An Investigation into the Influence of Dietary Saturated Fat and Quercetin Supplementation on Adiposity, Macrophage Behavior, Inflammation, and Non-Alcoholic Fatty-Liver Disease

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An Investigation into the Influence of Dietary Saturated Fat and Quercetin Supplementation on Adiposity, Macrophage Behavior, Inflammation, and Non-Alcoholic Fatty-Liver Disease

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Abstract

The overall objective of this dissertation was to examine the degree to which manipulation of the saturated fat content of a high-fat diet influenced obesity-related outcomes as well as to see if the naturally-occurring flavonoid, quercetin, could attenuate the resulting obesity and related metabolic and inflammatory side effects. Specifically, these studies examined 1) the influential role of saturated fat on macrophage function, inflammation, and other obesity-related comorbidities, including non-alcoholic fatty-liver disease, and 2) the potential benefits of quercetin supplementation. Overall, results from these studies suggest that adiposity, macrophage behavior, inflammation, insulin resistance, and non-alcoholic fatty-liver disease progression can be greatly affected by dietary SF content. However, it was found that these outcomes are not necessarily proportional to the percentage of SF in the diet; a diet most closely mimicking the standard American diet (12% and 40% of overall calories from saturated fat and total fat, respectively) led to the greatest adiposity, macrophage infiltration into adipose tissue, and insulin resistance, whereas diets composed of 6% (6%-SF) and 24% (24%-SF) of total calories from SF, but an equivalent level of overall calories from fat (40%), produced lower levels of these variables with the 24%-SF diet resulting in the least degree of insulin resistance and hepatic lipid accumulation. Further, contrary to the findings of others, the anti-inflammatory flavonoid, quercetin, had no effect at mitigating adipose tissue inflammation, hepatic steatosis, or improving body composition or fasting blood
glucose levels in a HFD-induced obesity model. Future studies are needed to better understand how the interaction between high-fat feeding and quercetin supplementation influences physiological processes.
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Introduction

The structure of this dissertation is "manuscript style". Thus, each aim of this dissertation corresponds to an individual journal article representing a chapter. Consequently, each journal article contains its own abstract, methods, results, and discussion section as well as references, figure legends, and figures according to the mandatory manuscript structure requested by the journal to which each article has been, or will be, submitted.

Review of the Literature

According to the World Health Organization (WHO), it is estimated that 1.5 billion people worldwide are overweight and at least 500 million are obese (1). Consequently, there has been a rise in obesity-related health problems including, non-alcoholic fatty-liver disease (NAFLD), cardiovascular disease, the metabolic syndrome, diabetes, degenerative diseases, and cancer (1, 2). Genetic predispositions, physical inactivity, and consumption of a high-fat diet (HFD) can all lead to the development of obesity. However, given the global acceptance and availability of energy dense foods, chronic ingestion of diets high in fat is arguably the leading contributor. As a result, significant research has been geared towards better understanding the link between high-fat-diet-induced obesity (HFDIO) and chronic disease risk as well as investigating nutrition-based interventions that may be used to combat the obesity epidemic.
It is now widely accepted that HFDIO can lead to a chronic state of low-grade inflammation, which is thought to be one of the main contributors to obesity-related diseases (3-5). This chronic inflammation is largely mediated through quantitative and functional alterations in white adipose tissue macrophages (ATMs) (6, 7). For example, it has been reported that approximately 45-60% of adipose tissue (AT) cells express the macrophage marker EMR1 (F4/80) in obese mice, whereas only 10-15% of cells from lean mice express this marker (6). In addition, ATMs exhibit a pro-inflammatory, classical phenotype (M1) in obese mice, while those from lean mice have an alternatively-activated, anti-inflammatory phenotype (M2) (7). These changes not only lead to increased inflammation but also to dysregulation of metabolic homeostasis; infiltration and polarization of macrophages in AT has been linked to lower plasma adiponectin levels as well as insulin and leptin resistance (8-11).

In addition to promoting a pro-inflammatory environment, obesity also leads to ectopic lipid accumulation, particularly in the liver. Although the liver is not meant to store excessive adipose tissue, under conditions in which energy intake exceeds energy use, the liver is a principal site for fat deposition leading to the development of NAFLD. NAFLD is the most common cause of chronic liver disease in the world, affecting more than 30% of the adult population in the United States alone (12, 13). NAFLD has been linked to cardiovascular morbidity and hepatocellular carcinoma (14).

While the association between HFD-induced obesity and adipose tissue (AT) and hepatic inflammation has been clearly recognized, there is a fundamental gap in understanding the relative contribution of different types of fatty acids (FAs) to these responses. Saturated fatty acids (SFAs) have received significant research attention as a
The inflammatory effects of SFAs are thought to be largely mediated by their capacity to serve as ligands for toll-like receptor-2 and -4 (TLR-2 and TLR-4); binding of SFAs to TLR-2 and/or TLR-4 on various cell types, in particular macrophages and adipocytes, results in the induction of pro-inflammatory gene transcription via activation of nuclear factor kappa-B (NFκB), the c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK) signaling cascades (10, 15, 16). Consistent activation of these pathways results in a chronic state of inflammation and subsequent insulin resistance (IR) (10, 17, 18). In addition, SFAs, in general, are more obesogenic than other FAs; long-chain saturated fatty acids (LCSFAs, > C12:0) are not as efficiently oxidized as unsaturated fatty acids (USFAs), and thus are more likely to be stored as AT (19, 20). Given the preponderance of evidence that supports a role of saturated fat (SF) on macrophage-mediated inflammation and metabolic dysfunction in high-fat-diet-induced obesity, it is surprising that there have been no dose response studies to more clearly evaluate their specific role in these processes.

