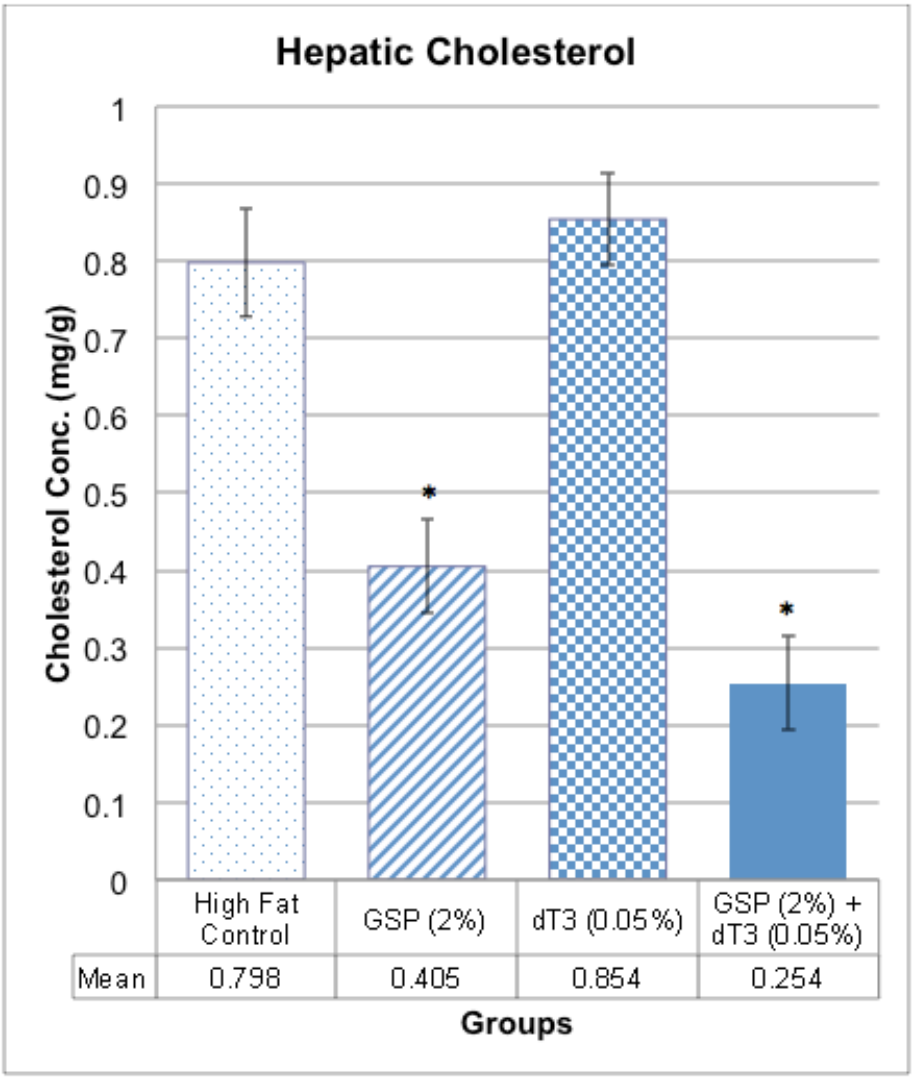

Figure 3. The effect of treatment diets on hepatic cholesterol concentration in C57BL/6J male mice fed grape seed polyphenol or ∂-tocotrienol, alone and combined.

Hepatic triglycerides were significantly lower in the GSP + ∂T3 combination diet group compared to both the high fat control and ∂T3 diet groups. There were no significant differences in hepatic triglycerides among the high fat control, GSP diet and ∂T3 diet groups (Figure 4).

