

2010

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The effect of drugs and nutraceuticals on the prevention and treatment of non-alcoholic fatty liver disease using rats as a model for humans

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Nutritional Sciences (Animal Nutrition)

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2010

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CHAPTER I:

General Introduction

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), or hepatic steatosis, is a disorder recently identified as a major health concern in developed countries with a high incidence of obesity and the metabolic syndrome. NAFLD is a condition that is recognized worldwide because, not only is it seen in developed countries, it is also seen in developing countries because of their high incidence of malnutrition and starvation. With an estimated one-third of the world's population having hepatic steatosis, research into the disease has skyrocketed over the last few decades.

Simply put, NAFLD is an accumulation of lipid in the liver and it occurs because of an imbalance in liver lipid homeostasis. The consequences of the progression of the disease can be daunting, as liver cirrhosis and failure is not an uncommon result. Understanding the pathogenesis of this disease, a mechanism that is still under investigation, will be key in identifying potential prevention and treatment strategies.

Many different dietary interventions and compounds have been proposed for the treatment of NAFLD, but the research into their effectiveness is sketchy at best. Very few human trials studying these treatments utilize the most accurate method for assessing liver damage and improvement – the liver biopsy. Most human trials utilize noninvasive techniques that are not as reliable, and, therefore, these studies are inherently flawed. To accurately determine

the effectiveness of the interventions under observation, liver biopsies need to be performed or animal models need to be utilized more often.

This thesis presents original research that utilizes an animal model to compare the effectiveness of different proposed drugs and nutraceuticals on the prevention and treatment of NAFLD.

THESIS ORGANIZATION

At the end of this chapter, a comprehensive review of the present literature on NAFLD is presented. This literature review will describe the definition and diagnosis of NAFLD, its prevalence worldwide, its proposed pathogenesis, and potential interventions and compounds that have been identified for its prevention and treatment. Chapter II presents original research outlining the effects of different drugs and nutraceuticals on the prevention of NAFLD. Chapter III presents original research describing a novel nutritional protocol for the treatment of NAFLD, specifically focusing on lifestyle changes and nutraceutical supplementation. Chapter IV discusses the overall conclusions from this thesis work, recommendations for future research, and personal acknowledgements.

REVIEW OF LITERATURE

INTRODUCTION

Non-alcoholic fatty liver disease was first described by Ludwig *et al.* in 1980 as a condition with histological similarities to alcoholic liver disease but observed in patients without a history of extensive alcohol intake [1]. These clinicians coined the term “non-alcoholic steatohepatitis” to describe this disease, as the livers were characterized by “fatty change” or

accumulation of triacylglycerol in hepatocytes and inflammatory infiltrates. For the first decade, non-alcoholic steatohepatitis was mostly described in obese and/or diabetic women. In 1994, Bacon *et al.* described observing non-alcoholic steatohepatitis in patients with a “different clinical profile” [2]. The disease was found in non-obese, non-diabetic men and women who had normal blood glucose and lipid profiles and tested negative for hepatitis C. This study also described distinct liver cirrhosis in 15% of the patients, which was the first demonstration that liver failure is a potential consequence of non-alcoholic steatohepatitis.

Research into non-alcoholic steatohepatitis (NASH) has profoundly expanded in the last 15 years. To this end, the term non-alcoholic fatty liver disease (NAFLD) was coined and is now well-accepted to better describe the spectrum of disease. The disease ranges from simple steatosis, or “fatty change”, without inflammation to steatosis with inflammation and fibrosis [3].

This review of literature will discuss the prevalence of NAFLD and its associated disorders, the current proposal on the pathogenesis of NAFLD, and potential preventatives and treatments for NAFLD.

NAFLD AND RELATED DISORDERS

Definition and Diagnosis of NAFLD

Non-alcoholic fatty liver disease is basically an “umbrella” term used to describe a wide spectrum of disease presentation observed in patients without a history of significant ethanol, hereinafter termed alcohol, consumption. The definition of “significant” alcohol intake has been debated for many years, and a wide variety of definitions have been used. In early case

reports, NAFLD was only used to describe disease in patients with no alcohol consumption or alcohol consumption less than 10 g/week [1, 4]. Over the years, that definition has become more realistic, with consumption up to 20 and 30 g/day for women and men, respectively, falling under the diagnostic criteria for NAFLD [5, 6]. Another important concept to consider would be the impact of lifetime alcohol consumption, an issue that has yet to be specifically addressed. To date, there has been no standardized, definitive alcohol consumption recommendation for the diagnosis of NAFLD. Interestingly, recent evidence has surfaced demonstrating a protective effect of modest wine drinking (~10 g/day or 5 oz of wine/day) on the prevalence of suspected cases of NAFLD [7].

The spectrum of NAFLD can be divided into four different types or categories based on histological hepatocellular characteristics. The current categories were first described by Matteoni *et al* [3]. Each type then can be graded based on the amount of hepatocytes affected established from a scheme outlined by Brunt *et al* [8]. Table 1 below summarizes these diagnostic criteria.

There are many limitations associated with the diagnosis of NAFLD. The most obvious limitation is the lack of consensus on the definition of the limit of alcohol consumption that can be considered NAFLD, as previously discussed. The second limitation focuses on the pathogenesis of NAFLD and its “silent” nature. Most patients with NAFLD are asymptomatic on presentation. If symptoms are present, they are very nonspecific, such as fatigue or pain in the central or right upper quadrant of the abdomen. The third limitation surrounds the specificity and sensitivity of the non-invasive techniques that are used to aid the diagnosis of NAFLD.

Table 1: Diagnostic Criteria for NAFLD

Category/Type	Histological Characteristics	Clinical Information
Type 1	Simple steatosis	Generally non-progressive
Type 2	Steatosis + lobular inflammation	Benign and not considered to be NASH
Type 3	Type 2 characteristics + ballooning degeneration	Considered NASH without fibrosis and may progress to liver cirrhosis
Type 4	Steatosis + ballooning degeneration + Mallory bodies + fibrosis	Considered NASH with fibrosis and may progress to liver cirrhosis/failure
Grading		
Grade 1	Fat droplets in <33% of hepatocytes	
Grade 2	Fat droplets in 33-66% of hepatocytes	
Grade 3	Fat droplets in >66% of hepatocytes	

Liver biopsy is the most definitive method for diagnosis, but no specific recommendations for the use of liver biopsies in suspected cases of NAFLD have been made. In 2002, the American Gastroenterological Association and the American Association for the Study of Liver Diseases came out with a joint medical position statement for NAFLD. The statement discussed the usefulness of liver biopsies in the diagnosis of NAFLD but, instead of recommending liver biopsies, they simply state that the ramifications of not doing a biopsy should be discussed with the patient [9].

In the place of liver biopsies, physicians rely on serum enzyme markers indicative of hepatocellular damage, especially alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [10]. ALT and AST are enzymes located in the cytosol of hepatocytes and, with membrane damage, will leak out of the cells into liver sinusoids and then into systemic circulation. ALT is specific for the liver, but AST is not a specific marker

for liver damage because it is found in both liver and muscle cells. These enzymes are rarely found in the bloodstream at activities higher than five times the upper normal limits.

Consequently, they are not necessarily correlated with the extent of liver damage [11]. In a study conducted by Yano *et al.* in 2001, the sensitivity of ALT and AST tests to detect fatty liver was 35.7%, which they found to be inferior to the use of the body mass index [12].

Additionally, the entire spectrum of NAFLD has been described in patients with normal ALT activities [13].

Ultrasound also is used commonly in medical practice to diagnose NAFLD. Its reliability, however, is often questioned. The diagnosis of NAFLD via ultrasound is exceptionally subjective. Each diagnostician will interpret the ultrasound images differently, introducing interobserver variability, and the same diagnostician, when revisiting the same image some time later, may not come to the same conclusion, introducing intraobserver variability. A study conducted by Graif *et al.* demonstrated an incorrect diagnosis of fatty liver in 33% of cases, and the ultrasound was only able to detect NAFLD if it was a grade 2 or 3, as more than 33% of the liver volume must be affected with “fatty change” for detection [14].

Additional research is needed for the development of more noninvasive, reliable, and accessible diagnostic methods for NAFLD. As more information into the pathogenesis of NAFLD is elucidated, more potential diagnostic tests may be identified.

Prevalence of NAFLD

The diagnostic limitations of NAFLD were discussed previously. Regardless, NAFLD is now considered to be the most common form of liver disease [15] but the diagnostic

restrictions must be kept in mind when considering prevalence statistics.

In a population-based study by Browning *et al.* in 2004, it was determined that one-third of the general population in the United States has excessive lipid accumulation in their liver [16]. In that study, 79% of those with NAFLD had normal serum ALT activities. The frequency of NAFLD varied by ethnic group, with Hispanics having the highest incidence, which is likely linked to their higher incidence of obesity. Interestingly, African Americans had the lowest incidence, even though their incidence of obesity is high as well. These findings have been corroborated by studies conducted by several other research groups and are now thought to have a genetic basis, being associated with specific alleles [17, 18]. To lend more evidence to the undiagnosed prevalence of NAFLD, a study of healthy, young adult, live liver transplant donors found that 20% of them had some form of NAFLD [19].

Obesity and the Metabolic Syndrome

According to the 2007-2008 National Health and Nutrition Examination Survey (NHANES) report, 68% of Americans are overweight or obese [20], which translates to about 148 million adults. According to the 2003-2006 NHANES report, the prevalence of obesity in children in the United States is 12-17% [21], translating to approximately 1.3 million children. It is estimated that about 53% of obese children have NAFLD [22]. Obesity is a risk factor for developing the metabolic syndrome, which has a prevalence in the United States of 25-41.3% [23]. Among researchers and physicians alike, NAFLD is gaining recognition as part of the metabolic syndrome [24]. In a study conducted in 2003 by Marchesini *et al.*, it was found that 88% of adult patients with NAFLD had at least one feature of the metabolic syndrome, with one-third of them having the complete metabolic syndrome and being more prone to

severe liver disease [25]. The complete metabolic syndrome is defined by the National Institute of Health as having at least three of the five following criteria:

1. Abdominal circumference
 - Men \geq 102 cm
 - Women \geq 88 cm
2. Triglycerides \geq 150 mg/dl
3. High-density lipoprotein (HDL) cholesterol
 - Men $<$ 40 mg/dl
 - Women $<$ 50 mg/dl
4. Blood pressure $>$ 130/85 mm Hg
5. Fasting glycemia \geq 100 mg/dl

Recent research has most closely correlated NAFLD with increased central obesity [26, 27] and body mass index. Hepatic and peripheral insulin resistance has been well-established as a hallmark feature of NAFLD, but its presence is not a necessity, even when associated with obesity. In a study of obese individuals with normal glucose regulation, it was demonstrated that increased adipocyte size and low adiponectin concentrations were significant predictors of increased hepatic lipid content [28]. Peripheral insulin resistance leads to NAFLD via increased peripheral lipolysis because of the lack of suppression by circulating insulin concentrations. This results in an increased delivery of free fatty acids to the liver. The liver works to compensate for the increased delivery by increasing beta-oxidation and exportation of very low density lipoproteins (VLDLs). That capacity, however, can be overwhelmed and the free fatty acids will be shunted into triacylglycerol formation and storage in hepatocytes. It is important to note that in over-fed animals insulin resistance in the liver and adipose tissue precedes the impairment of insulin sensitivity in skeletal muscle [29].

With this information, it is clear that NAFLD has a high prevalence in developed populations worldwide and its prevention and treatment will become crucial to the management of

obesity and the metabolic syndrome.

Negative Energy Balance

In a study conducted by Moller *et al.*, it was demonstrated that a prolonged 36 hour fast increases the lipid content in hepatocytes by 156%, whereas a fast of 12 hours had no effect on intrahepatic lipid [30]. The mechanism that explains this finding encompasses the balance between the delivery of fatty acids to the liver and the ability of the liver to export or utilize them. This observation has important implications for the existence of NAFLD in disorders associated with a negative energy balance.

Protein-calorie malnutrition can cause NAFLD [31]. According to the Food and Agriculture Organization of the United Nations, approximately 923 million people in the world are malnourished and it serves as a significant cause of childhood mortality. The liver steatosis seen in protein-calorie malnutrition was well studied and characterized in the 1970s [32, 33] but has lost popularity over the past few decades. Treatment of NAFLD because of malnutrition by way of dietary rehabilitation has mixed results. Some studies demonstrate a complete reversal of liver damage with dietary intervention [34], whereas others demonstrate slow mobilization of hepatic lipids, with 25% of cases showing no improvement at all [35].

In addition to malnutrition, patients with cachexia are also prone to NAFLD. Cachexia is defined as physical wasting, with the loss of weight and muscle mass, caused by disease. Cachexia is seen in patients with advanced cancer, AIDS, and other progressive disorders. In cachexia, the patients eat adequate amounts of food but are not able to absorb the nutrients

well. The anticipation and management of NAFLD in these cases may be important for the quality of life of the patients.

Individuals with anorexia nervosa, or those suffering from starvation, also develop NAFLD. There have been several case reports of patients with anorexia nervosa developing severe liver steatosis and sometimes liver failure as a result [36, 37]. NAFLD can be part of the refeeding syndrome seen during the treatment of patients with anorexia nervosa [38]. Very gradual refeeding of the patient is the best way to avoid the refeeding syndrome and any associated fatty liver damage.

Rapid weight loss is another obvious contributor to NAFLD. New surgical interventions for obesity are the cases in which this is most commonly seen. These techniques include, but are not limited to, restrictive bowel surgery, gastric bypass, or biliopancreatic diversion.

Unfortunately, many of the individuals undergoing these procedures already will have some form of NAFLD and the condition can be exacerbated by these surgeries. The progression of NAFLD to NASH with fibrosis has been demonstrated, with some patients needing liver transplants [39-41].

Overall, when considering the prevalence of NAFLD, it is important to consider all of the conditions that can result in its development. It has been suggested that by the year 2050, two-thirds of the population of the world will be malnourished, be it the result of obesity or undernourishment and starvation. These thoughts clearly define the vast impact that NAFLD has on the world today and the impact it will have in the future.

PATHOGENESIS OF NAFLD

Brief insights into the pathogenesis of NAFLD have been discussed previously in regards to obesity, insulin resistance, and negative energy balance. Regardless of the underlying cause, the basic mechanism is always the same. Liver lipid homeostasis is dependent on the balance of the following four aspects associated with free fatty acids: delivery, exportation, beta-oxidation, and storage. If the delivery of free fatty acids to the liver exceeds its ability to export or oxidize them, the liver will use the free fatty acids in triacylglycerol formation and storage. This imbalance causes accumulation of lipid in the liver, which is known as simple steatosis.

Simple Steatosis

The origin of the excess free fatty acids delivered to the liver is of interest in identifying potential risk factors for NAFLD development. It is important to note that, in cases of NAFLD, the confounding factor of a potential systemic impairment of free fatty acid incorporation into lipids, leading to increased delivery of free fatty acids to hepatocytes, has been ruled out [42]. In general, the excess of fatty acids can be induced by an increased release of fatty acids from peripheral or central adipocytes, excess lipid in the diet, and/or increased endogenous synthesis in the liver. In a study by Donnelly *et al.*, 59% of the triacylglycerol in the liver originated from non-esterified fatty acids in the blood, 26.1% arose from de novo lipogenesis, and 14.9% came from the diet [43].

Because non-esterified fatty acids account for the largest percentage of fatty acids used in triacylglycerol synthesis in the liver, their important role in the development in NAFLD

should be discussed. In regards to the increased mobilization of fatty acids from adipocytes, animal and human studies have demonstrated a higher contribution of visceral, or central, adipose tissue [44]. Speculation behind this observation suggests that these fatty acids are more easily mobilized, being less sensitive to the lipolysis-suppressing effects of insulin [45], and are placed in the portal circulation where the liver has immediate access to them.

Associated with this concept is the “single gateway hypothesis”, a hypothesis that links liver glucose and lipid metabolism and visceral adiposity. In this hypothesis, the influx of fatty acids into the liver from visceral adipose tissue stimulates glucose production and secretion by the liver, thereby suppressing the effects of insulin [46]. With the increase in blood glucose, the pancreas will secrete insulin, resulting in hyperinsulinemia, in an attempt to normalize blood glucose values. This process continues until the pancreas is unable to keep up with the insulin demand. Therefore, hepatic insulin resistance, as a result of visceral adiposity, may be the primary defect in the development of insulin resistance associated with obesity [28, 47].

The liver itself has an incredible capacity to store lipids. In fact, short-term fat feeding can lead to an approximate three-fold increase in rat liver triacylglycerol content without increasing skeletal or muscle fat content [46]. The question has been raised, however, as to which of the remaining two aspects of liver lipid homeostasis, exportation or beta-oxidation, are most affected or impaired in subjects with NAFLD. It has been demonstrated that fatty acid beta oxidation in the liver is not impaired in NAFLD patients [42]. The exportation of VLDLs, however, can be impaired in NAFLD cases as a result of decreased hepatic production of apolipoprotein B100, a protein incorporated into VLDLs and required for their

exportation [48]. This decrease in production has been associated with a specific genetic polymorphism in some cases of NAFLD [49] and with a decreased availability of the lipotropic factors required for formation of apolipoprotein B100, an observation commonly seen in malnutrition or starvation cases [50]. To summarize, most evidence supports the theory that an increase in free fatty acid flux into the liver, which exceeds the ability of the liver to secrete VLDLs, leads to the development of simple steatosis.