Due to the fact that western countries are plagued by diseases resulting from obesity, it has become the goal of scientists to come up with relatively inexpensive therapeutic interventions to help mitigate HFD-enhanced chronic inflammation in order to improve quality of life and decrease obesity-related deaths. Recently, scientists have utilized nutraceutical interventions to examine the effects that plant-derived metabolites have on obesity outcomes. There exists promising research that the naturally occurring flavonoid, quercetin, possesses anti-inflammatory properties and an anti-obesogenic effect when supplemented along with a HFD (21-27). However, the mechanism(s) by
which quercetin elicits its positive effects and the influence that quercetin has on macrophage behavior is still poorly understood. Thus, further investigations are necessary to better grasp the mechanism(s) by which quercetin produces its therapeutic benefits.

The overall objective of this dissertation was to examine the degree to which manipulation of the SF content of a HFD influenced obesity-related outcomes as well as to see if quercetin supplementation could attenuate the resulting obesity and related metabolic and inflammatory side effects. Specifically, these studies examined 1) the influential role of saturated fat on macrophage function, inflammation, and other obesity-related comorbidities and 2) the potential benefits of quercetin supplementation.

**Obesity & Adipose Tissue Dysfunction**

Obesity has quickly become a major health concern not only in the United States, but throughout the world. Moreover, it seems that the rate of obesity is on the rise. In 1980, as little as thirty years ago, the rate of obesity in the adult population was half (15%) of what it is now (30%). Even more worrisome, is that WHO projects that by 2015 there will be approximately 2.3 billion and 700 million overweight and obese individuals, respectively.

The health risks associated with obesity not only affect the individual suffering from obesity, but they also negatively impact the economy. It is estimated that obesity costs the economy of the United States more than 120 billion dollars annually (1). The 120 billion dollar stress on the economy is due many factors, including but not limited to: an increase in health care services that are needed as a direct result of the escalating rate of obesity and decreased productivity resulting from missed work time and premature
death. Additionally, taxpayers are burdened with high health care costs, which impacts the price of goods and employee raises (2).

Obesity is problematic as the condition is characterized by a chronic state of low-grade adipocyte inflammation linked to adipose tissue dysfunction. Whether the adipose tissue dysfunction precedes the low-grade inflammatory state, or vice versa, is debatable. Nonetheless, adipose tissue dysfunction and the associated low-grade inflammation have been shown to increase the rate of mortality and are characterized by a circular cascade of interconnected events including, but not limited to, an increase in adipocyte size and number, endoplasmic reticulum stress, adipose tissue hypoxia, increased infiltration of pro-inflammatory macrophages into adipose tissue, an increase in adipocyte, pre-adipocyte, and macrophage TLR-4 and TLR-2 protein content, an increase in circulating pro-inflammatory cytokines, insulin resistance, and a decrease in mitochondrial function (3-9).

Because western countries are plagued by an obesity epidemic, and thus an increased chronic disease risk, it has become the goal of scientists to uncover the mechanisms responsible for dysfunctional adipose tissue and the role that certain fatty acids have in promoting this state.

**Dietary Fat and Obesity**

It is practically impossible to narrow down the cause of obesity to one single factor. In reality, the high prevalence of obesity is due to several interacting factors. These factors include genetics, lifestyle choices (diet and physical activity), as well as the environment (access to parks, social networks, weather, etc.) (10-12). However, given
the global acceptance and availability of energy dense foods, chronic ingestion of diets high in fat is arguably the leading contributor.

Dietary fat consumption is necessary for various physiological functions, and has been shown to play a major role in preventing chronic conditions such as cardiovascular disease (13). However, high consumption of dietary fat has been associated with excessive energy intake, which has been strongly linked to the increasing rate of obesity within the United States (14). With this being said, research has shown that fat matters, but total caloric intake is more important (15). From the late 1950’s to the year 2008, it is estimated that caloric intake, per person, has increased from roughly 1900 kcal to 2,661 kcal – a 761 kcal increase over 58 years (16). This increase in caloric intake is strongly linked with an increase in the consumption of high-fat foods; according to the USDA, the average American in the year 2000 consumed 287% more cheese, 41% more meat, and 67% more added fats and oils than the typical American did on an annual basis in the 1950’s.

**Standard American Diet and Fat Consumption**

In order to educate individuals about nutrient consumption, the United States Department of Agriculture (USDA) and the Institute of Medicine created daily dietary guidelines for individuals to follow. These institutes recommend that fat intake be between 20 – 35% of total caloric intake. With regard to carbohydrate intake, carbohydrates should make up 45-65% of total caloric intake. Finally, protein intake should be 10-35% of total caloric intake. Thus, when examining the standard American diet, one will see that, Americans, in general, consume the three macronutrients
(carbohydrates, fat, and protein) within the recommended distribution range: the standard American diet consists of carbohydrate, total fat, and protein consumption to be 50%, 33%, and 15%, respectively, of total caloric intake (17). However, although these percentages fall within the Dietary Guidelines for Americans (DGA), the types of each macronutrient, particularly in the case of fats, do not fall within the DGA. For instance, it is recommended that the majority of fat consumption come from polyunsaturated and monounsaturated fats and for saturated fat to make up less than 10% of the total calories consumed. However, in reality, Americans consume 11% of total calories in the form of saturated fat. In addition, the ratio of monounsaturated to polyunsaturated fat consumption is roughly 2:1, with monounsaturated fatty acid consumption and polyunsaturated fat consumption making up 12% and 7%, respectively of total caloric intake (17).

Recently a significant amount of attention has been given to polyunsaturated fatty acids and the ratio between Omega-6 polyunsaturated fatty acids to Omega-3 polyunsaturated fatty acids. In general, Omega-6 polyunsaturated fatty acids are considered to be pro-inflammatory fatty acids, whereas Omega-3 polyunsaturated fatty acids are considered to be anti-inflammatory fatty acids (18). A high ratio of Omega-6/Omega-3 fatty acid consumption has been associated with the promotion of many chronic diseases, including cancer (18). Ideally, the ratio of Omega-6/Omega-3 fatty acid consumption should be 1/1 as is seen commonly within the populations of wild animals and our human ancestors (18-22). However, in the United States, the average consumption of Omega-6/Omega-3 fatty acids is 15-20/1, a far cry from the ideal ratio (18).
Challenges of Dietary Studies

In humans, there are many drawbacks to long-term nutrition studies. Although such studies can be well designed, high attrition rates and lack of dietary compliance are two of the biggest problems associated with long-term dietary studies. For instance, several 6-month studies involving low-carbohydrate diets had attrition rates of 43%, 60%, and 36% (23). Similarly, the attrition rate to a long-term, low-fat diet of roughly 19,000 women was 86% in the Women’s Health Initiative Dietary Modification Trial (15). Furthermore, it is not plausible to measure repeated multiple physiological markers (e.g. metabolism, organ function, precise food intake, etc.) over the long term. This is the reason why you will find few credible long-term dietary studies performed in humans.