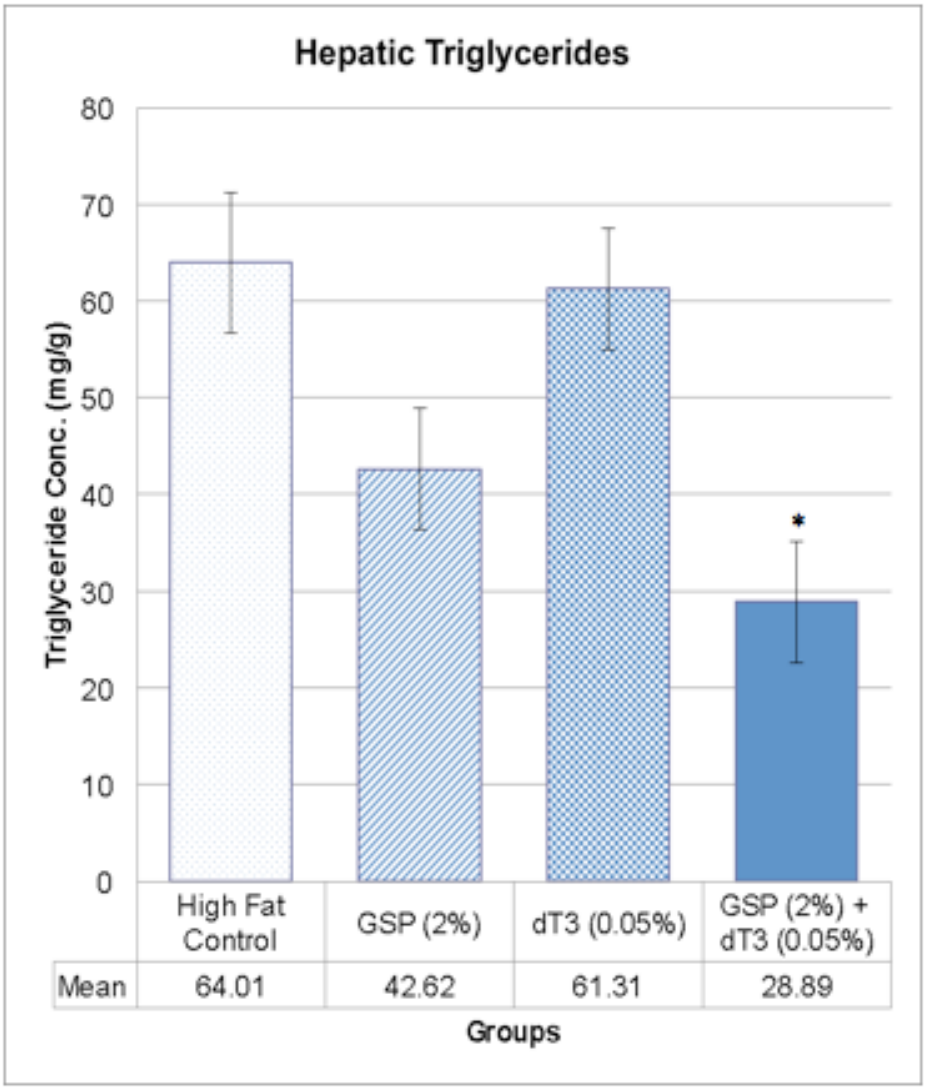

Figure 4. The effect of treatment diets on hepatic triglyceride concentration in C57BL/6J male mice fed grape seed polyphenol or ∂-tocotrienol, alone and combined.

Hepatic vitamin E was significantly lower in the GSP + ∂T3 combination diet group compared to both the high fat control and ∂T3 diet groups. There were no significant differences in hepatic vitamin E between the high fat control diet, GSP diet and ∂T3 diet groups (Figure 5). Results represent α-tocopherol isomer of vitamin E. The other isomers were not detected.