Progression of NAFLD to NASH

The next important concept to consider is how simple steatosis further develops into NASH and potential liver cirrhosis and failure. In 1998, Day and James described a “two-hit” theory for the development of NASH, which is now widely accepted [51]. The “first hit” is the accumulation of triacylglycerol in the liver, or simple steatosis, which can result from a variety of factors, as discussed above. Hepatic lipid accumulation does not universally result in hepatocellular injury [52], indicating the importance of secondary insults for the development of NASH. These secondary insults, though not fully understood, are referred to as the “second hit”. Oxidative stress, lipid peroxidation, mitochondrial dysfunction, and pro-inflammatory cytokines play an important role in this aspect of the progression of NAFLD.

When free fatty acids accumulate in the liver, they have hepatotoxic properties, a process named lipotoxicity [53]. These fatty acids have the capacity to stimulate the cytochrome P450 system [54], which leads to lipid peroxidation [55, 56], causing hepatocellular damage. Chronic oxidative stress undoubtedly depletes the cell of its antioxidant defenses [57], such as vitamin E and glutathione, and excessive reactive oxygen species (ROS) accumulate in the hepatocyte. It has been well documented that oxidative stress is generally increased in

hepatic steatosis, and the production of ROS is positively correlated with the severity of NAFLD [58, 59].

Accumulation of ROS in the hepatocyte contributes to insulin resistance [60] and stimulates the production of pro-inflammatory cytokines, especially tumor necrosis factor-alpha (TNF- α) [61]. It is interesting to note that differences in serum TNF- α concentrations is a distinguishing feature between patients with NASH and patients with NAFLD [62]. TNF- α activates caspases, which, in turn, activate cellular apoptosis [63]. TNF- α also is known to activate inhibitor kappa kinase beta (IKK- β), which activates nuclear factor kappa beta (NF- $\kappa\beta$). NF- $\kappa\beta$ induces TNF- α production, resulting in a positive feedback loop that attenuates insulin resistance and hepatocellular damage [64]. Recent evidence, however, indicates that TNF- α may not be essential for the stimulation of inflammation as NF- $\kappa\beta$ may play a more important role [65].

In addition to pro-inflammatory cytokines, there are other aspects of NAFLD that contribute to the development of NASH. Specific types of lipids stimulate inflammation and apoptosis differently, and their accumulation in the liver is linked to the extent of NASH progression [66, 67]. Recently, it has been demonstrated that free fatty acids sensitize hepatocytes to low levels of normal cholestasis, advancing liver damage via bile-induced apoptosis [68].

Intrahepatic cholesterol concentrations also serve as a stimulator of hepatic inflammation [69]. The liver is also subject to exposure to bacterial endotoxin directly from the gastrointestinal tract via the portal circulation. The accumulation of hepatic triacylglycerols potentiate the effects of the endotoxin and enhance the hepatic inflammatory response [70].

Irrespective of which activator of inflammation or potentiator of liver damage plays the most significant role in NASH development, the end result is an influx of inflammatory cells into the liver. These inflammatory cells add to the liver damage during their response to the initial stimulation, perpetuating a chronic inflammatory response. The liver damage activates hepatic stellate cells from their quiescent state. Hepatic stellate cells are responsible for collagen deposition and eventual fibrosis of the liver [71].

The final factor that will be discussed in this review of literature regarding the progression of NAFLD to NASH is its relationship to genetics. Several studies have indicated a familial component to the progression of NASH [72, 73]. Certain adiponectin single nucleotide polymorphisms are linked with the severity of NASH [74], and polymorphisms in the TNF- α gene at position -238 result in increased susceptibility to the development of NASH [75]. As research continues, it is certain that genetics will be extensively linked to the progression of NASH.

Overall, this review barely scratches the surface of the extensive amount of information being generated in regards to the pathogenesis of NAFLD and NASH. Its purpose was to provide an overview and to highlight some of the more current theories. There are still major gaps in our knowledge regarding the etiology of NAFLD and its progression. All-in-all, the disease is multi-factorial via interaction with metabolic and immunologic pathways that seem to be regulated by environmental factors and potential genetic predispositions.

PREVENTION AND TREATMENT OF NAFLD

Currently, there is no consensus on guidelines for the management of patients with NAFLD

or NASH. Proposed treatment regimens need to focus on the underlying causes and potentiators of NAFLD. These include:

- Interventions that target the metabolic syndrome
 - Limit fat accumulation in the body
 - Decrease insulin resistance
 - Preserve pancreatic beta cell mass and function
 - Improve dyslipidemia
- Interventions that target oxidative stress
- Interventions that target hepatic fibrosis

Reduction of Body Weight

Patients with NAFLD are commonly overweight or obese and have a lower resting metabolic rate accompanied by excessive energy intake [76]. So, theoretically, reduced caloric intake, exercise, and weight loss should improve NAFLD. Until recently, no randomized controlled trials had been conducted to directly demonstrate the effects of weight loss on NAFLD [77]. In overweight individuals, weight reduction of 10% or more corrects elevated aminotransferase activities and decreases hepatomegaly [78]. With this evidence, the American Gastroenterological Association recommends an initial weight loss goal of 10% of baseline weight within a six-month period if the patient's body mass index exceeds 25 kg/m² [79]. It is imperative that this weight loss be gradual, at a rate of 1-2 pounds per week, as rapid weight loss, which was previously discussed, can exacerbate NAFLD.

Weight loss should be achieved by decreasing caloric intake and increasing physical exercise, as both have been demonstrated to independently and additively improve insulin resistance [80]. There is an inverse correlation between the presence of NAFLD and a patient's physical fitness level [81]. The extent of caloric restriction and the dietary

guidelines recommended must be considered on a case-by-case basis, but a caloric deficit of 500-1000 calories per day would seem reasonable. A study published by Huang *et al.* identified histological improvement in liver characteristics in 60% of NAFLD patients counseled to follow a 1400 kcal/day diet for 12 months [82]. The remaining patients had no change in their grade of NAFLD. Recently, dietary and lifestyle intervention has been studied for the treatment of NAFLD and findings corroborate the beneficial effects of weight loss [83-86].

Few popular diets have been studied for their impact on hepatic steatosis. According to a review article written by Zivkovic *et al.*, potential hepatic steatosis improvement only has been studied with the Mediterranean diet and the Atkins diet [87]. The Mediterranean diet is characterized as having a lower glycemic index, higher fiber content, and higher monounsaturated fatty acids, compared with the “typical American diet”, and has been demonstrated to improve hepatic steatosis. The Atkins diet, however, exacerbates NAFLD because of its ability to induce rapid weight loss, especially within the induction period. The South Beach Diet, with an induction phase consisting of very little carbohydrate, also can induce rapid weight loss, and it is speculated to have the same deleterious effects on NAFLD as the Atkins diet.

There are also medical options for therapeutic weight loss. Orlistat, marketed as Alli in the United States, is a weight loss medication approved by the Food and Drug Administration that is a reversible inhibitor of gastric and pancreatic lipase. Orlistat is taken with meals and functions to block the absorption of dietary triacylglycerols by approximately 30%.

Unfortunately, its consistent effectiveness against NAFLD development has not yet been

demonstrated, as studies have provided mixed results. Some studies demonstrate a significant positive impact of Orlistat on hepatic steatosis [88] whereas other studies do not demonstrate any added effect of Orlistat in comparison to simple weight loss [89]. Overall, the potential beneficial effects of weight loss medications have yet to be determined.

Surgical therapy for weight loss is commonly applied to patients with a body mass index greater than 35 kg/m² and other complications associated with obesity. The jejunoileal bypass procedure, commonly done in the 1960s and 1970s to achieve weight loss via malabsorption, was commonly associated with liver failure [90] and has been replaced by current gastric bypass and gastric banding techniques. In a study conducted by Dixon *et al.*, laproscopic adjustable gastric band placement was used to induce weight loss in 36 obese patients. It was reported that 82% of subjects had resolution or remission of NASH, 9% had improvement in their grade of NASH, and 9% of patients remained unchanged [91]. These findings have been corroborated by several other recent studies [92-94]. Once again, the potential rapid weight loss associated with these bariatric procedures can be contraindicative of a treatment for NAFLD and their long-term effectiveness has yet to be evaluated, specifically in regards to complications associated with weight gain or malnutrition.

Regardless of the method for weight loss utilized, long-term support of these patients is critical to the successful treatment of NAFLD. Individuals undergoing a weight loss regimen have a high incidence of relapse, even if bariatric surgery has been performed, and require continued nutritional and emotional support. This type of weight loss and subsequent management is a lifestyle change and adapting to lifestyle changes is profoundly difficult.

Improvement of Insulin Resistance

Because NAFLD is found in approximately 50% of patients with diabetes mellitus, agents improving insulin resistance are obvious choices for NAFLD treatment and prevention. The main recommended protocol for the improvement of insulin resistance was just discussed – simple weight loss. Several pharmacological compounds, however, can be used to treat the insulin resistance seen in type II diabetes mellitus. These compounds are termed insulin-sensitizing agents.

Metformin

Metformin (N-1,1-dimethylbiguanide) is a biguanide, or guanidine derivative, that is most commonly prescribed for the treatment of type II diabetes mellitus. This drug is taken orally, has an oral bioavailability of 40-60%, is rapidly distributed in the body as it is not bound to plasma proteins, and is negligibly catabolized by the liver [95]. Metformin does not affect insulin output. It simply lowers glucose concentrations in the blood by improving hepatic and peripheral tissue sensitivity to insulin [96]. The decrease in fasting blood glucose seen in patients treated with metformin is highly correlated with a decrease in hepatic glucose production [97]. Decreased hepatic gluconeogenesis is a consequence of the activation of AMP-activated protein kinase [98, 99]. Experimental studies have shown that metformin increases the rate of anaerobic metabolism of glucose in the splanchnic bed [100]. The magnitude of this glucose disposal by the intestine is clinically significant in its effects at lowering blood glucose concentrations. Metformin also enhances peripheral insulin-mediated glucose uptake [101] via activation of AMP-activated protein kinase and subsequent translocation of GLUT4 in skeletal muscle [98].

The effect of metformin on body weight and adiposity varies considerably between studies. Metformin has been demonstrated to have anorexic effects, leading to greater weight loss than diet alone [102, 103], but other studies have demonstrated no significant effect of metformin on weight loss [104, 105]. In a recent meta-analysis, it was determined that there was no difference in weight loss between metformin and dietary treatment [106]. A recent study indicates that the anorexigenic effects seen in metformin administration are potentially mediated by an increase in hypothalamic leptin receptor expression and, consequently, an increased central sensitivity to leptin [107]. It is theorized also that the increase in glucose utilization by intestinal cells plays a significant role in the weight loss resulting from metformin administration. More information is needed to fully elucidate the effects of metformin on body weight.

Studies concerning the use of metformin for the prevention or treatment of NAFLD have demonstrated promising results. The first study involving the use of metformin for the treatment of hepatic steatosis was conducted by Lin *et al.* in 2000 using ob/ob mice. Metformin demonstrated an ability to reverse hepatomegaly, lipid accumulation, and serum ALT abnormalities [108]. These effects are most likely attributable to its ability to inhibit hepatic synthesis of TNF- α . Since 2000, metformin has continued to demonstrate its effectiveness at treating NAFLD in human trials [109-113], and its role at inducing weight loss may be important for those patients.

Rosiglitazone

Rosiglitazone is a second generation thiazolidinedione (TZD), which is a class of compounds that function as agonists of the peroxisome proliferator-activated receptor gamma (PPAR- γ).

PPAR- γ is highly expressed in adipocytes and its stimulation enhances the transcription of genes involved in free fatty acid uptake [114] and triacylglycerol synthesis and storage in adipocytes. By increasing adipose storage of triacylglycerol and promoting adipocyte differentiation, rosiglitazone administration results in a decrease in plasma free fatty acids and triacylglycerols and a redistribution of lipid from liver and muscle to peripheral adipocytes, which increases insulin sensitivity [115, 116]. Lessard *et al.* recently documented that rosiglitazone treatment restores AMP-activated protein kinase in skeletal muscle, thereby promoting insulin sensitivity [117].

Rosiglitazone also has demonstrated anti-inflammatory properties, resulting in a decrease in the production of pro-inflammatory cytokines such as TNF- α and interferon gamma (IFN- γ) [118, 119]. These effects are most likely mediated via the stimulation of adiponectin production and secretion by rosiglitazone [120, 121]. Adiponectin is an adipokine secreted by adipose tissue that plays an important role in glucose metabolism and insulin sensitivity. Hypoadiponectinemia is correlated strongly with insulin resistance and an increase in hepatic lipid content [122]. Decreases in plasma adiponectin concentrations will cause decreased glucose uptake, increased gluconeogenesis, and decreased fatty acid oxidation in the liver and skeletal muscles, ultimately leading to type II diabetes mellitus [123]. PPAR γ agonists, like rosiglitazone, directly stimulate the transcription and expression of adiponectin by adipocytes [124] and increase the metabolic activity of adipocytes [125]. These two actions directly cause increases in plasma adiponectin concentrations. Consequently, the stimulation of adiponectin secretion by rosiglitazone aids in the restoration of insulin sensitivity and decreases inflammation.

Overall, rosiglitazone has potential to serve as a treatment for NAFLD. Several studies have demonstrated significant improvement in liver histology with administration of rosiglitazone [126-129]. Some studies, however, contradict these findings, lending evidence to a lack of improvement of hepatic steatosis and a subsequent increase in oxidative stress with rosiglitazone dosing [130]. It is also important to note that the mechanism of action of rosiglitazone directly stimulates adiposity; so, its usefulness in the treatment of NAFLD associated with obesity may be limited [131-133].

Improvement of dyslipidemia

Because NAFLD is frequently associated with disordered lipid homeostasis, lipid-lowering drugs serve as potential treatment options for this disease. Gemfibrozil is a fibrate, a known PPAR- α agonist, whose main effects are to decrease plasma triglycerides and increase plasma high density lipoproteins (HDL) via stimulation of HDL synthesis [134, 135].

Fibrates stimulate lipoprotein lipolysis [136] and decrease VLDL secretion [137], consequently decreasing the concentration of triglyceride-rich lipoproteins in serum.

Fibrates increase fatty acid uptake by the liver via induction of fatty acid transport protein and acyl-CoA synthetase activity, increasing the transport of fatty acids across the cell membrane and esterifying them to keep them in the cell, respectively [138]. Hepatic triacylglycerol production, however, is decreased with gemfibrozil administration. This effect can most likely be attributed to stimulation of hepatic beta-oxidation and inhibition of hormone-sensitive lipase in adipose tissue, leading to a lower availability of fatty acids for triacylglycerol synthesis and storage in the liver [139]. A growing body of evidence supports a concurrent induction of beta-oxidation and lipogenesis in the liver, which is proposed to

have a protective effect on hepatocytes, especially if the excess lipid is stored in triacylglycerols containing mostly monounsaturated fatty acids [140, 141].

Fibrate effects on liver lipids are not well-documented. Some studies report gemfibrozil administration has no effect on hepatic lipid concentrations [142], and other studies demonstrate a stimulation of cholesterol synthesis in the liver [142, 143]. Large, randomized trials on the use of fibrates in the treatment of NAFLD have not been conducted. Of the human trials published to date, none of them utilized liver biopsy as a designated measurement of endpoint. Serum enzyme markers were used instead; so, no definitive conclusions can be made from the available data [144].

Mitigating Oxidative Stress

In patients with NAFLD, antioxidant therapy may be useful in preventing the progression from simple steatosis to NASH because of their characteristic depletion of antioxidants during the “second hit” of the disease. Several antioxidants have been evaluated for their effectiveness at treating NAFLD but alpha-tocopherol, or vitamin E, has been studied most extensively.

Vitamin E is a lipid-soluble, potent antioxidant that is known to inhibit lipid peroxidation and suppress inflammatory cytokines, such as TNF- α . There is evidence of decreased plasma vitamin E concentrations in NAFLD patients, although the degree of depression is not correlated with the severity of the disease [145]. Unfortunately, its effectiveness at preventing and/or treating NAFLD has not been well substantiated. In studies comparing the effects of weight loss alone with the effects of weight loss and concurrent administration of

vitamin E, results do not indicate superiority of vitamin E treatment over dietary intervention alone [86, 111, 146-148]. There are studies, however, that demonstrate an improvement in liver histology or serum enzyme activities with vitamin E treatment [149-151]. Overall, the limitations of the aforementioned studies lie in their endpoint determinations of the effects of vitamin E. Very few of these studies utilized liver biopsies as an endpoint as the majority rely on liver enzyme markers in the serum. The limitations of those assessment techniques were discussed previously in the diagnosis section of this review. More randomized controlled trials need to be conducted, with liver biopsy endpoints, to sufficiently address the effects of vitamin E on NAFLD.