Based on this evidence, animal models are better for long-term dietary interventions when examining the physiological outcomes and mechanisms of various dietary interventions, as a majority of possible confounding factors can be controlled. However, even when utilizing animal models, dietary studies still pose a challenge. For instance, when trying to control for various macro- and micronutrient ratios within an iso-caloric diet, the manipulation of one macronutrient, or sub-set of macronutrients, results in an uncontrollable alteration of another. This is the inherent nature of nutrition studies. Because of this, with any dietary alteration, it is difficult to pinpoint the macronutrient(s) responsible for any given outcome. Thus, it is imperative that as much care is taken to control for as many factors as possible when designing studies centered around nutrient manipulation.

Non-Alcoholic Fatty-Liver Disease (NAFLD)
NAFLD is characterized by evidence of hepatic steatosis (as determined by imaging or histology) with “no causes of secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication, or hereditary disorders” (24). The disease presents itself in 70-80% of diabetic and obese patients and is the principal cause of hepatological clinical referrals in the United States (24, 25).

NAFLD is a single term describing two pathological conditions. The first condition, known as hepatic steatosis, is characterized by the accumulation of excess fat in the liver. Hepatic steatosis precedes the second condition, non-alcoholic steatohepatitis (NASH), which is typified not only by hepatic steatosis, but also by hepatic inflammation and hepatocellular damage.

Hepatic steatosis is a product of a long-term imbalance between caloric intake and energy expenditure. When caloric intake exceeds energy expenditure, excess energy is stored in the form of triglycerides in adipocytes. Although adipocytes have a significant capacity to store energy, a prolonged overindulgence in energy intake without a sufficient matching energy expenditure can result in adipose tissue dysfunction (26). Adipose tissue dysfunction is characterized by many factors including, an increase in adipocyte size and number, adipocyte secretion of pro-inflammatory cytokines, as well as the promotion of a pro-inflammatory environment and ectopic fat deposition, including the liver (26, 27). As lipids continue to accumulate in the liver, the liver eventually becomes insulin resistant, inflamed, and fibrotic (NASH), leading to an increased risk of mortality (24).

Mounting evidence suggests that HFDs promote NAFLD (28, 29). Further, it seems the type of fat consumed in the diet can greatly affect the development of NAFLD
both in vitro and in vivo models provide evidence that diets higher in saturated fat induce endoplasmic reticulum (ER) stress, modulate mitochondrial metabolism, promote an inflammatory environment, and invoke liver damage (25, 30, 31). However, there have been no controlled studies, which have examined the effects of HFDs different in the percentage of saturated fat on NAFLD.

Although NAFLD is a relatively common disease within the United States and throughout the world, there are limited therapeutic interventions for this condition. Weight loss through lifestyle interventions has proven successful at perturbing NAFLD (24, 32), however, it is difficult for individuals to successfully maintain long-term weight loss. In addition to exercise and diet modifications, such drugs as thiazolidinediones and statins have been shown to attenuate hepatic steatosis and steatohepatitis (32), however, the long-term safety and effectiveness of these drugs is unknown.

**Saturated Fat**

SFAs are FAs that have no double bonds between any of the carbon atoms in the FA chain. Thus, each carbon atom is “saturated” with hydrogen atoms. Various foods found throughout nature contain abundant amounts of SF. These foods include oils, such as coconut, cottonseed, and palm kernel oil, as well as foods rich in animal fats (e.g. cheese, fatty meats, cream, etc.). The majority of SF consumed by the typical American stems from animal foods.

Increased SFA consumption was first implicated as a risk factor for cardiovascular disease (CVD) based upon studies conducted in the 1960s-70s as this augmented consumption was shown to increase low-density lipoprotein (LDL) cholesterol more than any other nutrient except for trans fatty acids (33). For this reason,
both the WHO and the U.S. Dietary guidelines and the American Heart Association recommend that consumption of SF should be limited to 10% and 7% of total caloric intake, respectively (34).

It was not until recently that it was discovered that SFAs are capable of serving as ligands for TLR-4 and TLR-2, leading to the induction of pro-inflammatory gene transcription via nuclear factor kappa-B (NF-κB) activation (35, 36). TLR-4 and TLR-2 are part of a family of trans-membrane receptors that recognize conserved molecular patterns found on exogenous and endogenous pathogens and are ubiquitously expressed on cells found throughout the body (37). TLRs serve as an integral part of the innate immune system; once coupled to their specific ligand, TLRs help to activate immune system responses in order to protect the host from infection.

Consistent activation of the TLR-NF-κB pathway can lead to insulin resistance and a chronic state of inflammation, which has been shown to stimulate metabolic diseases and cancer development and contribute to adipose tissue dysfunction (3, 9, 38-40). Additionally, although the rate of saturated fat oxidation increases with decreasing carbon number, the long-chain saturated fatty acids (LCSFAs, > C12:0), are not as efficiently oxidized as unsaturated fatty acids, and thus are more likely to be stored as adipose tissue, elevating the risk of excess fat-mass gain and ectopic fat deposition (41, 42). It is common knowledge that white adipose tissue (WAT) is the main storage site for fatty acids in the form of triglycerides. The fatty-acid composition of WAT is primarily dependent on the fatty acid composition of the diet (43). Thus, individuals who consume a diet that is higher in saturated fat, such as the typical American diet, will have
WAT that consists of a larger percentage of saturated fat than an individual consuming a diet that is significantly lower in saturated fat.