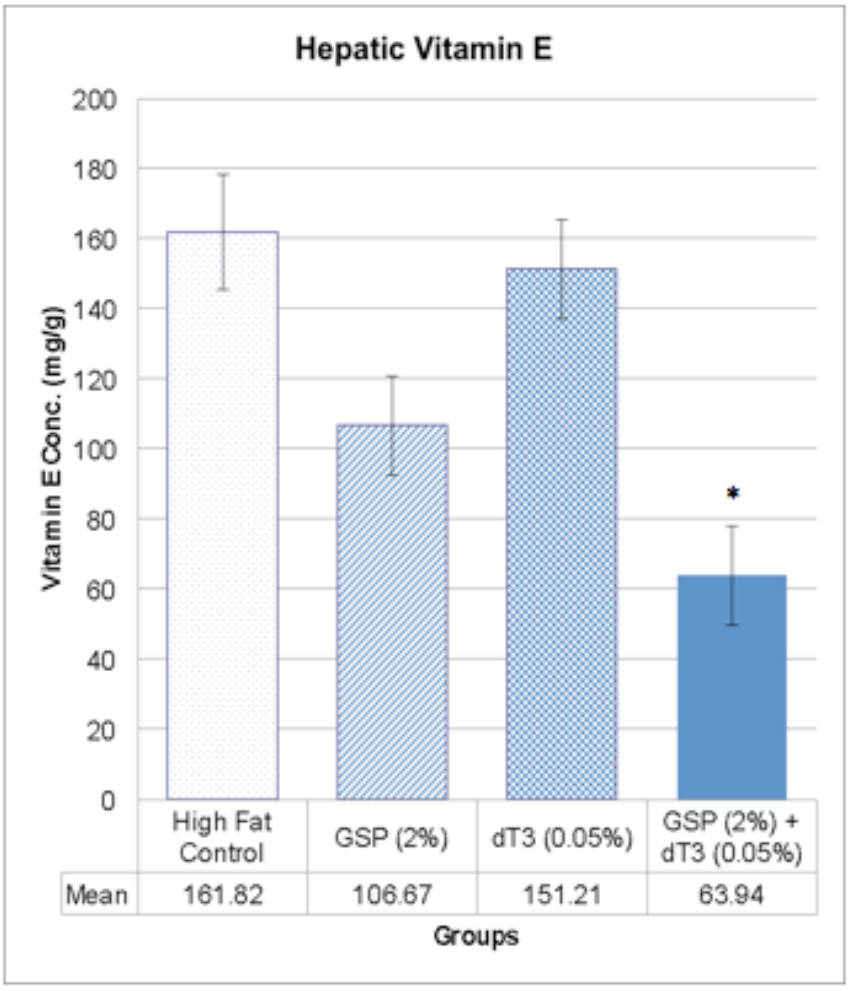

Figure 5. The effect of treatment diets on hepatic vitamin E concentration in C57BL/6J male mice fed grape seed polyphenol or ∂-tocotrienol, alone and combined.

There were no significant differences in serum triglycerides or free fatty acids among the four groups of mice. However the serum triglycerides in the GSP diet group was 13% lower than the high fat control (Table 3).

Table 3. Mean serum triglyceride and free fatty acid levels in C57BL/6J male mice. Values are means ± SEM.

	Mean Serum Levels	
Groups	Triglyceride (mg/dL)	FFA (nmol/μL)
High Fat (Control)	68.9±6.6	0.537±.1
GSP 2%	59.7±6.1	0.579±.1
∂T3 0.05%	73.5±6.1	0.703±.1
GSP (2%)+ ∂T3 (0.05%)	77.6±6.1	0.466±.1

GSP, Grape Seed Polyphenol; ∂T3, ∂-tocotrienol

Serum cholesterol was significantly lower in the GSP diet and GSP+∂T3 combination diet groups compared to both the high fat control diet group and ∂T3 diet group. There were no significant differences in serum cholesterol between the high fat control group and the ∂T3 diet group (Figure 6).

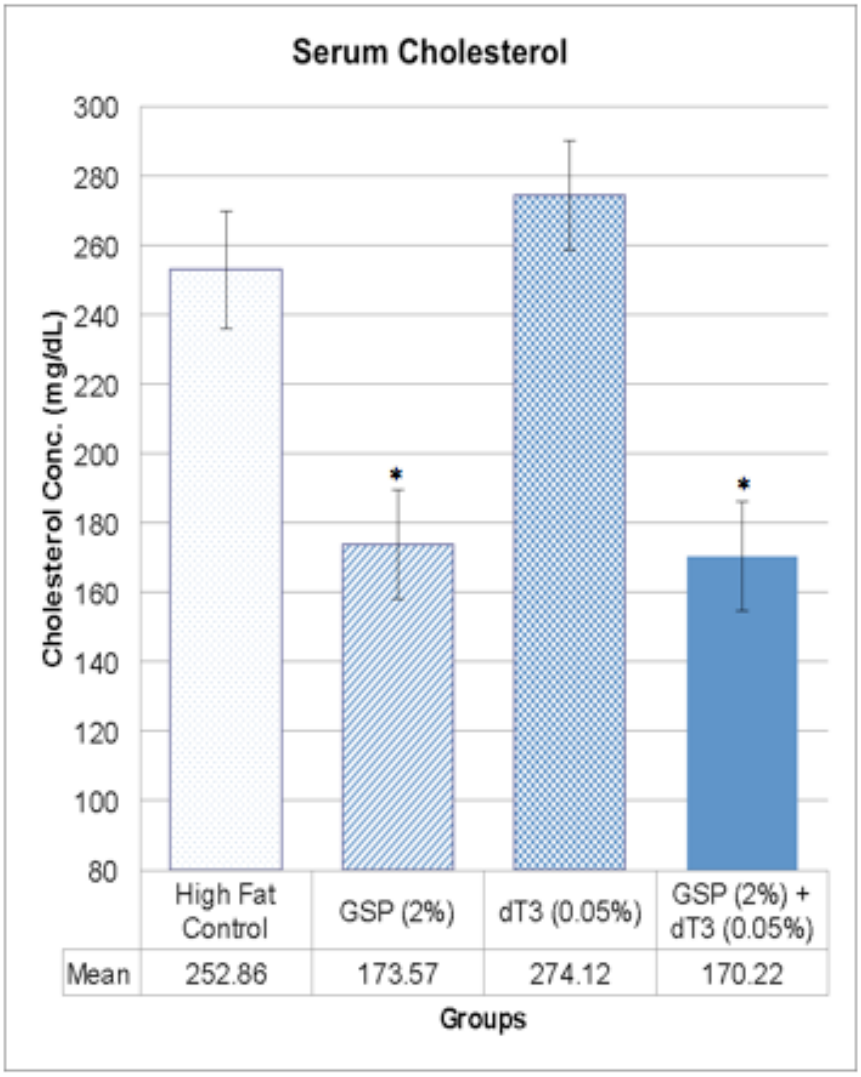

Figure 6. The effect of treatment diets on serum cholesterol concentration in C57BL/6J male mice fed grape seed polyphenol or ∂-tocotrienol, alone and combined.