Hepatocellular Protection

Cytoprotective compounds, such as ursodeoxycholic acid and taurine, have been identified as potential preventative and treatment strategies for NAFLD. These compounds are thought to work by preventing apoptosis and decreasing the production of pro-inflammatory cytokines [152].

Taurine is known for its ability to conjugate with bile acids and aid in lipid digestion, but it also has other homeostatic functions in the body. It acts to stabilize membranes, neutralize toxins and free radicals, and modulate the calcium concentrations within body cells [153].

As with antioxidant therapy, the benefits of taurine supplementation on NAFLD are also unclear. Some studies show detrimental effects of taurine on liver lipid accumulation [154], some show no additional benefit of taurine [155], and others show limited protective effects of taurine against hepatocellular damage and liver lipid accumulation [156, 157].

Consequently, there is potential for the use of taurine in the treatment of NAFLD, with a

variety of studies demonstrating positive effects on liver histology or serum enzyme markers [158-160]. These beneficial outcomes of taurine administration have been shown to be mediated by the ability of taurine to inhibit lipid peroxidation, improve glucose and lipid metabolism, decrease the synthesis of TNF- α , and promote synthesis of adiponectin [152]. Very few studies have been conducted with taurine supplementation in humans, as most of the aforementioned studies utilized animal models. It is clearly evident that more research into the effect of taurine on NAFLD in humans is needed.

CONCLUSIONS

NAFLD is becoming a common finding associated with obesity and the metabolic syndrome and is now recognized as a common cause of liver disease and dysfunction. Malnutrition, starvation, and rapid weight loss are other potential causes of NAFLD. With this in mind, the high prevalence of NAFLD worldwide is unquestionable and actions need to be taken to identify and evaluate potential prevention and treatment strategies.

Current research has identified many potential targets for NAFLD therapy, ranging from pharmaceuticals to nutraceuticals. The effectiveness of these therapies is questioned and weight loss remains the most recommended protocol for managing NAFLD and NASH. Identifying superior therapeutic strategies is dependent on the elucidation of the mechanisms of action behind the effects of these agents on NAFLD and the comparison of their effectiveness. Prevention and treatment strategies for NAFLD will need to be considered on a case-by-case basis; so, it is unlikely that a universal protocol will be identified.

The purpose of the work presented in the remaining chapters of this thesis was to evaluate and compare the effectiveness of regularly prescribed drugs and several nutraceuticals commonly taken as dietary supplements on the prevention and treatment of NAFLD using rats as a model for humans.

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CHAPTER II:**Evaluation of the Effectiveness of Different Pharmaceuticals and Nutraceuticals on the Prevention of Non-Alcoholic Fatty Liver Disease in Rats****ABSTRACT**

Background/Objective. The incidence of obesity and the metabolic syndrome is increasing in the United States, and non-alcoholic fatty liver disease (NAFLD) is gaining recognition for its relationship with these disorders. With this association, identifying potential preventatives for NAFLD is crucial. The purpose of this study was to evaluate and compare the effectiveness of the pharmaceuticals gemfibrozil, metformin, and rosiglitazone, and the nutraceuticals taurine and vitamin E, on the prevention of NAFLD induced by a choline-deficient diet using rats as a model for humans.

Design. Sixty-one, 11-week-old, male Sprague Dawley rats were fed a 10 kcal% from fat standard or choline-deficient diet for 56 days. Within each diet group, rats were assigned to either no additional treatment (control) or oral administration of gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg).

Results. The 10 kcal% from fat choline-deficient diet did not induce significant liver triacylglycerol accumulation that was detectable with the quantification methods used in this study. With no detection of NAFLD, rats within a treatment were pooled to determine the drug or nutraceutical effects on measured parameters. There were no treatment differences in

liver characteristics compared with the control, with the exception of increased liver triacylglycerol content seen with vitamin E administration (Δ 0.17 $\mu\text{g}/\mu\text{g}$ lipid, $p=0.0159$), but there were significant differences between the treatment groups. Overall, the metformin and rosiglitazone treatment groups had the lowest liver lipid and triacylglycerol concentrations. The use of these drugs may be limited as rosiglitazone increased adiposity compared with all other treatment groups ($p\leq 0.0002$) and metformin resulted in a lower growth rate. Taurine administration was not beneficial. Gemfibrozil and vitamin E treatment groups had higher liver lipid percentages than the rosiglitazone and metformin treatment groups ($p<0.01$), but the mechanisms behind these actions cannot be elucidated from this study.

Conclusions. This study stresses the importance of dietary considerations in the prevention of NAFLD and, amongst the potential preventatives studied, administration of metformin shows the most promise for the prevention of NAFLD in the population.

INTRODUCTION

According to the 2007-2008 National Health and Nutrition Examination Survey (NHANES) report, 68% of Americans are overweight or obese [1]. Obesity is a risk factor for developing the metabolic syndrome, which has a prevalence in the United States of 25-41.3% [2]. Non-alcoholic fatty liver disease (NAFLD) is gaining recognition as part of the metabolic syndrome and one of the most common liver disorders today [3]. With its association to these disorders, NAFLD has an estimated prevalence in the general population of 17-33% [4] and can lead to liver inflammation and fibrosis, known as non-alcoholic

steatohepatitis (NASH) and, eventually to liver cirrhosis. These statistics support the urgency for identifying agents that are effective at preventing NAFLD development.

The exact mechanism behind NAFLD development is still unknown. The “two-hit” theory is used commonly to describe the general progression of NAFLD [5]. In short, the accumulation of triacylglycerols in hepatocytes serves as the “first hit”. The “second hit” comes from cytokine production, inflammation, and hepatocellular damage. At the “second hit”, the disease is referred to as NASH but not all cases of NAFLD will progress to NASH. With this pathogenesis and its associated risk factors, certain drugs and nutraceuticals can be identified as potential protective compounds.

For efficacy against the metabolic syndrome, three drug classes are superior: biguanides, thiazolidinedione (TZDs), and fibrates. Metformin is a biguanide that has proven to be effective in the treatment of type 2 diabetes mellitus and has shown promise in the treatment of NAFLD [6]. It has insulin-sensitizing effects [7] and improves weight loss because of its anorexigenic effects [8, 9]. Rosiglitazone is a TZD that serves as an agonist for the peroxisome proliferator-activated receptor gamma (PPAR γ). Rosiglitazone is an insulin-sensitizing drug, and it also increases plasma adiponectin concentrations [10]. Gemfibrozil is a fibrate that is mostly prescribed for the treatment of dyslipidemia and serves to lower serum triacylglycerol and cholesterol concentrations and improves the ratio of high density lipoproteins to low density lipoproteins (HDL/LDL) [11].

Nutraceuticals are gaining popularity as potential preventatives or therapeutic compounds by the general public. Approximately 40% of Americans consume some type of dietary supplement [12], and 36% of Americans consume a dietary supplement that contains vitamin

E [13]. With its antioxidant properties and its prevalence in the supplement market, vitamin E has been identified for use with NAFLD. Its use is most suggestive for the prevention or treatment of the “second hit” in the pathogenesis of NAFLD.

Taurine is another potential compound for use against NAFLD because of its cytoprotective effects. Taurine is known for its ability to conjugate with bile acids and thereby aid in lipid digestion, but it also has other homeostatic functions in the body. It acts to stabilize membranes, neutralize toxins and free radicals, and modulate the calcium concentrations within body cells [14].

The purpose of this current study was to evaluate and compare the effectiveness of the aforementioned pharmaceuticals and nutraceuticals on the prevention of NAFLD induced by a choline-deficient diet in rats. We hypothesized that the choline-deficient diet would induce NAFLD and that co-administration of the pharmaceuticals or nutraceuticals would prevent the accumulation of triacylglycerols in the liver compared with control rats.

MATERIALS AND METHODS

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University. A total of 61, 11-week-old, male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in this study. The rats were individually housed in solid bottom shoe box cages with contact bedding and a wire top. The rooms were kept at a temperature of 21-23°C and set on an automatic 12 hour:12 hour light:dark cycle. The rats were allowed to acclimate to the environment for one week with free access to water and a standard rodent diet (2018, Teklad Diets, Madison, WI). Sections of polyvinyl chloride

(PVC) pipes were placed in the cage of each rat for environmental enrichment. Rat health and body weights were monitored and recorded daily.

Dietary treatments and preparation. Following the acclimation period, the rats (327-336 g) were assigned randomly to either a 10 kcal% fat standard diet or a 10 kcal% fat choline-deficient diet (D12450B and D05010401, Research Diets, Inc., New Brunswick, NJ). All diets had additional medium chain triglyceride (MCT) oil (SciFit, Tulsa, OK) added to balance for the lipid content of the gemfibrozil treatment. Rats were given free access to the diets, and the diet compositions are listed in Table 1. Rats in each diet group were assigned randomly to either no additional treatment (control) or one of the five drug or nutraceutical treatments, gemfibrozil, metformin, rosiglitazone, taurine, or vitamin E. There were five rats in each choline-treatment combination (n=5) with the exception of choline-deficient control in which there were six rats (n=6). All of the treatments were given for 56 days and added to the diet for oral administration, with dosages recalculated weekly.

Gemfibrozil (Sigma-Aldrich, Saint Louis, MO) was prepared in a 200 mg/mL MCT oil solution and administered p.o. at 34 mg/kg of body weight [15]. Metformin (1,1-dimethylbiguanide hydrochloride, Sigma-Aldrich, Saint Louis, MO) was prepared in a 300 mg/mL water solution and administered p.o. at 500mg/kg of body weight [16]. Rosiglitazone (rosiglitazone maleate, ChemPacific Corporation, Baltimore, MD) was prepared in a 7 mg/mL water solution and administered p.o. at 3 mg/kg of body weight [17]. Taurine (Acros Organics, Fair Lawn, NJ) was prepared in a 225 mg/mL water solution that was heated to maintain solubility and stability. Taurine was administered p.o. at 520 mg/kg of body weight [18]. Vitamin E (alpha-tocopherol, Sigma-Aldrich, Saint Louis, MO) was administered p.o.

at 200 mg/kg of body weight [19]. The rats were fed prior to the dark cycle, and food intakes were recorded daily.

Measurement of blood hormones and metabolites. Blood samples were collected from each rat prior to the beginning of the dark cycle on days 0 (baseline), 28, and 56 of the study. The day 0 and day 28 blood samples were collected from the saphenous vein by using cone-shaped rodent restraint bags (Harvard Apparatus, Holliston, MA). Blood was collected in potassium EDTA and clotting activator Microvette capillary blood collection tubes (Sarstedt Inc., Newton, NC). The blood sample collected on day 56 of the study was a terminal sample collected via cardiac puncture, following carbon dioxide inhalation. These terminal blood samples were collected in potassium EDTA Monoject tubes (Tyco Healthcare Group, Mansfield, MA) and silica clot activator Vacutainer tubes (BD, Franklin Lakes, NJ). All blood samples were stored on ice until processing. Plasma and serum samples were centrifuged at 4°C at $3,000 \times g$ for 10 minutes and stored at -20°C until analysis.

Plasma insulin concentrations were measured by using a commercially available rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO). The lower limit of detection was 0.1 ng/mL, and the intra-assay coefficient of variation was <5%. Plasma adiponectin concentrations were measured by using a commercially available adiponectin radioimmunoassay kit (Linco Research, St. Charles, MO). The lower limit of detection was 1 ng/mL, and the intra-assay coefficient of variation was <5%. Plasma samples also were analyzed for glucose concentrations by using a commercially available enzymatic kit (Pointe Scientific, Inc., Canton, MI).

Serum samples were analyzed for total cholesterol and triacylglycerol concentrations by using commercially available enzymatic kits (Pointe Scientific Inc., Canton, MI). Serum samples also were analyzed for non-esterified fatty acid (NEFA) concentrations by using a commercially available enzymatic kit (NEFA-C kit, Wako Chemicals USA, Inc., Richmond, VA).

In all assays, samples from rats in each treatment group were processed together in order to balance any possible inter-assay effects.

Evaluation of carcass and liver characteristics. On day 56 of the study, rats were euthanized with carbon dioxide and exsanguination via cardiac puncture, followed by decapitation. The liver was collected from each rat, rinsed in 0.15 M NaCl, homogenized, and shock frozen in liquid nitrogen. The liver samples were stored at -80°C until analysis. The rat livers were analyzed for total lipid concentrations by using a wet tissue lipid extraction procedure [20]. The extracted liver lipids were analyzed for total triacylglycerol concentrations by using a commercially available enzymatic kit (Pointe Scientific Inc., Canton, MI) following solubilization with Triton X-100 [21].

The carcasses were eviscerated and skinned following liver removal. The carcasses, minus skin, feet, head, tail, and entrails, were collected for compositional assays. The carcasses were stored at -20°C until analysis. Rat carcasses were weighed and then ground in a heavy duty food grinder (Oster, Boca Raton, FL). Most bones were ground or extracted during the grinding process, and bone pieces were not used in analyses conducted on the ground carcass samples. Ground carcasses were analyzed for dry matter by placing one gram of sample in an oven at 100°C for 24 hours. Protein content of the carcasses was approximated by using a

micro-Kjeldahl procedure to determine nitrogen content [22]. Total protein was approximated by using a factor of 6.25 times the nitrogen content. Carcass lipids were analyzed by using a 2:1 chloroform:methanol (v:v) modified Folch procedure [23].

STATISTICAL ANALYSIS

All statistics were calculated by using the GLIMMIX procedure of SAS (v9.2, SAS Institute, Inc., Cary, NC). Repeated-measures ANOVA was used to analyze longitudinal blood data accounting for blocks with treatment, diet, day, and interactions as model effects.

Longitudinal covariance was modeled as first-order autoregressive, AR(1). When day by treatment and/or diet interactions were significant, pair-wise comparisons were conducted within-days across treatments and/or diets with the slicediff statement and the Tukey-Kramer method of multiple comparison corrections was employed. Within treatment and/or diet comparisons against baseline were calculated by comparing subsequent values against the day 0 (baseline) value (slicediff=control). Carcass and liver characteristics were compared by controlling for blocks and modeling diet, treatment, and interactions. When significant effects were determined in the model, the significant terms were analyzed by using pair-wise comparisons, adjusted for multiple comparisons with the Tukey-Kramer method. Average daily food intake was modeled by repeated measures ANOVA by correcting for days within blocks and blocks and modeling diet, treatment, and the interaction. Growth curves were modeled similar to the methods of Eskridge and Stevens [24]. Briefly, cubic polynomials were fit to each rat over the 56 days and parameters were compared among treatments, diets, and diet-by-treatment interactions, accounting for the effects of group. Pair-wise comparisons of significant model terms were calculated and p-

values of multiple comparisons were corrected by the methods of Tukey and Kramer. A p-value ≤ 0.05 was considered significant.

RESULTS

The objective of this study was to evaluate and compare the effectiveness of metformin, gemfibrozil, rosiglitazone, taurine, and vitamin E on the prevention of NAFLD induced by a choline-deficient diet. The 10 kcal% from fat choline-deficient diet did not induce significant liver triacylglycerol accumulation that was detectable with the quantification methods used in this study. With no detection of NAFLD, rats within a treatment were pooled to determine the drug or nutraceutical effects on measured parameters.

Liver characteristics. There were no main or interaction effects of dietary choline content on measured liver characteristics. Table 2 summarizes the measured liver characteristics. Rats receiving metformin had significantly lower liver lipid percentages compared with rats receiving gemfibrozil ($\Delta 0.51\%$, $p=0.0053$) and vitamin E ($\Delta 0.45\%$, $p=0.0180$). Rats in the rosiglitazone treatment group had significantly lower liver lipid percentages compared to rats in the gemfibrozil ($\Delta 0.46\%$, $p=0.0018$) and vitamin E ($\Delta 0.51\%$, $p=0.0066$) treatment groups. Liver lipid percentages in the gemfibrozil treatment group were also significantly higher than the liver lipid percentages in the taurine group ($\Delta 0.43\%$, $p=0.0310$). Liver triacylglycerol concentrations for rats in the vitamin E treatment group were significantly higher than those for rats in the control ($\Delta 0.17 \mu\text{g}/\mu\text{g lipid}$, $p=0.0159$), metformin ($\Delta 0.21 \mu\text{g}/\mu\text{g lipid}$, $p=0.0020$), and rosiglitazone ($\Delta 0.22 \mu\text{g}/\mu\text{g lipid}$, $p=0.0014$) treatment groups.

Carcass characteristics. Terminal carcass weights, without skin, head, feet, tail, and entrails, were measured in this study (Table 3). There were no main or interaction effects of choline on the determined carcass characteristics. The average carcass weight for rats in the rosiglitazone treatment group was significantly higher than that of rats in the gemfibrozil (Δ 17.42 g, $p=0.0470$) and metformin (Δ 27.04 g, $p=0.0004$) treatment groups. The vitamin E treatment group also had significantly higher average carcass weight than did the metformin treatment group (Δ 22.57g, $p=0.0042$).