Acute or chronic inflammation, obesity, IR and high-fat feeding are known to increase lipolysis and increase plasma free-fatty acid (FFA) levels (7, 44). Consequently, lipolysis of WAT comprised of a greater proportion of saturated fatty acids will lead to elevated circulating saturated FFAs, which may serve as ligands for TLR-4 and TLR-2, ultimately leading to pro-inflammatory gene expression. The pro-inflammatory proteins produced from these genes can then feedback into the circular inflammatory cascade, compounding adipose tissue dysfunction, augmenting the chronic inflammatory state, and increasing the risk of cancer development. Furthermore, high-fat feeding, leading to excess adipose tissue accumulation, has been shown to up-regulate TLR-4 expression on adipocytes, pre-adipocytes, and macrophages, thus enhancing the sensitivity of these cells for TLR-4 specific ligands, notably saturated fatty acids (7, 45).

**Quercetin**

Plants and plant-derived foods, such as fruits and vegetables, are comprised of thousands of non-caloric, naturally-occurring chemical compounds known as phytochemicals. It is believed that the health benefits derived from fruits and vegetables largely stem from these compounds, as upon consumption, phytochemicals are known to possess numerous functional qualities beneficial to the body (46). One of the most abundant phytochemicals found in plant-derived foods is quercetin. In fact, human consumption of quercetin in the diet is estimated to be 5-40 mg/day and as high as 500 mg/day in those individuals consuming a plant-based diet (46).
Quercetin, along with other phytochemicals, has been linked to the prevention of various diseases including cardiovascular disease (CVD), cancer, and NAFLD. Epidemiological research has provided evidence that an inverse relationship exists between phytochemical consumption and coronary heart disease and stroke (46). In addition, various animal and in vitro investigations have shown that quercetin has the capacity to serve as a mediator of cancer, CVD, and NAFLD development (46-54). Quercetin’s ability to serve as an anti-inflammatory, antioxidant, anti-proliferative, pro-apoptotic, and anti-angiogenic agent, as well as a regulator of metabolism, is believed to the basis for the positive effects elicited by the nutraceutical (46, 55).

Specifically, it has been quercetin’s capability to influence metabolic processes and its anti-inflammatory property, which have gained the attention of researchers examining obesity-related diseases. For instance, a study performed by Jung et. al. found that, in mice, a 32% HFD supplemented with .025% quercetin for 9 weeks reduced body weight, liver weight, and white adipose tissue weight compared to mice consuming a HFD without quercetin (53). In addition, it was found that quercetin altered the mRNA expression of genes regulating lipid metabolism. Along a similar note, in mice, Koboriet. al., also found that a HFD supplemented with .05% quercetin for 20 weeks hindered the development of hepatic steatosis, reduced visceral adipose tissue accumulation, oxidative stress, and improved glucose metabolism (52). These changes were linked to the normalization of mRNA expression of genes associated with regulating hepatic steatosis. These studies provide evidence that quercetin does possess anti-obesogenic properties, and these properties may be regulated by genes involved in lipid metabolism. However, investigations have also shown that quercetin supplementation can elicit mitochondrial
biogenesis both in the brain and in skeletal muscle (56, 57). Given that the mitochondria is the primary organelle involved in aerobic respiration and energy metabolism, it is plausible that quercetin’s anti-obesogenic property is related to the flavanoid’s influence on mitochondria.

Regarding quercetin’s anti-inflammatory property, Panchalet. al. found that a HFD supplemented with quercetin (.8 g/kg) reduced abdominal obesity and symptoms associated with the metabolic syndrome in rats (58). These findings were associated with a decrease in the concentration of NF-κB, leading the researchers to believe that a reduction in inflammation was partly responsible for quercetin’s anti-obesogenic effects. Ying et. al. also found that 14 days of daily oral administration of quercetin (30-60 mg/kg) given to gerbils on a HFD significantly reduced circulating levels of TNF-α and IL-6, two pro-inflammatory cytokines, while simultaneously positively influencing the lipid profile and decreasing hepatic lipid accumulation (54). These are just three examples of several studies to show that quercetin can mitigate HFD-induced inflammation (59-62). Although it is evident that quercetin does possess an anti-inflammatory property, the mechanism through which quercetin elicits its anti-inflammatory effects are still poorly understood. However, there is evidence indicating that quercetin tempers inflammation via modulation of the NF-κB and JNK pathways (63-66). Nonetheless, no studies have examined the influence of a HFD supplemented with quercetin on AT macrophage behavior.

Although strong evidence exists suggesting that quercetin possesses both anti-obesogenic and anti-inflammatory properties and may ameliorate the metabolic syndrome, at least one study performed by Stewart et. al., has shown that quercetin
supplementation may not improve insulin sensitivity (67). In Stewart’s study, over a period of 8 weeks, mice consumed HFDs supplemented with or without 1.2% quercetin. Insulin signaling in various tissues was examined using euglycaemic-hyperinsulinaemic clamps at 3 and 8 weeks. The researchers found that quercetin did not successfully mitigate IR. Unfortunately, inflammatory markers were not measured in this particular study, thus the authors were unable examine the link between quercetin supplementation, IR, and inflammation.

**Limitations of Previous Studies**

Although there is an overabundance of studies that have examined the influence of high-fat feeding on various physiological markers, they are many limitations to these investigations. Such limitations include: 1) the utilization of a single high-fat diet, 2) the utilization of a diet with greater than 50% of total calories from fat and or greater than 30% of total calories from saturated fat, 3) lack of control for various nutrients (e.g. ratio of MUFA:PUFA; omega-6:omega-3 FAs; protein:carbohydrate:fat, etc.), 4) and utilization of a single ingredient as the sole source of dietary fat (e.g. corn oil, beef tallow, milk fat, etc.).