Serum vitamin E was significantly lower in the GSP diet group compared to the high fat control group. There were no significant differences in serum vitamin E between the high fat control and the GSP + ∂T3 combination groups (Figure 7). Results represent α-tocopherol of vitamin E as the other isomers were not detected within the HPLC.

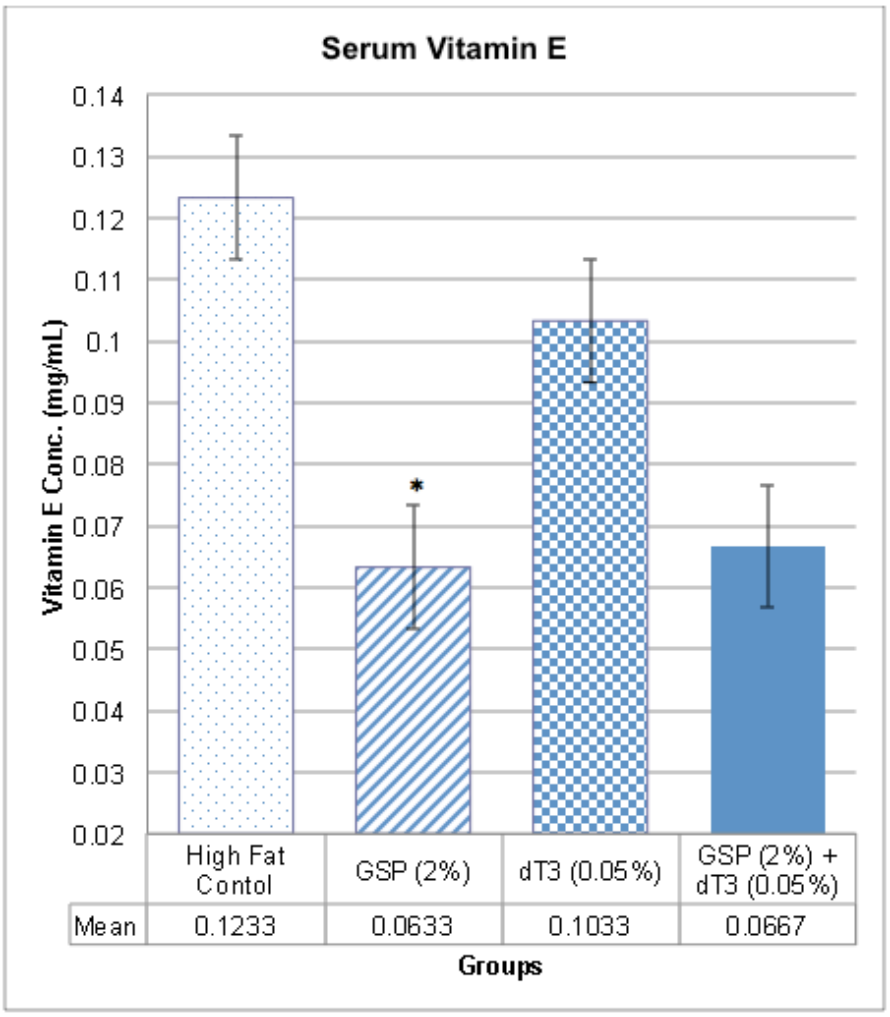

Figure 7. The effect of treatment diets on serum vitamin E concentration in C57BL/6J male mice fed grape seed polyphenol or ∂-tocotrienol, alone and combined.

CHAPTER V

DISCUSSION

The current study was conducted to determine whether grape seed polyphenol (GSP), ∂-tocotrienol (∂T3) or a combination of both GSP and ∂T3 (GSP+∂T3) with a high-fat diet, would improve lipid levels in C57BL/6J mice in a non-alcoholic steatohepatitis-like state. The NASH-like state was induced using a high-fat diet for 20 weeks. The current study is unique in that it is the first to test a combination of GSP and ∂T3 as a treatment for this mouse type.

The initial and final mean body weights were not significantly different among the four groups of mice. However, the final mean body weight of mice consuming the GSP diet alone or in combination with ∂T3 were 27 and 25% lower than the mice given the high fat control diet. It is possible that the addition of GSP to the diet was responsible for the lowered body weights observed in this study. Relative liver weight was significantly lower in the GSP diet and in the GSP + ∂T3 combination diet groups. Relative liver weight was dependent on the amount of free fatty acids being delivered to the liver. A manifestation of NASH is insulin resistance, which causes free fatty acids to move to the liver for storage, causing liver weight to increase in mice given a high fat control diet [3, 29].