Carcass dry matter had main effects of choline and treatment, but there were no interaction effects (Table 3). On average, rats fed the choline-deficient diet had a significantly lower dry matter percentage than rats fed the standard diet (28.53% versus 29.61%, respectively; $p=0.0221$). When considering treatment effects alone, the rosiglitazone treatment group had a significantly higher dry matter percentage than all other treatment groups ($p\leq 0.0014$).

There were main effects of treatment on carcass lipid percentage, but there were no main or interaction effects of choline (Table 3). Rats in the rosiglitazone treatment group had a higher average carcass lipid percentage on a wet basis than did rats in all other treatment groups ($p\leq 0.0002$).

Carcass protein content had only a main effect of treatment (Table 3). There were no main or interaction effects of choline. The rosiglitazone treatment group, on average, had significantly lower carcass protein percentage than did the control (Δ 1.54%, $p=0.0007$), metformin (Δ 1.40%, $p=0.0033$), and taurine (Δ 1.08%, $p=0.0397$) treatment groups. Rats in the rosiglitazone treatment group also had a tendency for a lower carcass protein percentage

than the gemfibrozil (Δ 1.04%, $p=0.0537$) and vitamin E (Δ 0.95%, $p=0.0918$) treatment groups.

Blood hormones and metabolites. There were no main or interaction effects of dietary choline content on blood hormones and metabolites, with the exception of non-esterified fatty acid (NEFA) concentrations in which there was no main choline effect but there was an interaction effect of choline and day.

There were no differences in baseline plasma adiponectin concentrations among treatments (Figure 1). Plasma adiponectin concentrations in rats receiving rosiglitazone were significantly higher than plasma adiponectin concentrations in all other treatment groups, and baseline (day 0), on days 28 and 56 ($p<0.0001$). Plasma adiponectin concentrations were significantly different from baseline for all treatment groups on day 56 ($p<0.0001$), lending evidence to the trend of increasing plasma adiponectin concentrations with increasing age [25, 26].

Baseline plasma insulin concentrations were not significantly different between treatment groups (Figure 2). On day 28, rats receiving rosiglitazone had significantly lower plasma insulin concentrations compared with those of the control (Δ 0.66 ng/mL, $p=0.0079$), gemfibrozil (Δ 0.74 ng/mL, $p=0.0028$), and taurine (Δ 0.82 ng/mL, $p=0.0006$) treatment groups. Plasma insulin concentrations on day 28 in the metformin treatment group were significantly lower than insulin concentrations in the gemfibrozil (Δ 0.60ng/mL, $p=0.0273$) and taurine (Δ 0.68 ng/mL, $p=0.0080$) treatment groups. Day 28 also demonstrated a decrease in insulin concentrations in the vitamin E treatment group in comparison to the gemfibrozil (Δ 0.56 ng/mL, $p=0.0494$) and taurine (Δ 0.64 ng/mL, $p=0.0156$) treatment

groups. On day 56, the only significant difference in plasma insulin concentrations was in the rosiglitazone treatment group, which was significantly lower than those in the taurine group (Δ 0.61 ng/mL, $p = 0.0228$). On day 28, plasma insulin concentrations were significantly higher than baseline in the control (Δ 0.70 ng/mL, $p < 0.0001$), gemfibrozil (Δ 0.88 ng/mL, $p < 0.0001$), and taurine (Δ 0.89 ng/mL, $p < 0.0001$) treatment groups. On day 56, plasma insulin concentrations were significantly higher than baseline in the gemfibrozil (Δ 0.53 ng/mL, $p = 0.0062$), taurine (Δ 0.62 ng/mL, $p = 0.0015$), and vitamin E (Δ 0.38 ng/mL, $p = 0.0448$) treatment groups.

There were no significant differences in serum cholesterol concentrations at baseline among the treatment groups (Figure 3). On days 28 and 56, serum cholesterol concentrations for rats receiving gemfibrozil were significantly higher than those for rats in all other treatment groups and at baseline ($p < 0.0001$). On day 28, metformin and vitamin E groups had serum cholesterol concentrations that were significantly lower than baseline (Δ 19.40 mg/dL, $p = 0.0100$; Δ 16.23 mg/dL, $p = 0.0303$; respectively).

For serum triacylglycerol concentrations, there was no interaction effect between treatment and day. Serum triacylglycerol concentrations increased significantly from baseline on day 28 for all treatment groups (Δ 44.50 mg/dl, $p < 0.0001$). Data are not shown. There was no difference in serum triacylglycerol concentrations from day 28 to day 56 for all treatment groups. Serum triacylglycerol concentrations were not different among treatment groups throughout the study.

Baseline serum NEFA concentrations were not significantly different among treatment groups (Figure 4 A and B). There was a choline by day interaction driven by the difference

in NEFA concentrations between the two diets on day 56. On day 56, rats fed the choline-deficient diet had lower serum NEFA concentrations than did rats fed the standard diet (Δ 2.03 mg/dL $p=0.0084$). On day 28, serum NEFA concentrations were significantly higher in the gemfibrozil treatment group than in all other treatments ($p<0.0001$), except taurine ($p=0.0532$). Rats receiving taurine had significantly higher serum NEFA concentrations compared with rats receiving rosiglitazone (Δ 4.79 mg/dL, $p=0.0058$). The rosiglitazone treatment group had significantly lower serum NEFA concentrations compared with the control treatment group (Δ 3.83 mg/dL, $p=0.0422$). On day 56, serum NEFA concentrations for the rosiglitazone treatment were significantly lower than the serum NEFA concentrations for the gemfibrozil (Δ 7.11 mg/dL, $p=0.0001$), metformin (Δ 3.88 mg/dL, $p=0.0453$), and taurine (Δ 4.79 mg/dL, $p=0.0058$) treatment groups.

There was no choline or treatment effect on plasma glucose concentrations. Data are not shown. There was an effect of day on plasma glucose concentrations, driven by higher concentrations measured on day 56 (Δ 51.05 mg/dL, $p<0.0001$). This effect can be attributed to the difference in the blood sample collection method, as the day 56 sample was taken during euthanasia via cardiac puncture. The observed hyperglycemia likely resulted from the physiological release of epinephrine in the rats because of the stress associated with the euthanasia process [27, 28].

Food intake and body weights. There were main and interaction effects of choline and treatment on average daily food intake. Figure 5 summarizes these effects. Differences between the standard diet and the choline-deficient diet, within a treatment, were evaluated. Rats fed the choline-deficient diet had significantly higher average daily food intake when

treated with gemfibrozil (Δ 1.32 g/day, $p < 0.0001$), taurine (Δ 1.27 g/day, $p < 0.0001$), and vitamin E (Δ 0.74 g/day, $p = 0.0012$). There were no significant differences in average daily food intake between the standard and choline-deficient diets for the control, metformin, and rosiglitazone treatment groups. Differences within the different diets among the treatments also were compared. Within the standard diet, the gemfibrozil, taurine, and vitamin E treatment groups had significantly lower average daily food intake than did the control, metformin, and rosiglitazone treatment groups ($p \leq 0.0036$). Within the choline-deficient diet, the control and vitamin E treatment groups had significantly lower average daily food intake than did all other treatment groups ($p \leq 0.0014$).

There were no main or interaction effects of choline on daily body weights. The growth curves are presented in Figure 6. There were no significant differences among treatment groups at baseline. The growth curves for all treatment groups were similar in shape, but the metformin group had a lower growth curve in comparison to other treatment groups.

Significant differences in the regression coefficients are listed in Table 4.

DISCUSSION

The purpose of this study was to evaluate and compare the effectiveness of pharmaceutical and nutraceutical administration on the prevention of non-alcoholic fatty liver disease (NAFLD). A 10 kcal% fat choline-deficient diet was used to induce NAFLD in this study. Phosphatidylcholine is an essential component of very low density lipoprotein (VLDL) synthesis and secretion from the liver, and it cannot be replaced in the structure of VLDLs by any other molecule [29]. Phosphatidylcholine can be synthesized utilizing dietary choline or dietary methionine. A choline-deficient diet will block one of the routes of synthesis for

phosphatidylcholine [30]. It has been demonstrated, however, that a choline-deficient diet in mice upregulates the synthesis of the rate-limiting enzyme involved in phosphatidylcholine synthesis from methionine as a compensatory mechanism [31]. Our results suggest that a 10% kcal from fat choline-deficient diet does not contain sufficient lipid to overwhelm those compensatory mechanisms and does not reliably induce fatty liver disease in rats.

Although our choline-deficient diet failed to induce quantifiable fatty liver disease based on our study methods, the nutraceuticals and drugs studied still had an effect on liver and carcass characteristics. Rosiglitazone was effective at lowering total liver lipid content, a change that can be attributed to a decrease in liver triacylglycerol concentrations. As an agonist of the PPAR γ , rosiglitazone directly stimulates the transcription and expression of adiponectin [32] and stimulates the metabolic activity of adipocytes [33], thereby increasing plasma adiponectin concentrations. Increases in plasma adiponectin concentrations have been linked to a decrease in hepatic lipid content [34], a finding that this study supports. Recent evidence also has linked rosiglitazone administration to an increase in adiposity [35, 36] and heart disease [37, 38]. The lower plasma NEFA concentrations with rosiglitazone treatment in the current study lends evidence to increased uptake of fatty acids into the adipocytes [39, 40]. In this study, rosiglitazone-treated rats had significantly higher carcass lipid content and lower protein content than did all other treatment groups, demonstrating the potential limitation of the use of rosiglitazone as a preventative for NAFLD and the metabolic syndrome.

Metformin administration was also effective at lowering liver lipid and triacylglycerol content in this study. Although the extent of its effects were not different from rosiglitazone,

it did not have adiposity side effects and will serve as a better preventative for NAFLD comparatively. The effects of metformin in this study can partially be attributed to its effects on body weight as evidenced by the decreased growth rate and terminal carcass weight. The lower growth rate seen in this study adds to existing evidence that part of the mechanism of action of metformin is weight loss [41-43].

Gemfibrozil, a PPAR- α agonist, did not have a preventative effect on liver lipid accumulation in these study conditions. The difference in liver lipid content between rosiglitazone and metformin and gemfibrozil cannot fully be attributed to increases in liver triacylglycerol content in this study. The synthesis of phosphatidylcholine, a major component of cell membranes, is increased with administration of gemfibrozil, resulting in the shunting of liver diacylglycerol into phosphatidylcholine instead of liver triacylglycerol [44]. Although liver phospholipid content was not measured in this study, this mechanism may explain the observed difference in liver lipid content with gemfibrozil administration.

Phosphatidylcholine content of hepatocyte membranes is decreased in NAFLD patients [45]; so, gemfibrozil may have protective effects on NAFLD development by simply preventing that deficit. Gemfibrozil is known also for its ability to decrease serum cholesterol concentrations while increasing serum HDL concentrations and decreasing VLDL and LDL concentrations [44]. In this study, gemfibrozil increased total serum cholesterol concentrations but, without the determination of serum HDL concentrations, the mechanism behind this effect cannot be elucidated.

Taurine is known for its cytoprotective and antioxidant properties [14]. In this study, taurine administration did not demonstrate any added benefit compared with the control diet. This

finding is consistent with other studies in which taurine had no effect on the prevention of NAFLD [46, 47]. Taurine also has proven to be detrimental during preventative administration through increasing liver triacylglycerol content [18]. The limitations of this study concerning the mechanism of action of taurine must be discussed. The beneficial effects of taurine administration on hepatic lipid accumulation during challenge with ethanol or obesity have been demonstrated previously [48, 49]. NAFLD was not induced in this study; so, the cytoprotective and antioxidant effects of taurine may not be evident without this challenge. Therefore, this study design may be limited in its ability to determine any potential preventative benefits of taurine on the development of NAFLD.

Vitamin E has been studied as a treatment for NAFLD with little consistent evidence as to its efficacy or its superiority to dietary intervention [19, 50, 51]. Because of the “two-hit” theory for the development of NAFLD, the antioxidant properties of vitamin E would seem beneficial for the prevention of the disease. Interestingly, this study demonstrates the opposite effect, as the vitamin E group had the highest accumulation of liver triacylglycerol, an amount significantly different from the control. To the best of our knowledge, this is the first study to identify this potential side effect of vitamin E administration. Many of the human studies conducted with vitamin E as a preventative or treatment for NAFLD do not utilize liver biopsies and rely solely on enzyme markers, especially alanine aminotransferase and aspartate aminotransferase activities, to determine the effects of this nutraceutical. These enzyme markers are not always reliably correlated with the extent and severity of NAFLD development [52, 53]. It has been documented that all stages of NAFLD can be seen in patients with normal alanine aminotransferase activities [54]. With this flaw, there is

potential for vitamin E to negatively impact the prevention or treatment of NAFLD. More trials with vitamin E will need to include liver biopsies and subsequent quantification of liver lipids to further explore the potential detrimental effects of vitamin E observed in this study.

Although there were differences in efficacy amongst the treatment groups, it should be noted that none of the treatments were significantly different from the control in terms of liver characteristics, with the exception of the increased liver triacylglycerol content seen with vitamin E administration. The effectiveness of the diet at preventing liver lipid accumulation lends evidence to the importance of dietary intervention or modification in the prevention of NAFLD. According to the American Gastroenterological Association and the American Association for the Study of Liver Diseases, gradual weight loss of 10% of body weight with the addition of aerobic exercise is the most clinically relevant protocol for the improvement of NAFLD [55]. The efficacy of dietary intervention is well-documented [56-59]. Although, the long-term benefits of dietary intervention may be limited due to a lack of compliance within the human population, its consistent effects in the prevention of NAFLD lend support to its superiority.

CONCLUSIONS

Overall, although NAFLD was not induced, rosiglitazone administration had positive effects on the prevention of lipid accumulation in the liver. Its capability of increasing adiposity, however, limits its use. Gemfibrozil and the nutraceuticals taurine and vitamin E did not prove to be effective in the prevention of NAFLD. In fact, there is evidence that vitamin E administration increases lipid accumulation in the liver. Further research needs to be done to elucidate the mechanism behind that effect. In this study, metformin, the gold standard for

treatment of the metabolic syndrome, proves to be the best option for the prevention of NAFLD development amongst the drugs or nutraceuticals examined in this study, but it is not superior to the effects of diet modification alone.

ACKNOWLEDGEMENTS

This research was supported by the Iowa State University Center for Designing Foods to Improve Nutrition (CDFIN) through funds obtained from the United States Department of Agriculture. Appreciation is extended to Michelle Bohan Brown for the authorship of this grant and to Andrew Brown for his help with statistical analyses. Thank you to the students of the Nutritional Physiology Group of the Animal Science Department at Iowa State University for their assistance with data collection and analyses.

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Table 1: Diet Compositions

Diet	Standard Diet¹	Choline-Deficient Diet²
	kcal%	kcal%
Protein	20.0	20.0
Carbohydrate	70.0	70.0
Fat	10.0	10.0
Ingredients		
	gram	gram
Casein, 80 Mesh	200	200
L-Cystine	3	3
Corn Starch	315	315
Maltodextrin 10	35	35
Sucrose	350	350
Cellulose, BW200	50	50
Soybean Oil	25	25
Lard	20	20
Mineral Mix, S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H ₂ O	16.5	16.5
Vitamin Mix, V10001	10	10
Choline Bitartrate	2	0
Additions³		
	gram	gram
Medium chain triglyceride (MCT) oil	0.05	0.05

¹ D12450B, Research Diets, Inc., New Brunswick, NJ² D05010401, Research Diets, Inc., New Brunswick, NJ³ Medium Chain Triglyceride Oil, SciFit, Tulsa, OK

Table 2: Liver Characteristics¹

Liver Component	Control	Gemfibrozil	Metformin	Rosiglitazone	Taurine	Vitamin E
Lipid (%)	3.61 ± 0.09 ^{ab}	3.89 ± 0.10 ^a	3.37 ± 0.10 ^b	3.32 ± 0.10 ^b	3.46 ± 0.10 ^{bc}	3.83 ± 0.10 ^{ac}
TAG (μg TAG/μg lipid)	0.218 ± 0.035 ^a	0.244 ± 0.036 ^{ab}	0.180 ± 0.036 ^a	0.172 ± 0.036 ^a	0.308 ± 0.036 ^{ab}	0.390 ± 0.036 ^b

¹Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, the rats in each diet were pooled to determine the treatment effects. Values are means ± pooled SEM; n=10 for all treatments except for control (n=11). Means in a row with superscripts without a common letter are significantly different, p<0.05.

Table 3: Carcass Characteristics¹

Carcass Component	Control	Gemfibrozil	Metformin	Rosiglitazone	Taurine	Vitamin E
Weight (g)	229.43 ± 3.94 ^{ab}	222.33 ± 4.10 ^{ac}	212.71 ± 4.10 ^a	239.75 ± 4.10 ^b	228.70 ± 4.10 ^{ab}	235.28 ± 4.10 ^{bc}
Dry matter² (%)	28.11 ± 0.54 ^a	27.51 ± 0.56 ^a	28.52 ± 0.56 ^a	32.58 ± 0.56 ^b	28.46 ± 0.56 ^a	29.21 ± 0.56 ^a
Lipid (% on wet basis)	7.07 ± 0.63 ^a	6.82 ± 0.66 ^a	7.01 ± 0.66 ^a	12.68 ± 0.66 ^b	7.87 ± 0.66 ^a	8.15 ± 0.66 ^a
Protein (% on wet basis)	19.60 ± 0.24 ^a	19.10 ± 0.25 ^{ab}	19.46 ± 0.25 ^a	18.06 ± 0.25 ^b	19.14 ± 0.25 ^a	19.02 ± 0.25 ^{ab}

¹Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, the rats in each diet group were pooled to determine the treatment effects. Values are means ± pooled SEM; n=10 for all treatments except for control (n=11). Means in a row with superscripts without a common letter are significantly different, p<0.05.