The reader will notice that the majority of previous studies have been limited by lack of control for various factors related to diet. This is the exact reason why the diets that will be utilized in this dissertation have been carefully selected and manipulated to not only mimic the composition of the Standard American Diet, but also to control for as many potential outcome-influencing factors as possible.
References


Chapter II

Influence of Dietary Saturated Fat Content on Adiposity, Macrophage Behavior, Inflammation, and Metabolism: Composition Matters

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ABSTRACT

We examined the effects of three high-fat diets differing in the percentage of total calories from saturated fat (SF) (6%, 12%, and 24%), but identical in total fat (40%), on body composition, macrophage behavior, inflammation, and metabolic dysfunction in mice.

Diets were administered for 16 weeks. Body composition and metabolism (glucose, insulin, triglycerides, LDL-C, HDL-C, total cholesterol) were examined monthly.

Adipose tissue (AT) expression of marker genes for M1 and M2 macrophages and inflammatory mediators (TLR-2, TLR-4, MCP-1, TNF-α, IL-6, IL-10, SOCS1, IFN-γ) was measured along with activation of NFκB, JNK and p38-MAPK. AT macrophage infiltration was examined using immunohistochemistry. Circulating MCP-1, IL-6, adiponectin, and leptin were also measured. SF content, independent of total fat, can profoundly affect adiposity, macrophage behavior, inflammation, and metabolic dysfunction. In general, the 12%-SF diet, most closely mimicking the standard American diet, led to the greatest adiposity, macrophage infiltration, and insulin resistance (IR), whereas the 6%-SF and 24%-SF diets produced lower levels of these variables with the 24%-SF diet resulting in the least degree of IR and the highest TC/HDL-C ratio.

Macrophage behavior, inflammation and IR following HFDs are heavily influenced by dietary SF content, however, these responses are not necessarily proportional to the SF%.

Keywords: Saturated Fat, High-Fat Diet, Macrophages, Inflammation, Obesity
INTRODUCTION

According to the World Health Organization, it is estimated that 1.5 billion people worldwide are overweight and at least 500 million are obese (1). Consequently, there has been a rise in obesity-related health problems including, cardiovascular disease, the metabolic syndrome, diabetes, degenerative diseases, and cancer (1, 2). Genetic predispositions, physical inactivity, and consumption of a high-fat diet (HFD) can all lead to the development of obesity. However, given the global acceptance and availability of energy dense foods, chronic ingestion of diets high in fat is arguably the leading contributor. As a result, there has been a major emphasis on understanding the link between high-fat-diet-induced obesity and chronic disease risk. In this context, low-grade chronic inflammation has emerged as a key pathogenic link (68-70).

It is now widely accepted that high-fat-diet-induced obesity can lead to a chronic state of low-grade inflammation. This is largely mediated through quantitative and functional alterations in white adipose tissue macrophages (ATMs) (71, 72). For example, it has been reported that approximately 45-60% of adipose tissue (AT) cells express the macrophage marker EMR1 (F4/80) in obese mice, whereas only 10-15% of cells from lean mice express this marker (71). In addition, ATMs exhibit a pro-inflammatory, classical phenotype (M1) in obese mice, while those from lean mice have an alternatively-activated, anti-inflammatory phenotype (M2) (72). These changes not only lead to increased inflammation but also to dysregulation of metabolic homeostasis; infiltration and polarization of macrophages in AT has been linked to lower plasma adiponectin levels as well as insulin and leptin resistance (4, 6, 7, 73). While the association between high-fat-diet-induced obesity and macrophage-mediated
inflammation has been clearly recognized, there is a fundamental gap in understanding the relative contribution of different types of fatty acids (FAs) to these responses.

Saturated fatty acids (SFAs) have received the most attention for their ability to influence pro-inflammatory processes in high-fat-diet-induced obesity. These effects are thought to be largely mediated by their capacity to serve as ligands for toll-like receptor 2 and 4 (TLR-2 and TLR-4); binding of SFAs to TLR-2 and/or TLR-4 on various cell types, in particular macrophages and adipocytes, results in the induction of pro-inflammatory gene transcription via activation of nuclear factor kappa-B (NFκB), the c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK) signaling cascades (7, 35, 74). Consistent activation of these pathways results in a chronic state of inflammation and subsequent insulin resistance (IR) (7, 39, 40). In addition, SFAs, in general, are more obesogenic than other FAs; long-chain saturated fatty acids (LCSFAs, > C12:0) are not as efficiently oxidized as unsaturated fatty acids (USFAs), and thus are more likely to be stored as AT (41, 42). Given the preponderance of evidence that supports a role of saturated fat (SF) on macrophage-mediated inflammation and metabolic dysfunction in high-fat-diet-induced obesity, it is surprising that there have been no dose response studies to more clearly evaluate their specific role in these processes.

The purpose of this study was to examine the effects of three HFDs differing in the percentage of total calories from SF (6%, 12%, and 24% of total caloric intake), but identical in total fat (40%), on body composition, macrophage behavior, inflammation, and metabolic dysfunction in mice. We hypothesized that high dietary fat intake would
increase adiposity, macrophage infiltration, inflammation, IR, and impair the lipid profile, and that these effects would be augmented as the percentage of SF increased.

METHODS

Animals

Male C57BL/6 mice were bred and cared for in the animal facility at the University of South Carolina. They were housed, four-five per cage, maintained on a 12:12-h light-dark cycle in a low stress environment (22°C, 50% humidity, low noise) and given food and water ad libitum. Principles of laboratory animal care were followed, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Diets

At four weeks of age, mice were randomly assigned to 1 of 5 treatment diets (n=8-9/group): two control diets (AIN-76A, AIN-76A Mod) and three HFDs (6%-SF, 12%-SF, and 24%-SF) (BioServ, Frenchtown, NJ) (Table 2.1). The percentage of calories provided by each of the three macronutrients, the ratio of polyunsaturated:monounsaturated FAs (PUFA:MUFA), and the ratio of omega-6:omega-3 FAs were identical for the HFDs and were designed to be similar to the standard American diet (17, 18). The second control diet (AIN-76A Mod) was used in order to match the PUFA:MUFA and omega-6:omega-3 ratios of the HFDs.
Body weights, food intake, and body composition

Body weight and food intake were monitored weekly. Body composition was assessed every four weeks (weeks 4, 8, 12, 16, and 20). For this procedure, mice were placed under brief anesthesia (isoflurane inhalation) and were assessed for lean mass, fat mass, and body fat percentage via dual-energy x-ray absorptiometry (DEXA) (Lunar PIXIImus, Madison, WI).