The current study found no reduction in hepatic or serum cholesterol when ∂T3 was given alone. These results are similar to a study performed by Chin, et al., showing that when older adults were given TRF, their serum cholesterol

levels remained the same within 6 months of the study [20]. This may be due to an intake of a tocopherol and tocotrienol mixture. Tocopherols are prioritized to absorb before tocotrienols, therefore blocking tocotrienols from performing its cholesterol lowering function [8]. Using GSP in the current study presented significant decrease in cholesterol levels in both the liver and serum of the mice. Not only was GSP effective when used alone, but when added to ∂T3, hepatic and serum cholesterol were also lowered significantly. Two studies performed by Adisakwattana and Ngamukote, et al found that polyphenols in grape seeds prevent production of cholesterol esterase in the pancreas [29-30]. Grape seed polyphenol has the ability to bind with bile acids and excrete them from the system, further reducing cholesterol levels in the system. El-Adawi, et al., also observed a 32-42% decrease in serum cholesterol when grape seed extract was given to male mice [31]. The current study follows these trends, in that mice fed a grape seed polyphenol diet, alone and in combination, had significantly lower cholesterol levels in both serum and liver.

In the present study, the ∂T3 diet group had similar serum and hepatic triglycerides as mice consuming a high fat control diet. Interestingly, serum triglycerides were 31% higher in mice given the ∂T3 diet compared to the high fat control group. Our results are similar to a study performed by Chin, et al., showing that when older adults were given TRF, their serum triglyceride levels remained the same or increased within 6 months of the study [20]. Interestingly, when GSP was combined with ∂T3, liver triglyceride levels were reduced

significantly. This dramatic change in triglycerides with GSP supplementation demonstrates the power of this polyphenol in reducing effects of NASH. Zern, et al., observed the effect of grape polyphenol on triglycerides and cholesterol in female guinea pigs, and reported that grape polyphenols lowered plasma triglycerides by 39% [32]. Two years later Zern, et al., found that by giving pre- and postmenopausal women grape polyphenols, plasma triglycerides were lowered by 15% in premenopausal women, and 6% in postmenopausal women [33]. Del Bas, et al., suggested that the lowered plasma triglycerides in normolipidemic rats given grape seed procyanidin extract are attributed to the hepatic expression of a nuclear receptor called small heterodimer partner, which decreases action of ApoB [2008]. The lowered hepatic triglycerides occurring with GSP supplementation is attributed to its ability to prevent peroxidation of lipids in the liver, permitting the liver to reduce lipids in storage [24].

In the current study serum free fatty acids (FFA) were not different among the four groups of mice. This was the first study of its kind to measure free fatty acids in mice given GSP, ∂T3, and a combination of both treatments.

The liver can store more tocotrienols than any other organ [8, 35]. The reason is due to the liver being a highly perfused organ that allows vitamin E to be stored [35]. The current study found that hepatic vitamin E in mice given ∂T3, were similar to mice given a high fat control diet. Similar results were found in a study performed by Chin, et al., where long term supplementation of TRF increased levels of serum vitamin E [20]. In the current study, hepatic vitamin E

was significantly lower in the combination GSP + ∂T3 diet group, and serum vitamin E was significantly lower in the GSP diet group. Studies have found that grape polyphenol intake improves antioxidative activity in the blood and a variety of tissues, allowing vitamin E to be spared, essentially increasing the levels [36-37]. Lowered vitamin E is attributed to its antioxidant activity in having to decrease oxidative stress in the body [38]. In the current study, it is possible that vitamin E was used to fight all symptoms of the NASH-like state in the mice.

CHAPTER VI

SUMMARY & CONCLUSION

The purpose of this study was to investigate the effect delta-tocotrienol, grape seed polyphenol, alone and in combination, in C57BL/6J mice fed a western-like fat-diet induced non-alcoholic steatohepatitis-like state. The study investigated lipid levels and vitamin E levels in the liver and sera of the mice. This investigation was part of a larger study investigating histological changes in the same condition. The study examined the lipid profile and vitamin E levels in the liver and blood of mice. This study was part of a larger project investigating histological changes in this same mouse type with the same condition.

The current study consisted of two null hypotheses; the first stating that there would be no significant difference in hepatic cholesterol, triglyceride or vitamin E concentration between ∂-tocotrienol and GSP treatments in mice with non-alcoholic steatohepatitis-like symptoms . Results show that a mixture of GSP and ∂-tocotrienol given to mice reduced levels of cholesterol, triglycerides, and vitamin E significantly. Hepatic cholesterol was lower in those consuming a GSP diet and GSP + ∂T3 combination diet compared to the high fat control and ∂T3 diet group. Hepatic triglycerides were lower in the GSP +∂T3 combination diet group compared to high fat control and the ∂T3 diet group. Hepatic vitamin E was lower in the GSP + ∂T3 combination diet group compared to those in the high fat control and ∂T3 diet groups. Therefore, this hypothesis was rejected.