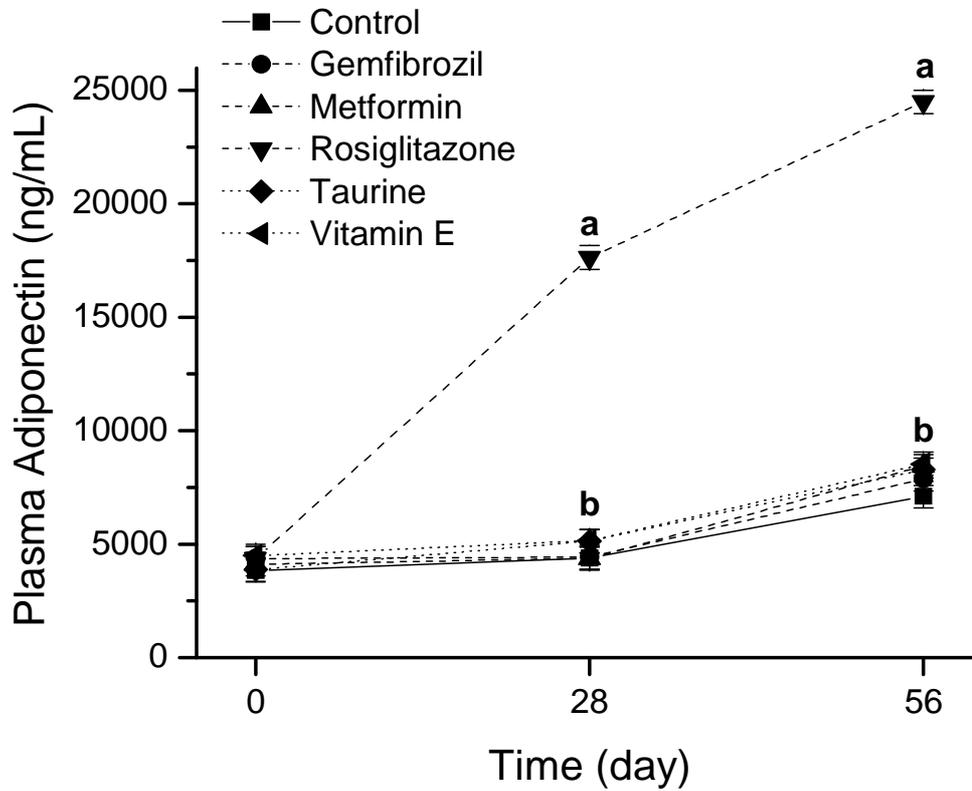
² Carcass dry matter also had a main choline effect but no interaction. Rats fed the choline-deficient diet had lower average dry matter than rats fed the standard diet (p=0.0221)

Table 4: Regression Coefficients for Cubic Growth Curves¹

Treatment	Intercept	Linear	Quadratic	Cubic
Control	325.72	5.2453 ^{ac}	-0.104 ^{ab}	0.000881 ^{ab}
Gemfibrozil	318.33	4.5771 ^{bc}	-0.08676 ^{ac}	0.000761 ^a
Metformin	316.67	3.7343 ^b	-0.07541 ^a	0.000685 ^a
Rosiglitazone	323.54	6.0113 ^a	-0.13 ^b	0.001213 ^b
Taurine	321.32	4.9876 ^{ac}	-0.09507 ^{ab}	0.000808 ^a
Vitamin E	326.49	5.3219 ^{ac}	-0.1176 ^{bc}	0.001095 ^{ab}

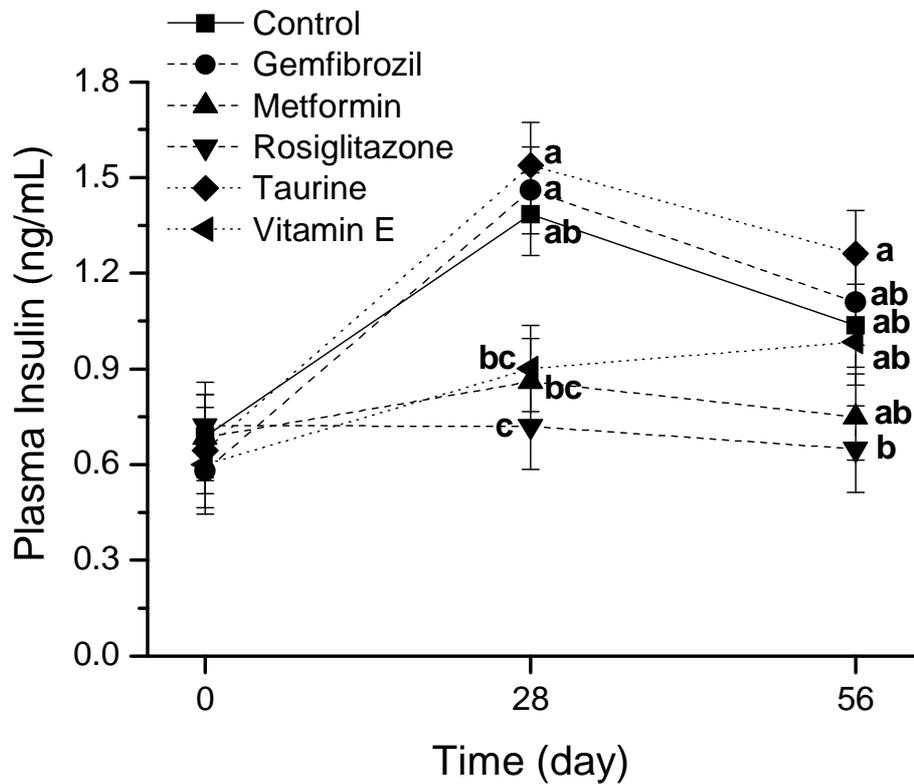
¹Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, the rats in each diet group were pooled to determine the treatment effects. n=10 for all treatments except for control (n=11). Rats were weighed daily and the above regression equations were fit to those data. Means in a column with superscripts without a common letter are significantly different, p<0.05.

Figure 1:



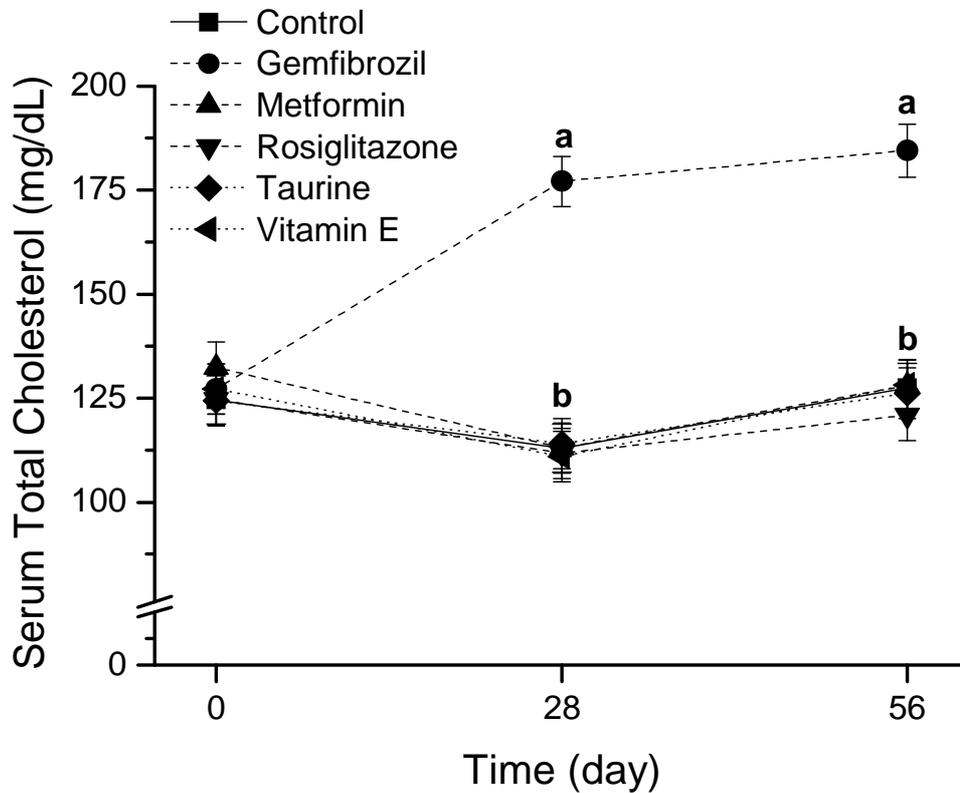
Drug and nutraceutical effects on plasma adiponectin concentrations. Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, data shown are pooled within treatments. Values are means \pm pooled SEM; n=10 for all treatments except for control (n=11). Data points within the same day without common letters are significantly different, $p < 0.05$.

Figure 2:



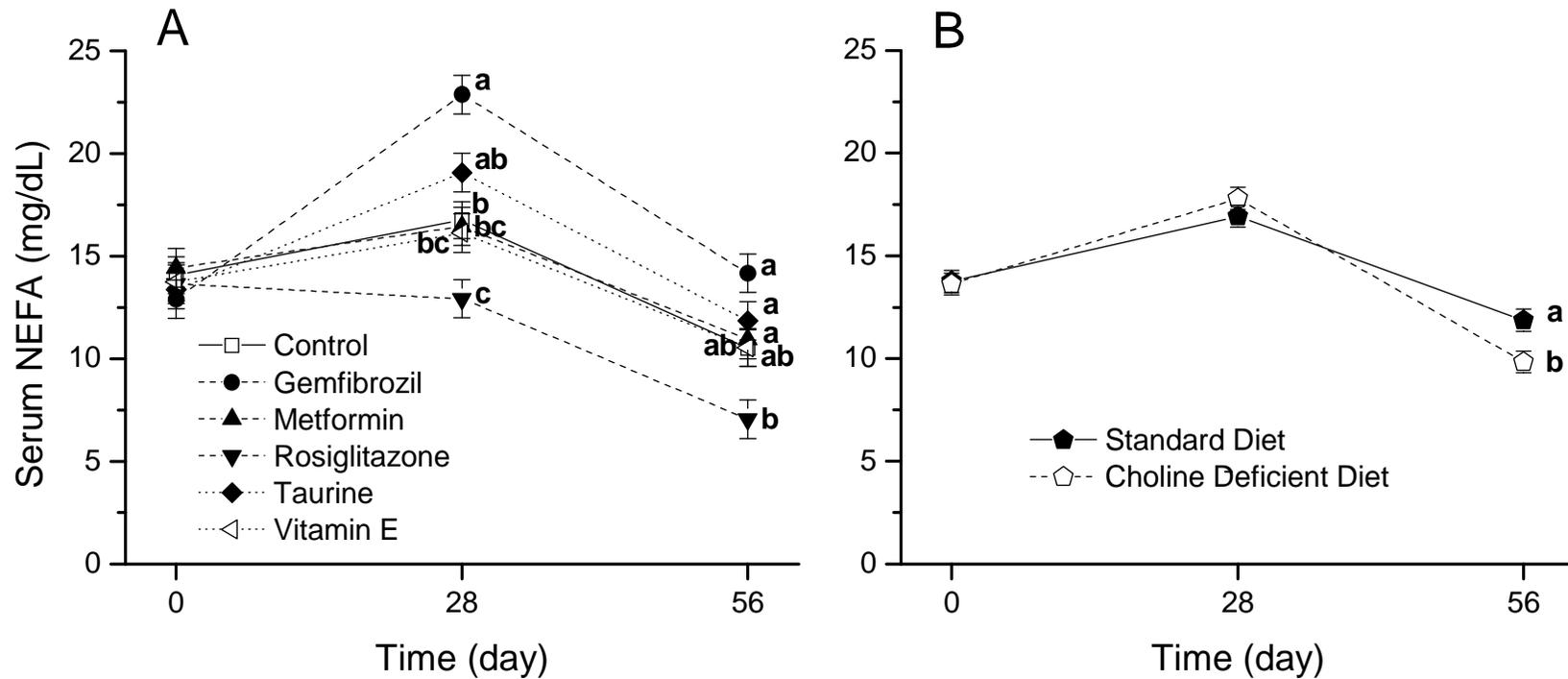
Drug and nutraceutical effects on plasma insulin concentrations. Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, data shown are pooled within treatments. Values are means \pm pooled SEM; $n=10$ for all treatments except for control ($n=11$). Data points within the same day without common letters are significantly different, $p<0.05$.

Figure 3:

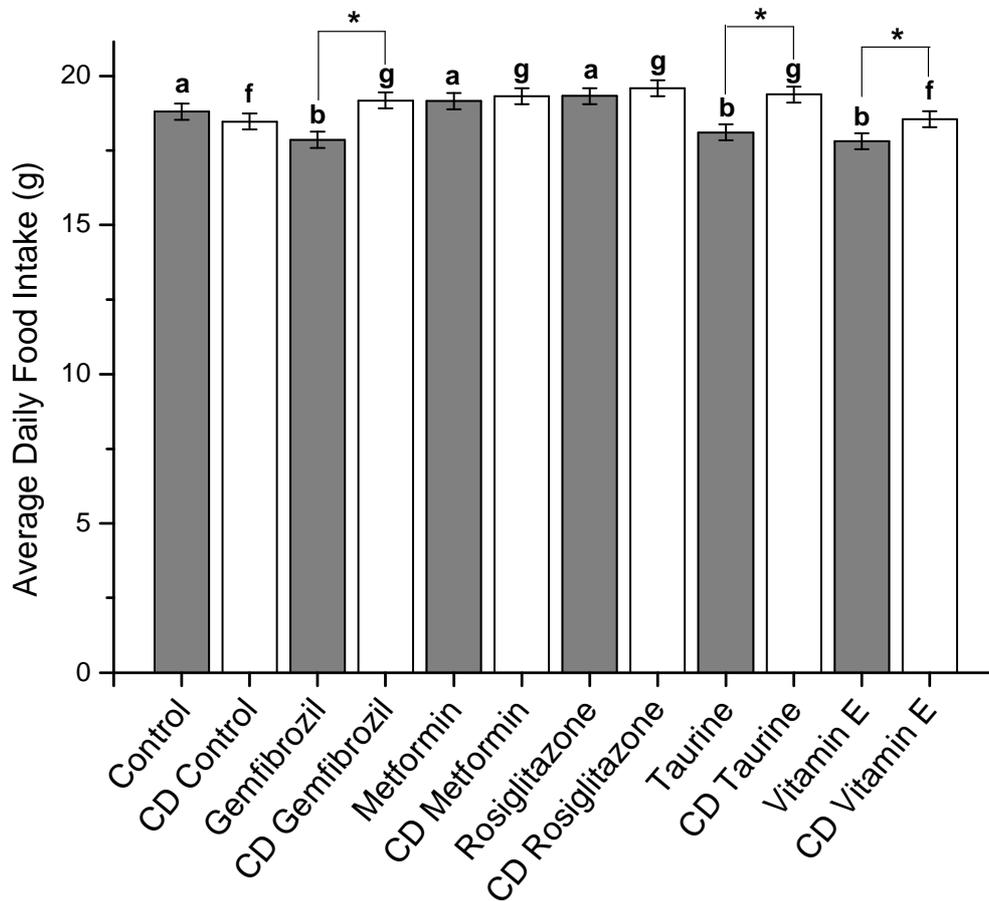


Drug and nutraceutical effects on serum total cholesterol concentrations. Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, data shown are pooled within treatments. Values are means \pm pooled SEM; $n=10$ for all treatments except for control ($n=11$). Data points within the same day without common letters are significantly different, $p<0.05$.