Metabolism

Plasma was assessed for fasting concentrations of glucose, insulin, total cholesterol (TC), HDL-C, and triglycerides at weeks 8, 12, 16 and 20 and for LDL-C at weeks 16 and 20. Blood samples were collected from the tip of the tail after a five-hour fast. Blood glucose concentrations were determined in whole blood using a glucometer (Bayer Contour, Michawaka, IN). Collected blood was centrifuged and plasma was aliquoted and stored at -80°C until analysis. Insulin concentrations were determined using an ELISA kit (Mercodia, Uppsala, Sweden) and colorimetric kits were used for plasma triglycerides (Pointe Scientific, Canton, Michigan), TC, HDL-C, and LDL-C (Genzyme, Kent, United Kingdom). Insulin resistance was estimated by HOMA index as follows: insulin resistance index = fasting insulin (μU/ml) x fasting glucose (mmol/l)/22.5 (75).

Tissue collection
At 20 weeks of age, mice were sacrificed for tissue collection. Epididymal, mesentery, and retroperitoneal fat pads were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin until analysis. Blood was collected from the inferior vena cava using heparinized syringes, and centrifuged at 4,000 rpm for 10 min at 4°C. Plasma was aliquoted and stored at −80°C.

**Adipocyte size and F4/80 immunohistochemistry**

At sacrifice, a portion of epididymal AT was excised from each mouse, fixed overnight in 10% formalin, dehydrated with alcohol and embedded in wax. Paraffin sections were stained with hematoxylin and eosin (H&E). The surface area of 100 adipocytes was determined (manual trace) and then averaged to represent mean adipocyte size for each mouse using Infinity Analyze software (Lumenera, Ottawa, ON). F4/80 staining was performed in epididymal AT using rat monoclonal antibody (Serotec, Raleigh, NC). Color detection was visualized with a Vectastain avidin-biotinylated enzyme complex detection kit (R&D Systems, Minneapolis, MN), and 3,3′-diaminobenzidine followed by counterstaining with hematoxylin.

**Western blots**

Epididymal AT was homogenized in radioimmunoprecipitation buffer (Sigma, St. Louis, MO), which included a protease inhibitor cocktail (Sigma, St. Louis, MO), and 1% glycerophosphate (100x), 0.5% sodium orthovanadate (1 mM), and 1% sodium fluoride (5 mM). The protein concentration was determined by the Bradford method (76). Western blots were performed as previously described using primary antibodies for
phosphorylated (Ser536) and total NFkB p65, phosphorylated (Thr183/Tyr185) and total JNK (Cell Signaling, Danvers, MA), and phosphorylated (Tyr182) and total p-38 MAPK (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (77). As there were no differences in the activation of any of the measured proteins between the two control diets (AIN-76A and AIN-76A Mod), these samples were combined to represent the “control” diets for these analyses.

**Gene expression**

Epididymal AT was homogenized under liquid nitrogen using a polytron and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy mini-spin columns (Qiagen, Valencia, CA). Quantification of epididymal AT gene expression for F4/80, CD11c, CD206, MCP-1, IL-6, SOCS1, TLR-4, TLR-2, TNF-α, IFN-γ and IL-10 (Applied Biosystems, Foster City, CA) were performed as previously described (78).

**Concentration of circulating markers of obesity and inflammation**

Plasma concentrations of leptin, adiponectin, IL-6, and MCP-1 were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

**Statistical analysis**

All data were analyzed using commercial software (SigmaStat, SPSS, Chicago, IL). Body weight, body composition outcomes, metabolic outcomes, the TC:HDL-C ratio, and the HOMA index were analyzed using a repeated measures two-way ANOVA. All other data were analyzed using a one-way ANOVA. Student-Newman-Keuls test
was used for all post-hoc analyses. Statistical significance was set with an alpha value of $P \leq 0.05$. Data are presented as mean ($\pm$SEM).

RESULTS

12%-SF consumption leads to heavier body weights, larger adipocyte size, and greater fat mass than any other diet

Body weights, fat pad weights, and average adipocyte size are presented in Figure 2.1. The mice consuming the 12%-SF diet had significantly elevated body weights compared to control-diet-fed mice starting at 11 weeks of age ($P \leq 0.05$), but this effect began later for 6%-SF and 24%-SF-fed mice (weeks 12 and 13 compared to AIN-76A and AIN-76A Mod, respectively) ($P \leq 0.05$). Further, the 12%-SF-fed mice had greater body weights than both 6%-SF-fed mice (starting at week 15) and 24%-SF-fed mice (starting at week 17) ($P \leq 0.05$).

For all fat-pad depots, the HFD-fed mice had enhanced fat mass compared to control-diet-fed mice ($P \leq 0.05$). Although there was no difference among HFDs with respect to total visceral AT, the 12%-SF-fed mice had a greater mesentery fat weight than both the 6%-SF and 24%-SF-fed mice ($P \leq 0.05$).

As expected, HFD consumption increased adipocyte size compared to control-diet consumption ($P \leq 0.05$). When comparing across HFD-fed mice, the 12%-SF-fed mice had the largest adipocyte size followed by 6%-SF and 24%-SF-fed mice, respectively ($P \leq 0.05$).