The second null hypothesis stated that there would be no significant difference in serum cholesterol, triglyceride, free fatty acid or vitamin E concentration between ∂-tocotrienol and GSP treatments in mice in a non-alcoholic steatohepatitis-like state. Results show that the GSP + ∂T3 combination diet consumed by mice reduced levels of serum cholesterol and vitamin E. Serum cholesterol was lower in the GSP diet and GSP + ∂T3 combination diet groups compared to the high fat and ∂T3 diet groups. Serum vitamin E was lower in the GSP diet group compared to those in the high fat control group. Serum triglycerides and free fatty acids were similar among the groups. Therefore, this hypothesis was rejected.

This study exhibited a trend that GSP alone and in combination with ∂-tocotrienol can improve conditions in mice with nonalcoholic steatohepatitis-like symptoms. Further research is necessary to fully understand the benefits of ∂T3.

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APPENDIX A
Procedure for Liver Tissue Extraction

Procedure for Liver Tissue Extraction
(Modified procedure of Hara A, Radin N, 1978)

PERFORM IN LOW LIGHT!

1. Mix 15mL hexane and 10 mL isopropanol together under a hood
2. ADJUST: Gather 250 mg of rat liver
3. Add 4.5 mL (or adjusted amount) of Hexane: Isopropanol to livers test tube.
4. Wait 60 seconds.
5. Homogenize the mixture using a Ultra turax machine (speed= 270-280 rpm) until smooth.
6. Close with cap, wrap in foil to prevent oxidation.
7. Store for 2 hours.
8. Filter mixture using filter paper into a new test tube (wet paper first).
9. Make up the volume to original volume with Hexane: Isopropanol
10. Aliquot into (5) 1 mL microfuge tubes.
11. Label and wrap all tubes in foil paper, keep in low light.
12. Keep one tube out to use in experiment.
13. Store aliquoted tubes at -80°C as soon as possible.

APPENDIX B
Procedure for Vitamin E Analysis

Procedure for Vitamin E Analysis

1. Gather mobile phase substances dichloromethane, methanol, acetonitrile.
2. De-gas all substances.
3. Connect the dichloromethane, methanol, and acetonitrile to the HPLC machine.
4. Set up method in program: 20% dichloromethane, 60% methanol, 20% acetonitrile.
5. Purge the machine for 5 minutes.
6. While machine is purging prepare samples (see Appendices E and I)
7. From the four tubes that were stored in -80ºC, pull out a liver sample filtrate that has not seen light for experimentation.
8. Purge the machine again for 5 minutes.
9. Place autosampler with samples into the machine
10. Run the machine.

HPLC Parameters
Wavelength= 292 nm
Mobile: methanol: acetonitrile: dichloromethane (60:20:20)
Flow rate: 0.8 mL/min
Column: C18

APPENDIX C
Procedure for the Determination of Hepatic Cholesterol

Procedure for the Determination of Hepatic Cholesterol
(Stanbio Cholesterol Liquicolor Kit #1010-225)

1. Turn water bath on: 37°C.
2. Use 20µl standard-200 and make triplicates.
3. Pipette 200 µl sample for each mouse in each group into their own tube.
4. Evaporate each tube under N_2 until dry.
5. Reconstitute with 200 µl of ethanol
6. Vortex for 1 minute.
7. Transfer all 200 µl to another tube, labeled transfer tube.
8. Wash two more times (steps 5-7); transfer tube will now have 600 µl.
9. Transfer 100 µl from the transfer tube to 3 fresh tubes.
10. Add 500µl TG reagent enzyme to blank, standards, and samples:

Tube	1 Blank	4 std 200	5 samples 1- 8
Reagent	500 µl	500 µl	500 µl
Std (200mg/dL)	-	20 µL	-
Sample of unknown	-	-	100 µL

11. Vortex all tubes.
12. Incubate all test tubes at 37°C for 10 minutes.
13. Plate 300 µl into wells.
14. Read at 500 nm within 60 minutes on a spectrophotometer.

Calculations:

1. 200µL filtrate (smpl)/4500µL hexane:isoproanol*250mg liver=11.11mg liver
2. $\frac{\text{600 µL (filtrate+ethanol)}}{\text{100 µL (sample in triplicates)}} = \frac{\text{11.11 mg Liver}}{X} = 1.85 \text{ mg Liver}$
3. Note: 200 mg/dL (Chol std)= 2 mg/mL= 2000 µg/mL Chol in std
4. 20µL std/500µl to change to mL*2000µg TG std/ mL=80µg TG in 20µL
5. $\frac{\text{600 µL (Filtrate+ethanol)= 80mg}}{\text{20 µL (std)}} = \frac{2.7\text{mg}}{X}$
6. Abs U/ Abs S X 80 µg (std)= X µg in 1.85 mg liver samples
7. X µg in sample/ 1.85 mg liver= Y µg/mg= Y mg Chol/ 1 g liver
8. Note: normal range is 2.5-4.5 mg TG/g liver in humans