Figure 4:

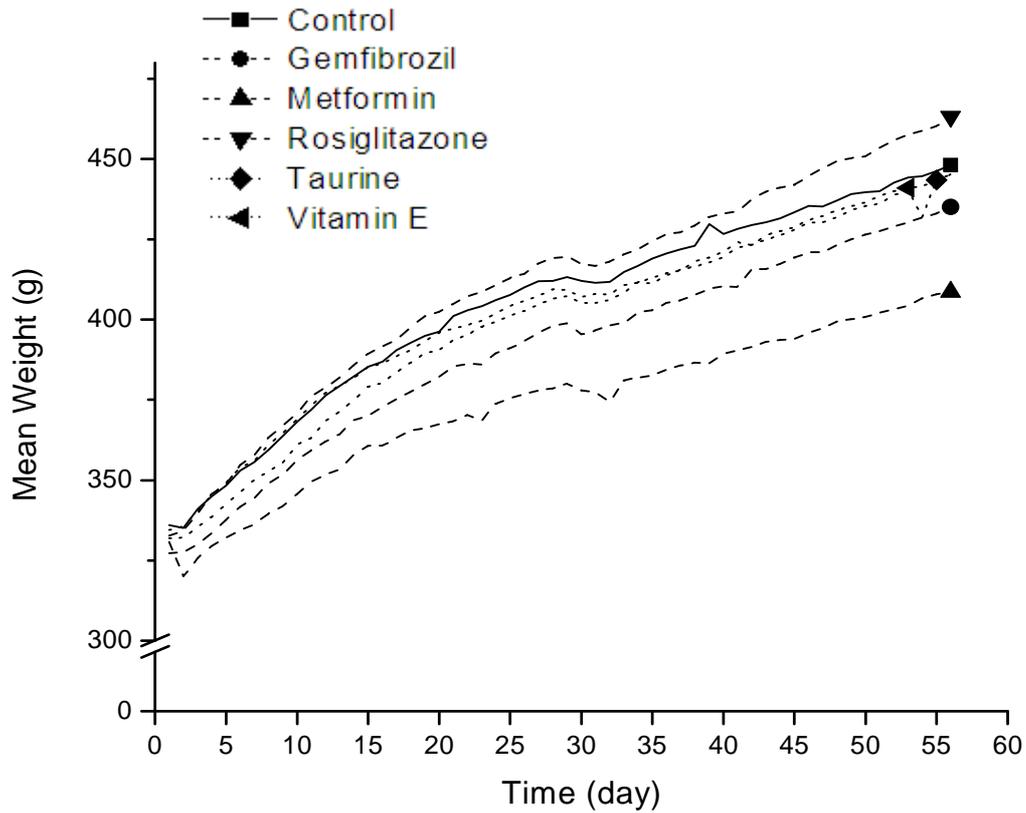


Drug and nutraceutical effects on serum non-esterified fatty acid (NEFA) concentrations. Rats were fed a standard or choline-deficient diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). **Panel A:** There were no main effects of choline; so data shown are pooled within treatments. Values are means \pm pooled SEM; n=10 for all treatments except for control (n=11). Data points within the same day without common letters are significantly different, $p < 0.05$. **Panel B:** There was an interaction effect of choline and day, driven by the difference in NEFA concentrations on day 56. Rats from each diet group were pooled over treatments. Values are means \pm pooled SEM; n=30 for standard diet group, n=31 for choline-deficient diet group. Data points within the same day without common letters are significantly different, $p < 0.05$.

Figure 5:

Diet and drug/nutraceutical effects on average daily food intake. Rats were fed a standard or choline-deficient (CD) diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were main and interaction effects of choline and treatment on average daily food intake. Values are means \pm SE; n=5 for all treatments except CD control (n=6). Differences between diets within a treatment group are denoted with a (*), $p < 0.05$. Values within a diet without common letters differ significantly; a/b denote differences within the standard diet, f/g denote differences within the CD diet; $p < 0.05$.

Figure 6:



Diet and drug/nutraceutical effects on growth curve. Rats were fed a standard or choline-deficient (CD) diet for 56 d without (control) or with gemfibrozil (34 mg/kg), metformin (500 mg/kg), rosiglitazone (3 mg/kg), taurine (520 mg/kg), or vitamin E (200 mg/kg). There were no main or interaction effects of choline; so, data shown are pooled within treatments. Values are means; n=10 for all treatments except for control (n=11).

CHAPTER III:**Intervention with a Low-Fat Diet and Nutraceutical Supplementation as a Treatment
for Non-Alcoholic Fatty Liver Disease in Rats as a Model for Humans****ABSTRACT**

Background/Objective. Non-alcoholic fatty liver disease (NAFLD) is becoming increasingly recognized as a common cause of liver dysfunction, and, with its close association with obesity and the metabolic syndrome, it is estimated that NAFLD affects one-third of the general population in the United States. With this high prevalence, identifying and comparing potential treatments for this disorder is crucial. The purpose of this study was to induce NAFLD and then compare the effectiveness of a standard diet alone or a standard diet supplemented with metformin, vitamin E, taurine, or a combination of vitamin E and taurine at treating the disease using rats as a model for humans.

Design. Sixty-three, 11-week-old, male Sprague Dawley rats were fed a 60 kcal% from fat, choline-deficient diet for 28 days to induce fatty liver disease. Following induction, 11 rats were euthanized to determine the extent of fatty liver development. The remaining rats were fed a 10 kcal% from fat standard diet alone or that same diet supplemented with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine.

Results. The choline-deficient diet used in this study induced an increase in liver lipid concentrations via accumulation of liver triacylglycerols. The rats did not demonstrate

insulin resistance or dyslipidemia after the induction phase. The standard diet, irrespective of supplementation, was sufficient to reverse the disease ($p < 0.002$). There was no additional benefit demonstrated with the drug or nutraceutical supplementation. The change in diet did not result in weight loss or changes in carcass adiposity, lending evidence to a greater importance of a lifestyle change over the importance of weight loss, especially for those NAFLD patients not demonstrating aspects of the metabolic syndrome. The effects of choline, however, cannot be assessed with this study design, and this change in nutritional state may have influenced the results of this study.

Conclusions. This study demonstrates the importance of dietary intervention and lifestyle change in the treatment of NAFLD, as those effects were enough to reverse the disease. More research is needed with this animal model to determine the potential effects of the change in dietary choline status on the results of this study.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is beginning to be recognized as one of the most common liver disorders seen today [1]. It is estimated that approximately one-third of the general population in the United States has excessive lipid accumulation in their liver [2]. With its close association to obesity and the metabolic syndrome, its prevalence in developed countries is unquestionable. There is currently no recommended treatment for the improvement or reversal of NAFLD. Many different treatments have been proposed, but there are few studies to demonstrate the superiority of one treatment over another.

Metformin is a biguanide that is one of the most commonly prescribed and recommended drugs for the management and treatment of type II diabetes mellitus [3] and it has been extensively studied for the treatment of NAFLD. In addition to pharmaceuticals, many physicians and patients are turning to nutraceuticals as potential treatments for a variety of disorders, including NAFLD. Vitamin E and taurine are two nutraceuticals that have been proposed as potential treatments for NAFLD based on their antioxidant and cytoprotective properties [4, 5].

The objective of this study was to induce NAFLD in rats through the administration of a high-fat, choline-deficient diet and then determine and compare the effects of a standard diet alone or a standard diet with supplementation of metformin, vitamin E, taurine, or a combination of vitamin E and taurine on the induced disease. We hypothesized that the choline-deficient diet would induce fatty liver [6, 7]. We also hypothesized that the dietary change would reverse the disease, with the supplementation of metformin or the nutraceuticals providing additional benefit.

MATERIALS AND METHODS

Animals

All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University. This study utilized 63, 11-week-old, male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN). Rats were individually housed in shoe box cages with a wire top and solid bottom lined with contact bedding. Environmental enrichment was provided by using sections of polyvinyl chloride (PVC) pipes placed in each

cage. The rooms were set on an automatic 12 hour:12 hour light:dark cycle and kept at 21-23°C. Upon arrival, the rats had a one-week acclimation period with free access to water and a standard rodent diet (2018, Teklad Diets, Madison, WI). Rat health was monitored daily, and rat body weights were averaged weekly.

Dietary treatments

Fatty liver induction phase. Following the acclimation period, all rats were fed an ad libitum high-fat (60 kcal% fat), choline-deficient diet (D05010403, Research Diets, Inc., New Brunswick, NJ) for 28 days to induce fatty liver disease (Table 1). Rats were fed immediately before the beginning of the dark cycle, and feed intake was recorded daily. On day 28, 11 rats were selected randomly to undergo euthanasia for evaluation of the extent of fatty liver development during the induction period. This induction group of rats allowed for the determination of an approximate liver lipid baseline for the remaining rats entering the treatment phase of this study.

Fatty liver treatment phase. Following the induction phase, the remaining 52 rats were taken off the high-fat, choline-deficient diet and fed a free access standard diet with 10 kcal% fat (D12450B, Research Diets, Inc., New Brunswick, NJ). Diet composition is listed in Table 1. The treatment phase of the study was conducted for 28 days. The rats were assigned randomly to the standard diet alone (control; n=10) or the standard diet supplemented with metformin (n=11), vitamin E (n=11), taurine (n=10) or a combination of vitamin E and taurine (n=10). All of the treatments were given for 28 days and added to the diet for oral administration, with dosages based on body weight, and recalculated weekly. Metformin (metformin hydrochloride, Spectrum Chemicals & Laboratory Products, Gardena,

CA) was administered p.o. at 500 mg/kg of body weight and prepared in a 300 mg/mL water solution [8]. Vitamin E (alpha-tocopherol, Sigma-Aldrich, Saint Louis, MO) was administered p.o. at 200 mg/kg of body weight [9]. Taurine (Acros Organics, Fair Lawn, NJ) was administered p.o. at 520 mg/kg of body weight [10] and prepared in a 225 mg/mL water solution that was heated to obtain solubility but maintain stability. The vitamin E and taurine treatment group received the dosages, as previously described, concurrently. The rats were fed prior to the dark cycle, and food intakes were recorded daily.

Blood sample collection

Fatty liver induction phase. All blood samples were collected before the beginning of the dark cycle. A baseline blood sample was collected from each rat (day 0) prior to the beginning of the induction phase. A blood sample was collected from each rat at the end of the induction phase (day 28), and prior to the beginning of the treatment phase. All blood samples, with the exception of the terminal blood samples, were collected via the saphenous vein by using cone-shaped rodent restraint bags (Harvard Apparatus, Holliston, MA). The blood was collected in potassium EDTA and clotting activator Microvette capillary blood tubes (Sarstedt Ind., Newton, NC). At the end of the induction phase, 11 rats were assigned randomly to be euthanized to determine the extent of fatty liver development. Those rats were euthanized via carbon dioxide inhalation and exsanguination via cardiac puncture that was immediately followed by decapitation. The blood samples obtained through cardiac puncture were collected in potassium EDTA Monoject tubes (Tyco Healthcare Group, Mansfield, MA) and silica clot activator Vacutainer tubes (BD, Franklin Lakes, NJ). All

blood samples were stored on ice until processing. Plasma and serum samples were prepared by centrifugation at 4°C at 3,000 × g for 10 minutes and stored at -20°C until analysis.

Fatty liver treatment phase. All blood samples were collected before the beginning of the dark cycle. During the treatment phase of the study, the remaining 52 rats had blood samples collected on day 14, which was midway through the treatment phase, and day 28, which was at the end of the treatment phase. All blood samples on day 14 of the treatment phase were collected via the saphenous vein utilizing rodent restraint cones (Harvard Apparatus, Holliston, MA). The blood was collected in clotting activator and potassium EDTA Microvette capillary blood tubes (Sarstedt Inc., Newton, NC). On day 28 of the treatment phase, the rats were euthanized via inhalation of carbon dioxide, exsanguination via cardiac puncture, and subsequent decapitation. For these terminal samples, blood was placed in potassium EDTA Monoject tubes (Tyco Healthcare Group, Mansfield, MA) and silica clot activator Vacutainer tubes (BD, Franklin Lakes, NJ). All blood samples were stored on ice until processing. Plasma and serum samples were prepared by centrifugation at 4°C at 3,000 × g for 10 minutes and stored at -20°C until analysis.

Analyses

Measurement of blood hormones and metabolites. Blood hormones analyzed included plasma adiponectin and insulin concentrations. Plasma adiponectin concentrations were measured by using a commercially available adiponectin radioimmunoassay kit (Linco Research, St. Charles, MO). The lower limit of detection was 1 ng/mL, and the intra-assay coefficient of variation was <5%. Plasma insulin concentrations were measured by using a commercially available rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO).

The lower limit of detection was 0.1 ng/mL, and the intra-assay coefficient of variation was <5%. Plasma samples also were analyzed for glucose concentrations by using a commercially available enzymatic kit (Pointe Scientific, Inc., Canton, MI).

Serum samples were analyzed for total cholesterol and triacylglycerol concentrations with commercially available enzymatic kits (Pointe Scientific Inc., Canton, MI). Non-esterified fatty acid (NEFA) concentrations were analyzed on the serum samples utilizing a commercially available enzymatic kit (NEFA-C kit, Wako Chemicals USA, Inc., Richmond, VA).

In all assays, samples from rats in each treatment group were processed together to balance any possible inter-assay effects.

Evaluation of liver and carcass characteristics. Following euthanasia, the liver was collected from each rat, rinsed in 0.15 M NaCl, homogenized, and immediately placed in liquid nitrogen. The liver samples were stored at -80°C until analysis for total lipid and triacylglycerol content. A wet tissue lipid extraction procedure [11] was used to determine total liver lipid concentrations. The liver lipid extracts were analyzed for total triacylglycerol concentration with a commercially available enzymatic kit (Pointe Scientific Inc., Canton, MI), after solubilization with Triton X-100 [12].

Following liver removal, the rat carcasses were collected for compositional analysis, minus skin, feet, head, tail, and entrails. The carcasses were stored at -20°C until analysis. Rat carcasses were weighed and then ground in a heavy duty food grinder (Oster, Boca Raton, FL). Most carcass bones were ground or extracted during the grinding process, and bone

pieces were not used in compositional analyses. Carcass dry matter was determined by desiccation in an oven at 100°C for 24 hours. Protein content of the carcasses was estimated by using a micro-Kjeldahl procedure to determine nitrogen content [13]. Total protein was approximated by using a factor of 6.25 times the nitrogen content. Total carcass lipids were analyzed by using a 2:1 chloroform:methanol (v:v) modified Folch method [14].

STATISTICAL ANALYSIS

Statistics were computed by using SAS software (V9.2, SAS Institute, Inc., Cary, NC). Blood data were analyzed as a repeated measures ANOVA during the treatment phase (day 0, 14, and 28) using the GLIMMIX procedure, with treatment, day, and their interaction as model effects. Blocks and the interaction between blocks and treatments were treated as random effects. The repeated measures were fit with a heterogeneous compound symmetry (type=CSH) covariance structure. Rat growth was analyzed by a similar repeated measures ANOVA, with body weights compared across weeks and treatments; a heterogeneous first order autoregressive covariance structure was used. Terminal data, including carcass and liver characteristics, were compared with treatments as fixed effects, and blocks and blocks-by-treatment interactions as random effects. For liver lipid data, the treatment effect was significant; so, the factorial treatment substructure was tested for interactions (control, control+vitamin E, control+taurine, control+vitamin E+taurine). No interaction was detected; so, contrasts were written to compare the main factorial treatments (vitamin E-containing or taurine-containing treatments) against each other, the metformin treatment, the control treatment, and the induction treatment. Average daily food intakes for the treatment groups were analyzed separately for the two phases by accounting for the effects of days and

blocks, and the interaction of days and blocks with treatments. A p-value ≤ 0.05 was considered significant. Tendencies for significance were considered to be $0.05 < \text{p-value} < 0.1$.

RESULTS

Liver and carcass characteristics. Liver characteristics analyzed are summarized in Table 2. There was no interaction effect of vitamin E and taurine on liver characteristics; so, the effects in the vitamin E and taurine combination group were pooled into the vitamin E and taurine groups for determination of treatment effects for vitamin E-containing and taurine-containing treatment groups. Liver lipid concentrations were significantly different from the induction group for all treatment groups ($p < 0.002$). The only difference between treatment groups was between the vitamin E-containing and metformin treatment groups, with the vitamin E-containing treatments having significantly higher liver lipid concentrations compared to the metformin treatment group ($\Delta 0.64\%$, $p=0.0472$). Liver triacylglycerol concentrations tended to be significant between the treatment groups ($p=0.068$), driven by the higher liver triacylglycerol concentrations in the induction group. Compared with the induction group, the rats receiving the standard diet, regardless of nutraceutical supplementation, had significantly lower liver triacylglycerol concentrations ($\Delta \sim 0.36 \mu\text{g}/\mu\text{g}$ lipid, $p=0.0058$). Liver weights were not significantly different between any of the treatment groups, including the induction group.

There were no significant differences between treatment groups, including the induction group, with respect to any of the carcass characteristics analyzed in this study (Table 3).

Blood hormones and metabolites. Differences between concentrations of plasma hormones and metabolites were only analyzed for the treatment phase of the study to determine the treatment effects.

There were no significant differences in plasma adiponectin concentrations (Figure 1) or plasma insulin concentrations (Figure 2) between the treatment groups but there was an effect of day. Mean plasma adiponectin concentrations over all treatments were significantly higher than day 0 on day 14 (Δ 1167.27 ng/mL, $p=0.0119$) and day 28 (Δ 1853.37 ng/mL, $p=0.0005$). Mean plasma insulin concentrations across all treatments was significantly higher on day 14 than all other days (Δ 1.98 ng/mL, $p<0.0001$).

Plasma glucose concentrations were not significantly different between treatment groups throughout the study, but there was an effect of day (Figure 3). This day effect was driven by the higher plasma glucose concentrations seen on the last day of the treatment phase (Δ 226.48 mg/dl, $p<0.0001$). This effect is likely attributable to the difference in circumstances surrounding the blood sample collection process, as this blood sample was taken during the euthanasia process via cardiac puncture.