Body composition analysis revealed a significant difference among groups ($P \leq 0.05$) (Table 2.2). Specifically, the 12%-SF-fed mice had a greater body fat percentage
compared to control-diet-fed mice (starting at 12 weeks) and 24%-SF-fed mice (16 weeks) (P≤.05). However, the 24%-SF and 6%-SF-fed mice did not differ from control-diet-fed mice until week 16. There were no significant differences in body fat percentages across the HFD-fed mice at 20 weeks-(P≤.05). On the other hand, at week 20, the 12%-SF-fed mice did have a greater fat mass than the 24%-SF and 6%-SF-fed mice (P≤.05). In general, lean mass increased over time for all groups, and by week 16, the HFD-fed mice exhibited greater lean mass compared to control-diet-fed mice (P≤.05).

It was not possible to calculate individual food intake as mice were housed 4-5/cage. However, in general, we did not observe any differences, among the HFD-fed mice, in weekly food intake (food consumed by mice in each cage/number of mice in cage) over the course of the study.

**ATM infiltration is greatest in 12%-SF-fed mice**

Expression of F4/80, CD11c (M1) and CD206 (M2) was greater with the consumption of the 12%-SF diet compared to all other diets (P≤.05), with the exception of no difference in CD206 between the 12%-SF and 6%-SF-fed mice (Figures 2.2A-C). Interestingly, we did not detect a difference in TLR-4 expression across the groups (data not shown), however, TLR-2 expression (Figure 2.2D) was increased with the consumption of the 12%-SF diet compared to all other diets, except the 6%-SF diet (P≤.05). We next confirmed inflammation and macrophage infiltration in AT via H&E and immunohistochemistry staining of F4/80, respectively. The 12%-SF-fed mice showed increased inflammation (Figure 2.2E) and accumulation of macrophages in the AT (Figure 2.2F) compared to control-diet-fed mice. Although the 6%-SF and 24%-SF-
fed mice also exhibited increased inflammation and macrophage infiltration, these effects were not as pronounced as in 12%-SF.

**Adipose tissue inflammation is influenced by the content of dietary fat**

There was no difference in the activation of p-38 MAPK among any of the diets (Figure 2.33A). Alternatively, the activation of JNK, was significantly increased in all HFDs (P<.05) (Figure 2.33B). However, only 12%-SF exhibited a higher degree of NFκB p65 activation (P<.05) (Figure 2.3C).

Consumption of all HFDs led to increased expression of MCP-1 in epididymal AT compared to control diets (P<.05) (Figure 2.4A). TNF-α, was increased in 6%-SF and 12%-SF-fed mice only (P<.05) (Figure 2.4B), but there were no differences in IL-6 (Figure 2.4C) or IFN-γ (Figure 2.4D) expression among any of the groups. Interestingly, SOCS1 expression was significantly decreased in the 24%-SF-fed mice only (P<.05) (Figure 2.4E). Regarding the anti-inflammatory cytokine, IL-10, we found increased expression in the 12%-SF-fed mice only (P<.05) (Figure 2.4F).

**Circulating leptin is greatest with the 12%-SF diet**

For leptin, not only did all HFDs exhibit significantly elevated plasma concentrations compared to control diets, but there were also differences among HFDs; the 12%-SF-fed mice had increased leptin levels compared to 6%-SF and 24%-SF-fed mice (P<.05) (Figure 2.5D). However, there were no significant differences across the groups for circulating levels of IL-6, MCP-1, or adiponectin (Figures 2.5A-C).
The 12%-SF diet leads to the greatest IR, followed by 6%-SF and 24%-SF diets, respectively.

Beginning at week 16, all three HFDs produced higher fasting blood glucose concentrations compared to control diets (P≤0.05), but by 20 weeks, only the 6%-SF and 12%-SF-fed mice had elevated fasting blood glucose concentrations (P≤0.05) (Table 2.3). And in fact, the 12%-SF-fed mice exhibited a higher fasting blood glucose in comparison with the 24%-SF-fed mice at this time (P≤0.05).

All HFD-fed mice had elevated insulin levels compared to control-diet-fed mice at 16 and 20 weeks (P≤0.05). However, at 16 weeks the 12%-SF-fed mice had increased levels compared to the 6%-SF and 24%-SF-fed mice (P≤0.05), and at 20 weeks, both the 6%-SF and 12%-SF-fed mice had elevated levels versus the 24%-SF-fed mice (P≤0.05).

Similar to insulin, HFD-fed mice had a greater HOMA index than control-diet-fed mice at 16 weeks (P≤0.05). However, at week 20, not only were the HFD groups different from the control-diet groups, but they were different from each other; the 12%-SF groups had the greatest HOMA score, followed by 6%-SF and 24%-SF groups (P≤0.05).

Changes in lipid profile are influenced by SF content

The 12%-SF-fed mice had elevated TC compared to control-diet-fed mice at week 8 (P≤0.05). By week 12 and 16, the 6%-SF and 12%-SF-fed mice had increased levels compared to the AIN-76A-fed mice (week 12) and AIN-76A-Mod-fed mice (week 16), respectively (P≤0.05). All HFD-fed mice had a greater plasma concentration of TC versus the control-diet-fed mice at 20 weeks (P≤0.05), but within the HFD groups, the 12%-SF group had significantly greater TC versus the 24%-SF group (P≤0.05).
In general, the plasma HDL-C concentration tended to be highest with the consumption of the 24%-SF diet. Statistically, mice consuming the 24%-SF diet exhibited significantly elevated HDL-C levels compared to the control-fed mice (week 8), 6%-SF-fed mice (week 16), and 12%-SF-mice (week 8, 16 and 20) (P≤.05). Interestingly, however, there were no significant changes in HDL-C concentrations for any of the groups over time. Both the 6%-SF and 12%-SF-fed mice had a greater TC/HDL-C ratio than all other mice at week 20 (P≤.05). LDL-C, measured at weeks 16 and 20 only, was elevated in all HFD-fed mice compared to control-diet-fed mice (P≤.05). Differences in triglycerides across the groups were detected at 20 weeks only; the 24%-SF-fed mice and AIN-76A-Mod-fed mice had the highest and lowest levels of triglycerides, respectively (P≤.05)