*Bold and underlined indicate that they could be altered depending
std: standard

APPENDIX D
Procedure for the Determination of Hepatic Triglycerides

Procedure for the Determination of Hepatic Triglycerides
(Stanbio Triglyceride Liquicolor Kit #2200-225)

1. Turn water bath on: 37°C.
2. Use 20µl standard-200 and make triplicates.
3. Pipette 50µl sample for each mouse in each group into their own tube..
4. Evaporate each tube under N_2 until dry.
5. Reconstitute with 50 µl of ethanol
6. Vortex for 1 minute.
7. Transfer all 50 µl to another tube.
8. Wash two more times (steps 5-7); transfer tube will now have 150 µl.
9. Transfer 10 µl from the transfer tube to 3 fresh tubes.
10. Add 500µl TG reagent enzyme to blank, standards, and samples:

Tube	1 Blank	4 std 200	5 samples 1- 8
Reagent	500 µl	500 µl	500 µl
Std (200mg/dL)	-	20 µL	-
Sample of unknown	-	-	10 µL

11. Vortex all tubes.
12. Incubate all test tubes at 37°C for 10 minutes.
13. Plate 300 µl into wells.
14. Read at 500 nm within 60 minutes.

Calculations:

1. 50µL filtrate (sample)/**4500**µL hexane:isoproanol***250** mg liver = **2.8mg** liver
2. 150 µL (filtrate+ethanol) = 2.8 mg Liver = .187 mg Liver
   10 µL (sample in triplicates) X
3. Note: 200 mg/dL (TG std)= 2 mg/mL= 2000 µg/mL TG in standard
4. 20µL std/500µl to change to mL*2000 µg TG std/ mL=80µg TG in 20µL
5. 150 µL (filtrate+ethanol)= 80µg =10.7µg
   20 µL (standard) X
6. **Abs U/ Abs S X 10.7 µg (std)= X µg in .187 mg liver samples**
7. **X µg in sample/ .187 mg liver= Y µg/mg= Y mg TG/ 1 g liver**
8. Note: normal range is 30 – 40 mg TG/g liver in humans

***Bold and underlined indicate that they could be altered depending on availability of mouse liver.**

APPENDIX E
Procedure for the Determination of Hepatic Vitamin E

Procedure for the Determination of Hepatic Vitamin E
(Modified procedure of Hara A, Radin N, 1978)

PERFORM IN LOW LIGHT

1. Label two sets of test tubes (12 X 75mm) and one set of HPLC sample vials.
2. Remove ATA internal standard from the -70ºC freezer.
3. Pipette 200 µL of sample extract into the first set off test tubes.
4. Pipette 50 µL of ATA internal standard (0.3 mg/mL concentration) into each tube.
5. Vortex for 1 minute.
6. Dry the hexane under nitrogen gas until completely dry.
7. Reconstitute with 200 µL of ethanol.
8. Vortex for 30 seconds.
9. Transfer 200 µL of the extracted sample into the HPLC sample vials and place in the autosampler.

HPLC Parameters

Wavelength: 292 nm
Mobile:methanol: acetonitrile:dichloroethane (60:20:20)
Flow rate: 0.8 mL/min
Column: C18

Calculation

1. 250 mg Liver= 0.25 g Liver homogenized in 4.5 mL buffer
2. Solution has 0.25 g/4.5 mL = 0.05566 g of Liver/ mL of solution
3. 50 µL injected into HPLC. 1000 µL/ 50 µL = 20 (the factor)

Hepatic Vitamin E = unknown X 20= # mg/ 0.0556 g of Liver

APPENDIX F
Procedure for the Determination of Serum Cholesterol

Procedure for the Determination of Serum Cholesterol
(Stanbio Cholesterol Liquicolor kit #1010-225)

1) Label test tubes: Blank, standard, sample (unknown). Work in duplicate.
2) Turn water bath on, set temperature to 37°C
3) Prepare standards in advanced
   a) S50: 12.5 µL std + 37.5 µL DI water
   b) S100: 25 µL std+ 25 µL DI water
   c) S200: 50 µL std+ 0 µL DI water
      *Store all for later use
4) Pipette 1 mL of reagent into all test tubes (blank, standard and samples)
5) Add 10 µL of DI water to blanks
6) Add 10 µL of standards to appropriate tubes
7) Add 10 µL of samples to appropriate tubes
8) Vortex each test tube
9) Incubate all tubes in water bath at 37°C for 5 minutes
10) Transfer 300 mL from each tube to the appropriate well on the Wells Plate.
11) Read plates at 500nm on spectrophotometer.