There were no changes in serum lipid concentrations during the induction phase, indicating that dyslipidemia was not induced with the high-fat diet used in this study. There were no significant differences between treatment groups with respect to serum cholesterol (Figure 4), triacylglycerol (Figure 5), or NEFA (Figure 6) concentrations throughout the study. There was a day effect associated with serum triacylglycerol and NEFA concentrations. Mean serum triacylglycerol concentrations across treatments gradually increased throughout the treatment phase of the study. From day 0 to day 14, mean serum triacylglycerol

concentrations increased by 33.16 mg/dl ($p < 0.0001$) and increased by 15.87 mg/dl from day 14 to day 28 ($p = 0.0042$). The day effect on serum NEFA concentrations was driven by the decrease seen on day 28 ($\Delta 6.63$ mg/dl, $p < 0.0001$).

Food intake and body weights. There were no significant differences between the treatment groups with respect to food intake and body weight throughout the study. The mean daily food intake and mean weekly body weights are presented in Figure 7. The food intake and growth curves had distinct patterns. Food intake gradually decreased for all animals during the induction phase, plummeted for several days after the diet change, then increased to eventually plateau. Mean weekly body weight gradually increased for all animals during the induction phase, dipped at diet change, and slowly increased to plateau.

DISCUSSION

The overall objective of this study was to induce non-alcoholic fatty liver disease and then compare the effectiveness of a standard diet alone and a standard diet supplemented with metformin, vitamin E, taurine, or a combination of vitamin E and taurine at treating the disease. The high-fat choline-deficient diet utilized in the induction phase of this study led to accumulation of triacylglycerols in the liver and the standard diet alone was sufficient to reverse this change. There was no added benefit in the treatment of the disease with supplementation of metformin or nutraceuticals.

It is important to note that the potential effects of dietary choline could not be determined by this study design. The standard diet fed in the treatment phase of this study contained an adequate amount of choline bitartrate. The rats progressed from a choline-deficient state to a

choline-sufficient state and this change may contribute to the effects seen in the liver characteristics. Without a standard, choline-deficient diet group, it is impossible to separate out the potential choline effects from the diet effects.

The gradual increase in serum triacylglycerol concentrations throughout the treatment phase appropriately correspond with the decrease in liver triacylglycerol. These effects are likely correlated with the increase in plasma insulin concentrations seen on day 14 of the treatment phase. This spike in insulin was anticipated as the rats underwent a diet change from a high-fat, low carbohydrate diet to a low-fat, high carbohydrate diet. This rise in plasma insulin concentration likely inhibited fatty acid oxidation in the liver and other tissues. This change would shunt the NEFAs delivered to the liver, as well as the stored triacylglycerols, into very low density lipoprotein (VLDL) formation and secretion, contributing to the increase in serum triacylglycerol concentrations [15]. Low-fat, high carbohydrate diets have also been shown to decrease the clearance of VLDLs from the blood, adding to the increase in serum triacylglycerols quantified [16, 17].

The changes in plasma glucose and insulin seen on day 56 of the study can be attributed to the euthanasia process. On this day, the blood was collected after anesthetic administration of carbon dioxide via cardiac puncture. The stress of carbon dioxide inhalation stimulated epinephrine release in the rats. Epinephrine is known to stimulate glycogenolysis [18] and gluconeogenesis [19] in the liver, causing a hyperglycemia, and suppresses insulin secretion [20]. A dramatic increase in plasma glucose concentrations and decrease in plasma insulin concentrations were identified in this study as a result of this physiological response to

euthanasia. Blood samples for accurate quantification of plasma glucose and insulin should not be taken during the euthanasia process.

For humans, body mass index has been found to be the only independent predictor of the degree of lipid accumulation in the liver, and it has a positive correlation [21]. Overall, considering many of the prevalence studies conducted on NAFLD, the median obesity rate in NAFLD human patients is approximately 71%. With most of the NAFLD subjects being obese, it is recommended for patients to have an initial weight loss goal of 10% of baseline body weight within a six month period if the patient's body mass index exceeds 25 kg/m^2 [22]. A recent study identified histological improvement in liver characteristics in 60% of NAFLD patients counseled to follow a 1400 kcal/day diet for 12 months [23]. There are studies that have corroborated the beneficial effects of dietary modifications on weight loss [24-27], and others that have demonstrated its superiority to other therapy options [28-30]. This study supports these findings.

The insignificant effects of metformin and nutraceutical supplementation with a standard diet lend convincing evidence as to the importance of dietary intervention in the treatment of non-alcoholic fatty liver disease. The diet in this study was not fed to induce weight loss. It was administered as an imitation of a "lifestyle change" for humans. Even with a lack of change in terminal carcass weight and carcass lipid content, in addition to a continual increase in weight throughout the treatment phase of the study, the simple change in diet was sufficient to drastically reduce the fatty liver. This observation demonstrates that some patients with NAFLD, especially those that do not demonstrate signs of the metabolic syndrome, may still

see beneficial effects on the disease by simply altering their diet to one of lower fat content, even if they do not lose weight.

CONCLUSIONS

The fatty liver induced in this study was reversed with a simple change in diet, and additional supplementation with metformin, vitamin E, or taurine demonstrated no additional benefit. This finding holds significant implications for the importance of “lifestyle changes” in the treatment of NAFLD in humans, irrespective of weight loss. More research, however, is needed to fully elucidate the mechanisms explaining the effects seen in this study, as the effects of a choline-sufficient state were not addressed with this rodent model of NAFLD.

ACKNOWLEDGEMENTS

This research was supported by the Iowa State University Center for Designing Foods to Improve Nutrition (CDFIN) through funds obtained from the United States Department of Agriculture. Appreciation is extended to Michelle Bohan Brown for her assistance with the authorship of this grant and to Andrew Brown for his help with statistical analyses. Thank you to the students of the Nutritional Physiology Group of the Animal Science Department at Iowa State University for their assistance with data collection and analyses.

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Table 1: Diet Composition

Diet	Induction Phase Diet¹	Treatment Phase Diet²
	kcal%	kcal%
Protein	20.0	20.0
Carbohydrate	20.1	70.0
Fat	59.9	10.0
Ingredients		
	gram	gram
Casein, 80 Mesh	200	200
L-Cystine	3	3
Corn Starch	0	315
Maltodextrin 10	125	35
Sucrose	68.8	350
Cellulose, BW200	50	50
Soybean Oil	25	25
Lard	245	20
Mineral Mix, S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H ₂ O	16.5	16.5
Vitamin Mix, V10001	10	10
Choline Bitartrate	0	2

¹ D05010403, Research Diets, Inc., New Brunswick, NJ² D12450B, Research Diets, Inc., New Brunswick, NJ

Table 2: Liver Characteristics¹

Liver Component	Induction	Control	Metformin	Vitamin E²	Taurine²	Vitamin E and Taurine²
Weight (g)	12.14 ± 0.82	12.83 ± 0.82	13.15 ± 0.82	13.68 ± 0.82	13.22 ± 0.82	14.50 ± 0.82
Lipid (%)	5.57 ± 0.23 ^a	3.81 ± 0.24 ^{bc}	3.45 ± 0.23 ^b	4.02 ± 0.23 ^c	3.67 ± 0.24 ^{bc}	4.15 ± 0.24
TAG (µg TAG/µg lipid)	0.538 ± 0.071 ^a	0.196 ± 0.073 ^b	0.181 ± 0.071 ^b	0.159 ± 0.071 ^b	0.179 ± 0.073 ^b	0.178 ± 0.073

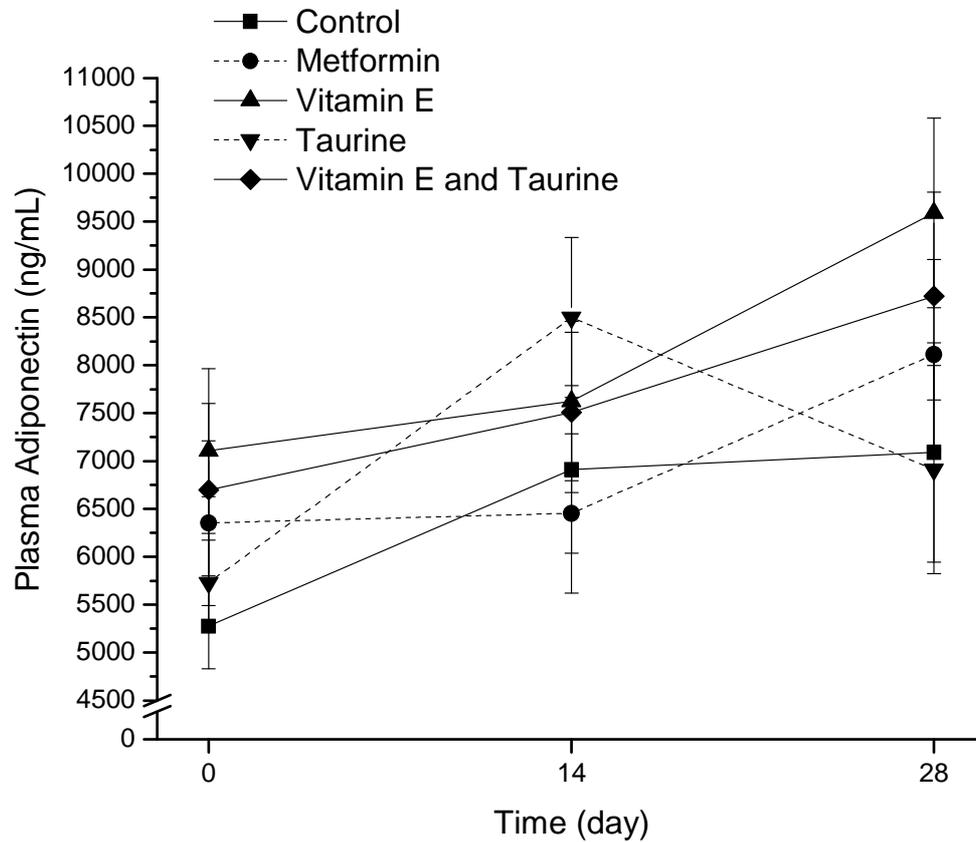
¹ Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Eleven rats were euthanized at the end of the induction phase to determine the extent of fatty liver induced. Remaining rats were fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. n=11 for the induction, metformin, and vitamin E groups but n=10 for the remaining treatment groups. Values are means ± pooled SEM. Means in a row with superscripts without a common letter are significantly different, p<0.05.

² There was no interaction effect of vitamin E and taurine. Effects in the combination group were pooled into the vitamin E and taurine groups for determination of those treatment effects.

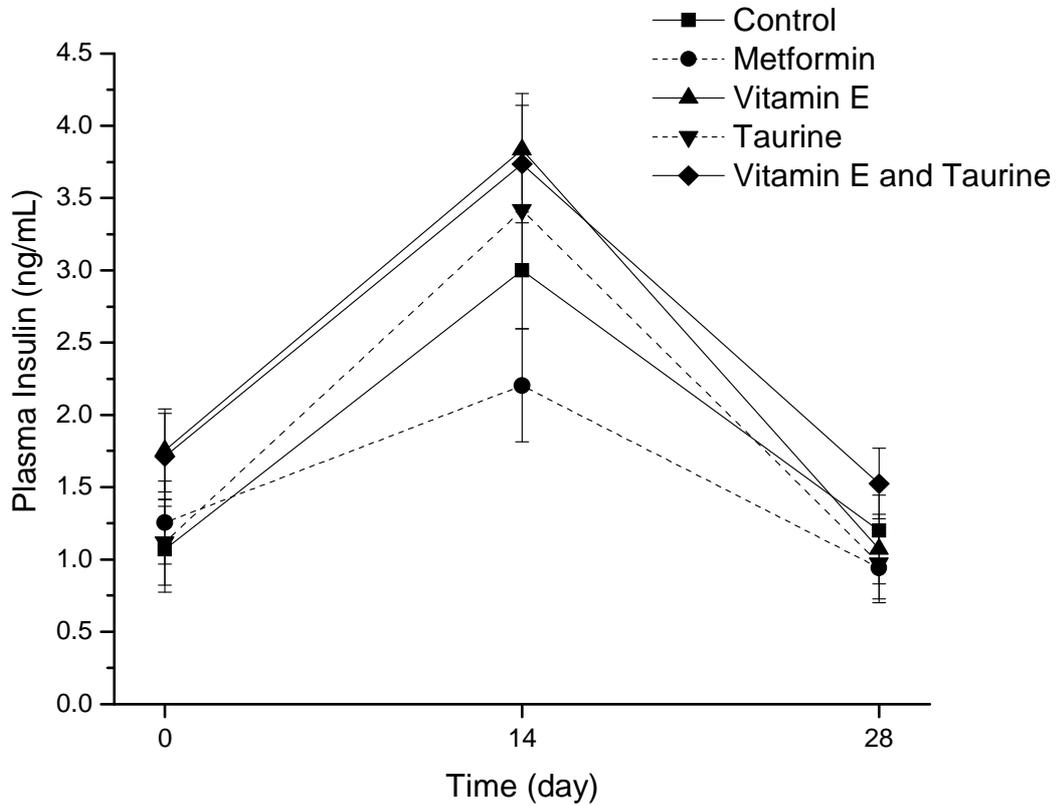
Table 3: Carcass Characteristics¹

Carcass Component	Induction	Standard Diet	Metformin	Vitamin E	Taurine	Vitamin E and Taurine
Weight (g)	217.49 ± 11.08	224.30 ± 11.15	222.17 ± 11.08	224.42 ± 11.08	235.03 ± 11.15	238.81 ± 11.15
Dry matter (%)	30.23 ± 1.05	29.33 ± 1.07	29.90 ± 1.05	20.19 ± 1.05	30.41 ± 1.07	29.37 ± 1.07
Lipid (% on wet basis)	8.30 ± 1.28	6.69 ± 1.30	6.60 ± 1.28	7.52 ± 1.28	8.21 ± 1.30	7.07 ± 1.30
Protein (% on wet basis)	19.31 ± 0.38	20.24 ± 0.40	20.18 ± 0.38	19.33 ± 0.38	19.59 ± 0.40	20.49 ± 0.40

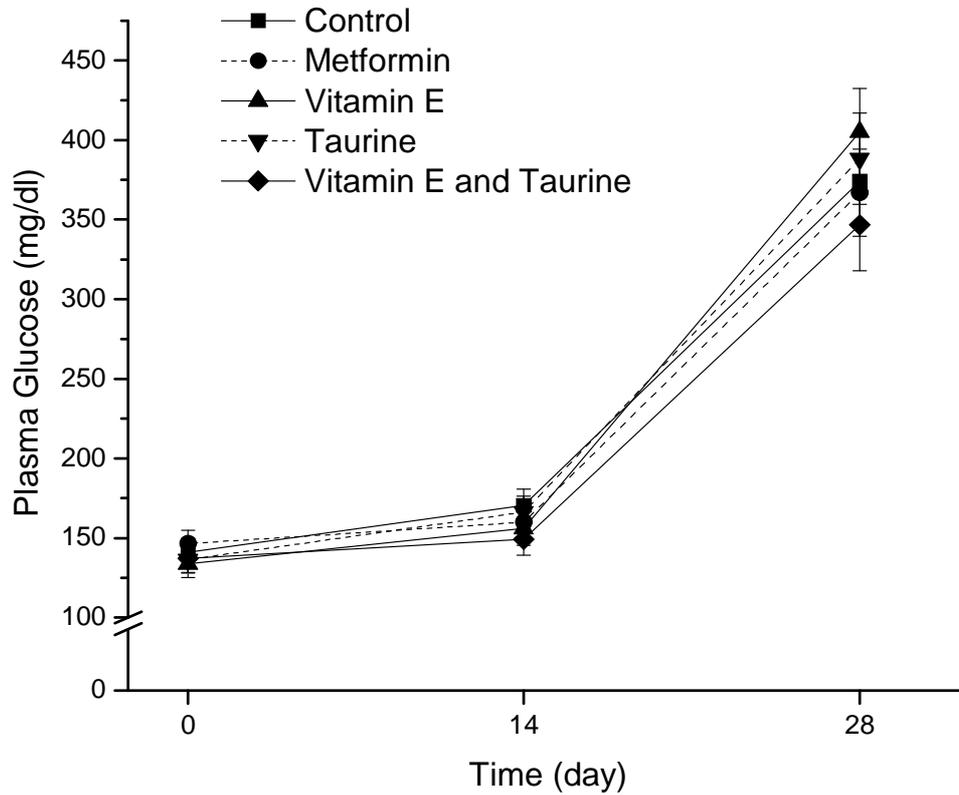
¹Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Eleven rats were euthanized at the end of the induction phase to determine the extent of fatty liver induced. Remaining rats were fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. n=11 for the induction, metformin, and vitamin E groups but n=10 for the remaining treatment groups. Values are means ± pooled SEM. There were no significant differences between the treatment groups with respect to carcass characteristics.