Calculation

$$\text{Serum Cholesterol (mg/dL)} = \frac{Au}{As} \times 200$$

Au-unknown
As- standard
200- concentration of the standard

APPENDIX G
Procedure for the Determination of Serum Triglycerides

Procedure for the Determination of Serum Triglycerides
(Stanbio Liquicolor Triglycerides Kit #2200-225)

1) Turn water bath on, set temperature to 37°C.
2) Label test tubes: Blank, standards, sample (unknown). Work in duplicate.
3) Prepare standards in advanced
   a) S50: 12.5 µL std + 37.5 µL DI water
   b) S100: 25 µL std+ 25 µL DI water
   c) S200: 50 µL std+ 0 µL DI water
      *Store all for later use
4) Pipette 1 mL of reagent into each test tube
5) Add 10 µL of DI water to blanks
6) Add 10 µL of standards to appropriate tubes
7) Add 10 µL of samples to appropriate tubes
8) Vortex each test tube
9) Incubate all tubes in water bath at 37°C for 5 minutes
10) Transfer 300 mL from each tube to the appropriate well on the Wells Plate.
11) Read plates at 500nm on spectrophotometer.

Calculation

$$\text{Serum Triglyceride (mg/dL)} = \frac{Au}{As} \times 200$$

Au-unknown
As- standard
200- concentration of the standard

APPENDIX H
Procedure for the Determination of Serum Free Fatty Acids

Procedure for the Determination of Serum Free Fatty Acids
(BioVision Catalog #K612-100)

PERFORM IN DARK
1. Turn on water bath to 37°

Preparation :
2. Dissolve ACS (Acyl-CoA Synthetase) reagent with 220 µL assay buffer by pipetting up and down.
3. Dissolve enzyme mix with 220 µL assay buffer by pipetting up and down.
4. Store at -20°C, and use within 2 months.
5. Reaction Mix Prep: Calculate so each blank, standard and sample will get 50 µL:

44 µL Assay Buffer
2 µL Fatty Acid Probe
2 µL Enzyme Mix
2 µL Enhancer
50 µL

Standard Curve (Colorimetric):
6. Add 0, 2, 4, 6, 8, 10 µL palmitic acid standard into mini test tubes individually.
7. Adjust volume to 50 µL/tube with Assay Buffer to generate fatty acid standard.

Method:
8. For liquid samples, different volume of samples can be directly added to each well, then bring up the volume to 50 µL/well with Assay Buffer.
9. Acyl-CoA synthesis: Add 2 µL ACS Reagent into all standard and sample tubes.
10. Vortex each test tube.
11. Incubate at 37°C for 30 minutes.
12. Add 50 µL of the Reaction Mix to each tube (standards, samples, blank).
13. Cover with foil to protect from light.
14. Incubate at 37°C for 30 minutes.
15. Transfer to plate.
16. Measure O.D. 570 nm for colorimetric assay.

Calculations:

Fatty Acid Concentration = Fa/Sv (nmol/µL or mM)
Fa is the Fatty Acid amount (nmol) in the well obtained from std curve.
Sv is the sample volume (µL) added to the sample well.

APPENDIX I
Procedure for the Determination of Serum Vitamin E

Procedure for the Determination of Serum Vitamin E
(Modified procedure of Hara A, Radin N, 1978)

1. Label three sets of test tubes (12X75mm) and one set of HPLC samples vials.
2. Remove ATA internal Standard from the -70ºC freezer.
3. Pipette 50 µL of sample extract into first set of test tubes.
4. Pipette 50 µL of internal standard (0.3 concentration) into each tube.
5. Pipette 50 µL of ethanol and vortex for 30 seconds.
6. Add 250 µL of hexane and vortex for 30 seconds.
7. Allow tubes to stand until there is a clear separation of two layers or centrifuge the tubes for 2 minutes.
8. Remove ~ 200 µL of the top layer and transfer to the second set of tubes.
9. Add an additional 250 µL of hexane and re-extract by vortexing for 30 seconds.
10. Repeat step 8 by removing ~250 µL of the top layer (total volume= ~450 µL).
11. Dry the hexane under nitrogen.
12. Reconstitute with 100 µL of ethanol and vortex for 30 seconds.
13. Transfer to third set of tubes.
14. Repeat steps 12 and 13 again.
15. Transfer 200 µL of the extracted sample into HPLC sample vials and place into the autosampler.
16. Place autosampler into the HPLC machine.
17. Run machine.

HPLC Parameters
Wavelength: 292 nm
Mobile:methanol: acetonitrile:dichloroethane (60:20:20)
Flow rate: 0.8 mL/min
Column: C18

APPENDIX J
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Authors: Melissa Mendez *
Received: 10 November 2013
E-mails: mmendez4@twu.edu
Submitted to special issue: Vitamin E Nutrition and Metabolism,
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