Figure 1:

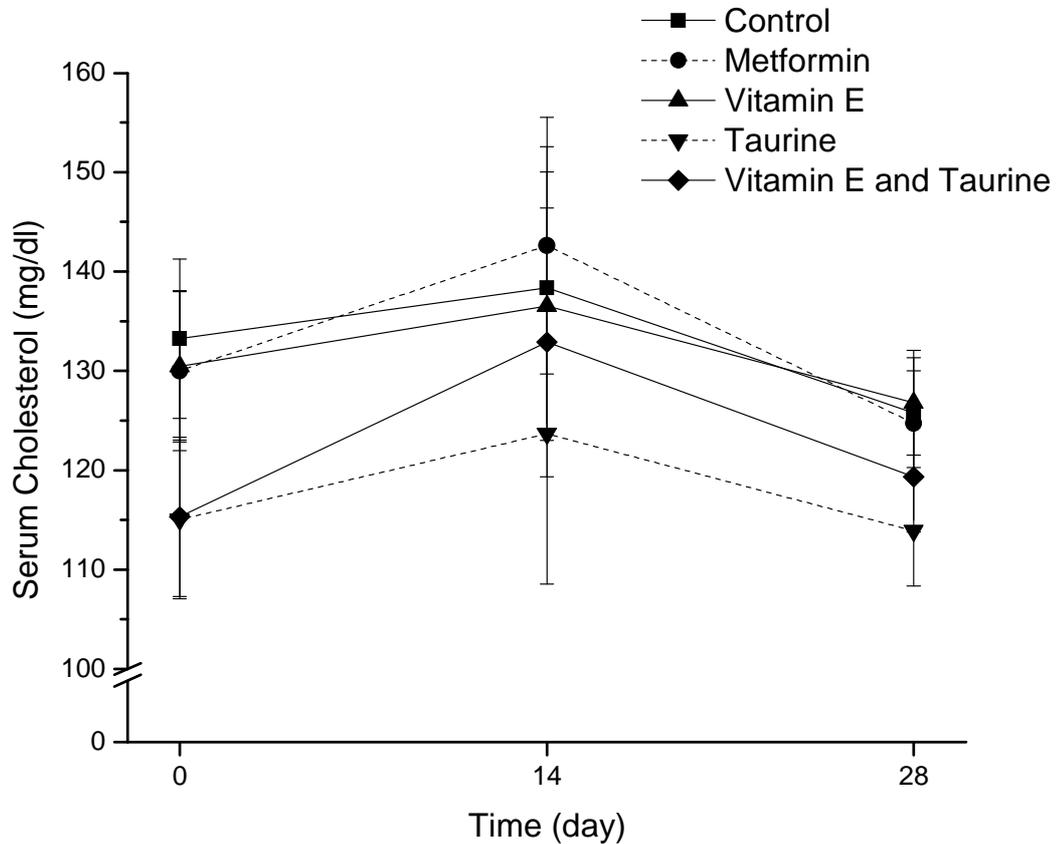
Effects of standard diet and metformin or nutraceutical supplementation on plasma adiponectin concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in plasma adiponectin concentrations between treatment groups throughout the treatment phase of the study.

Figure 2:

Effects of standard diet and metformin or nutraceutical supplementation on plasma insulin concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in plasma insulin concentrations between treatment groups throughout the treatment phase of the study.

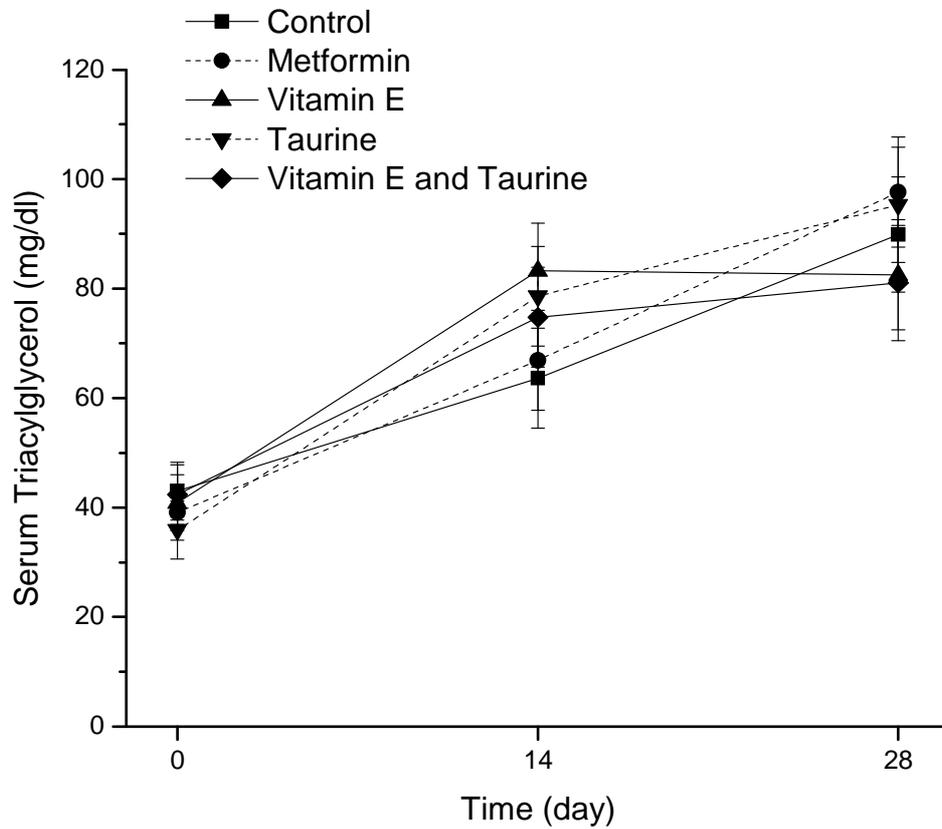
Figure 3:

Effects of standard diet and metformin or nutraceutical supplementation on plasma glucose concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in plasma glucose concentrations between treatment groups throughout the treatment phase of the study.

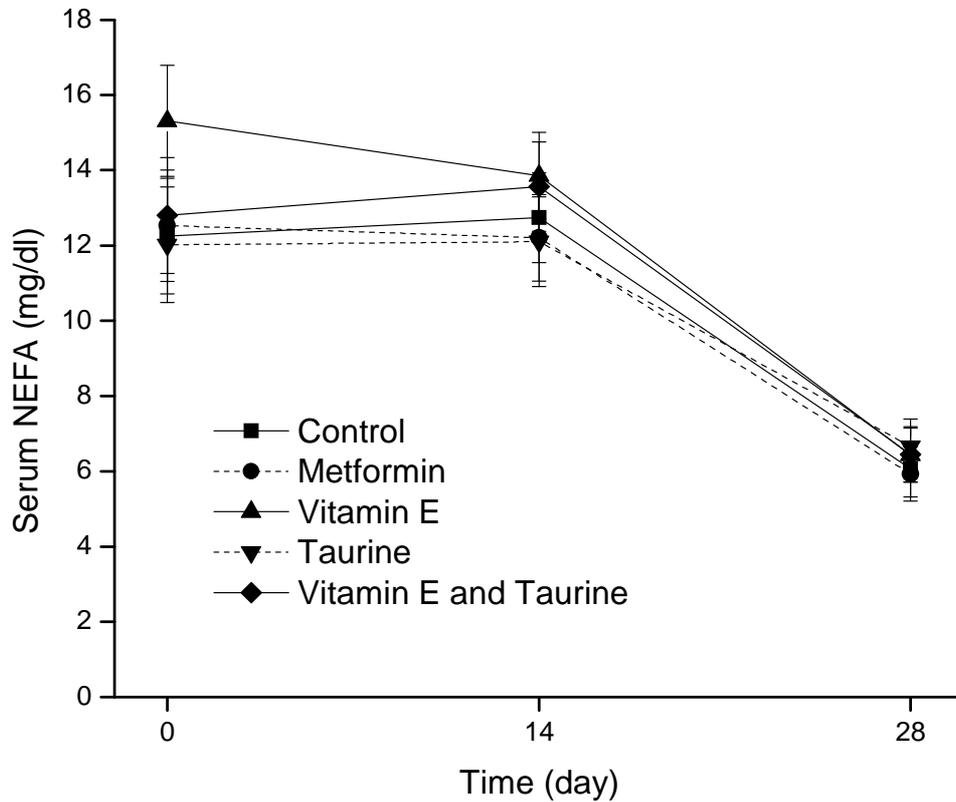
Figure 4:

Effects of standard diet and metformin or nutraceutical supplementation on serum cholesterol concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in serum cholesterol concentrations between treatment groups throughout the treatment phase of the study.

Figure 5:

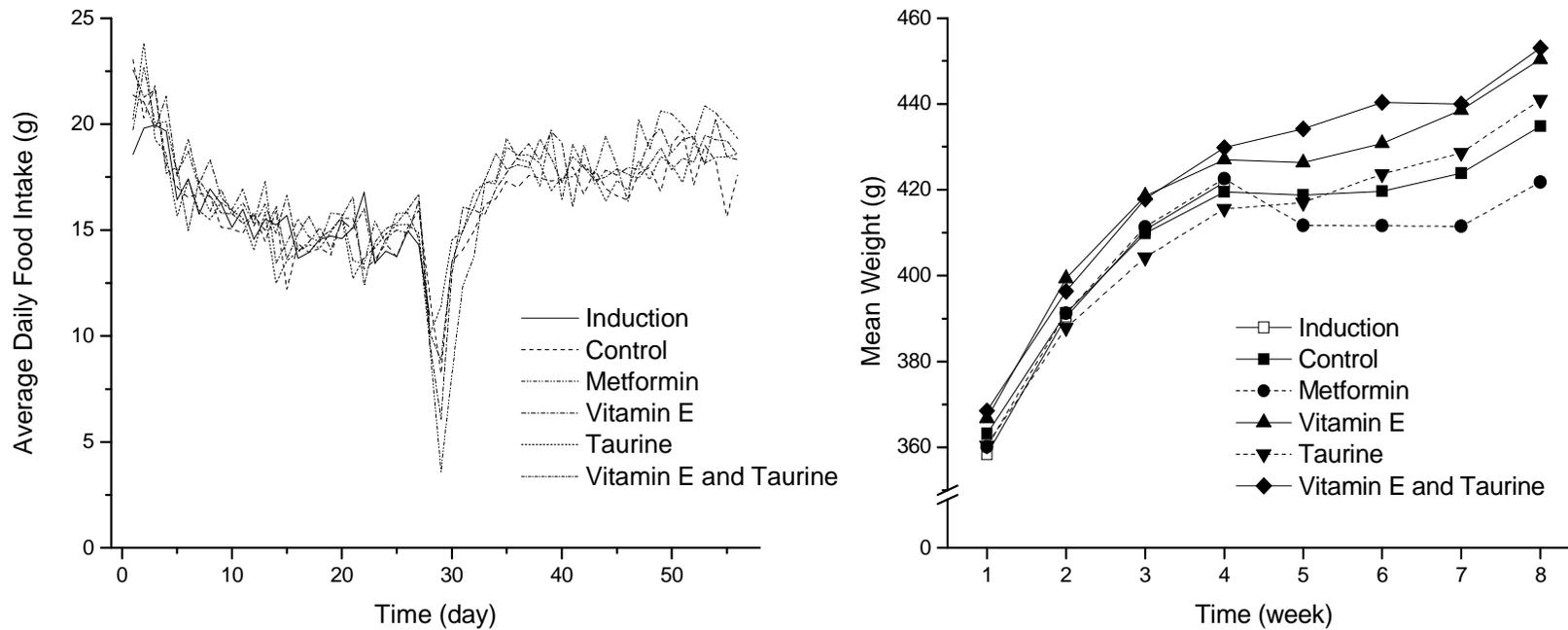


Effects of standard diet and metformin or nutraceutical supplementation on serum triacylglycerol concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in serum triacylglycerol concentrations between treatment groups throughout the treatment phase of the study.

Figure 6:

Effects of standard diet and metformin or nutraceutical supplementation on serum non-esterified fatty acid (NEFA) concentrations. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Rats were then fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. $n=11$ for the metformin and vitamin E groups but $n=10$ for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in serum NEFA concentrations between treatment groups throughout the treatment phase of the study.

Figure 7:



Effect of fatty liver induction and subsequent treatment on mean daily food intake and mean weekly body weights. Rats were fed a high-fat choline deficient diet for 28 days to induce fatty liver. Eleven rats were euthanized at the end of the induction phase to determine the extent of fatty liver induced. Remaining rats were fed a standard diet (control) or a standard diet with metformin (500 mg/kg), vitamin E (200 mg/kg), taurine (520 mg/kg), or a combination of vitamin E and taurine for 28 days. n=11 for the induction, metformin, and vitamin E groups but n=10 for the remaining treatment groups. Values are means \pm pooled SEM. There were no significant differences in food intake or body weight between treatment groups throughout the study.

CHAPTER IV:

General Conclusions

OVERALL CONCLUSIONS

Non-alcoholic fatty liver disease (NAFLD) is a disorder that will likely be endemic throughout the world in the next 50 years or so. Whether the disease is acquired through malnutrition or obesity, its management will be important to the quality of life of those afflicted by it. The amount of research concerning NAFLD has erupted in the last few decades but there is still much more that needs to be done. Effective prevention and treatment strategies must be identified if we are to be successful in mitigating the damage caused by this disease in future patients.

The information contained in this thesis compared the effectiveness of different pharmaceuticals and nutraceuticals on the prevention and treatment of NAFLD. When it comes to NAFLD, the most important aspect in the prevention and treatment of the disease is the diet of the patient. Initiating lifestyle changes, specifically in regards to diet, have beneficial effects on the disease, irrespective of potential weight changes. There is not one specific diet that would be recommended over another. Individuals will need to be assessed on a case-by-case basis. There will never be one universal strategy for the management of NAFLD.

Supplementation of the diet with the nutraceuticals vitamin E and taurine had no added benefit for the prevention or treatment of this disease. In fact, vitamin E may even cause

fatty liver when taken for preventative purposes. With the current organic movement and the drastic increase in public consumption of supplements, this is an important finding. These supplements may be beneficial in some aspects, but they can also be detrimental in others. All of these factors need to be considered by physicians, dieticians, and patients when identifying ways to prevent or treat certain disorders.

Future research is needed to address the potential adverse effects of nutraceuticals, specifically vitamin E, on the development of NAFLD. The mechanisms of action behind the pharmaceuticals and nutraceuticals used in these studies for this disease need to be elucidated and more studies need to be conducted to compare their effectiveness concurrently. Studies conducted utilizing human subjects need to include liver biopsies in order to get a more accurate representation of the treatment effects on liver histology, a distinction that is currently lacking in the present research.

ACKNOWLEDGEMENTS

My first thank you goes out to the people who made the grants and this thesis possible. First, I would like to extend my thanks and gratitude to Dr. Donald Beitz, Dr. Walter Hsu, and Dr. Michael Spurlock for their help with the content of this thesis. Secondly, I would like to thank Michelle Bohan Brown for spearheading the authorship of the first grant. Without that grant and inspiration, this thesis would not exist. Thirdly, I would like to thank Andrew Brown for his assistance with the statistical analysis. Without his help, there would be no results. Lastly, I would like to extend a special thank you to Dr. Donald Beitz for all of his support and guidance throughout my undergraduate and graduate career. I have learned so much from him, and he is someone I will admire and speak fondly of for the rest of my life.

These research studies would not have been successful without the help of many undergraduate, graduate, and veterinary students. I would like to thank the following people for their assistance during blood collection and euthanasia: A. Avdonina, M. Bohan Brown, A. Brown, Q. Duan, H. Gilliland, D. Goede, C. Grote, K. Korn, G. Mao, K. Noack, B. Noack, M. O'Neil, M. Osman, E. Richter, C. Schauer, A. Svoboda, W. Thomas, E. Wormley, A. Young, E. Yugo, and S. Zhang.

A special thank you goes out to Alyona Avdonina, Benjamin Noack, Erin Ritcher, Crystal Schauer, and Anthony Young. These individuals worked exceptionally long hours to help me complete the daily duties for the study and made the 7-hour process of treatment preparation possible. They were instrumental in all analyses and strove for the perfection I expected of them. I sincerely appreciate all of the physical and emotional support they gave to me throughout this trying process.

I would also like to thank Dr. Travis Knight and Dr. Leo Timms for their emotional support and encouragement. Dr. Knight and his family helped me see the light at the end of the tunnel and always made me smile. They are truly wonderful people, and I am thankful for everything they have done for me. Dr. Timms was always there for me, even through e-mail at 4 am, and helped me find a love for teaching. His advice, hugs, and lunches will never be forgotten.

It is with a swelling heart that I thank my two best friends, Michelle Bohan Brown and Andrew Brown, for absolutely everything. Their passion for research and life gave me the strength and courage to pursue this path. The support they provided is immeasurable, and I know I could not have done this without the two of them. They inspire me every day, and I love them both.

A final thank you goes out to my family for all of their support and patience these last few years. They were always there to lend an ear and reiterate that everything would be fine. I love you all.