DISCUSSION

HFDs are strongly linked with the accumulation of excess body fat, chronic inflammation, and metabolic perturbations, ultimately leading to poorer health outcomes. It is well known that ATMs play a central role in this relationship (79). However, the extent to which the FA composition of a HFD influences macrophage behavior and inflammation is still poorly understood; most of the available supporting literature is limited by the lack of control for various nutrients (e.g. ratio of MUFA:PUFA; omega-6:omega-3 FAs; protein:carbohydrate:fat, etc.), the utilization of a single ingredient as the sole source of dietary fat (e.g. corn oil, beef tallow, milk fat, etc.) and the absence of dose response studies. We examined the effect of three HFDs differing in the percentage of total calories from SF (6%, 12%, and 24%), but identical in total fat (40%), on adiposity
(absolute fat mass), macrophage phenotype, inflammation, and metabolism utilizing controlled diets consisting of various lipid-rich ingredients. Our findings indicate that manipulating the SF content, without changing the percentage of total calories from fat, has a profound effect on these outcomes. The 12%-SF diet, most closely mimicking the standard American diet, led to the greatest adiposity (absolute fat mass), macrophage infiltration, and IR. Although the 24%-SF diet increased adiposity and produced IR, it did not significantly increase macrophage infiltration, it led to a lesser degree of AT inflammation, and it did not raise the TC/HDL-C ratio.

Given that SFAs, in general, are less efficiently oxidized than USFAs, we hypothesized that adiposity would be greatest following consumption of the 24%-SF diet (41, 42). Interestingly, despite the fact that HFD-fed mice consumed similar kcals, the 12%-SF diet led to the greatest accumulation of fat and the largest adipocyte size followed by the 6% and 24%-SF groups, respectively. We also confirmed that plasma leptin was proportional to the degree of fat-mass accumulation as previously reported (6).

The FA composition of AT is primarily dependent on the FA composition of the diet (80). Therefore, lipolysis of white adipose tissue (WAT) that is comprised of a greater proportion of SFAs should lead to higher levels of circulating saturated free-fatty acids and subsequent activation of macrophages through binding to TLRs (7, 74). Given this, we expected that a diet higher in SF content would lead to greater macrophage infiltration and more pronounced inflammation. In agreement with previously reported literature (29), all HFDs increased expression of F4/80 as well as markers for M1 and M2 macrophages; interestingly, however, these reached statistical significance only in the 12%-SF group. The activation of macrophages in AT is thought to be mediated by TLRs;
both TLR-2 and TLR-4 have been implicated in HFD-induced macrophage activation and inflammation given their ability to bind saturated free-fatty acids (74, 81, 82). Consistent with the macrophage data, we show a statistically significant increase in TLR-2 expression only in the 12%-SF-fed mice, but surprisingly there was no significant up-regulation of TLR-4 in any of the HFD groups. To our knowledge, there have been no reports of an up-regulation of WAT TLR-2 expression without significant changes in WAT TLR-4 expression following HFD feedings. These results warrant further investigation into the role that individual TLRs play in HFD-induced inflammation, and conversely, the effect that varying the composition of HFDs has on TLR activation.

Because macrophages are thought to mediate their inflammatory processes through activation of various transcription factors (7, 35, 74), we next examined phosphorylated NFκB, JNK, and p38 MAPK and found that all three HFDs increased activation of JNK, whereas only the 12%-SF diet significantly increased NFκB activation. All HFD-fed mice exhibited an increase in mRNA expression of MCP-1 in AT, and the 6%-SF and 12%-SF-fed mice, but not the 24%-SF-fed mice, exhibited an increase in TNF-α mRNA expression. Surprisingly, IFN-γ, that has been shown to be up-regulated in WAT and play a key role in macrophage activation in HFD-induced obesity (83), was not statistically different across diets. This may be due to the specific measurement time-point, the composition of these novel diets, or most likely, an increase in factors that can regulate expression of IFN-γ. Additionally, we found no changes in AT IL-6 mRNA expression across groups. Although previous studies have shown AT IL-6 mRNA expression to be up-regulated as a result of high-fat feeding (84, 85), others have shown that this is not always the case (86). We also measured IL-10, an anti-
inflammatory cytokine, and found it to be up-regulated in the 12%-SF mice only, which is most likely a compensatory response to the increased inflammation (87). Interestingly, SOCS1, a negative regulator of inflammation (88), appeared to be down-regulated in all the HFDs but this reached significance in the 24%-SF group only. Circulating markers of inflammation (MCP-1, IL-6) were measured to determine whether they mirrored the observed changes in WAT as has previously been reported (85, 89). We found no significant increases in plasma MCP-1 or IL-6 for any of the HFDs. The most likely explanation for the discrepancies between our findings and those of others is the differences in the composition of the diets used.

Emerging evidence suggests that pro-inflammatory M1 macrophages may play a role in inducing IR, whereas alternatively activated M2 macrophages may help to maintain insulin sensitivity (84). Our data somewhat supports this hypothesis as the degree of M1 macrophage mRNA expression corresponds well with the level of IR; the 12%-SF diet resulted in the most severe IR, followed by the 6%-SF and 24%-SF diets, respectively. However, given that M1 macrophage quantification in this study was limited to mRNA expression of CD11c this association should be interpreted with caution. Adiponectin, well characterized as an anti-inflammatory adipocytokine, known to promote insulin sensitivity, has been shown to be inversely correlated with body fat accumulation (6). Interestingly, there were no changes in the concentration of plasma adiponectin across groups. It is likely that changes in adiponectin would have been observed if it had been measured in the WAT (90), however, our analysis was limited to plasma levels. Concerning lipid metabolism, all three HFDs increased TC and LDL-C levels. Of interest was the finding that although the 24%-SF diet increased LDL-C and
elevated plasma triglycerides, it also resulted in a higher HDL-C level, producing a more favorable TC/HDL-C similar to that of control diets.

A possible explanation for the discrepancies in adiposity (absolute fat mass), IR, inflammation, and the TC/HDL-C ratio between the 12%-SF-fed mice and the 24%-SF-fed may be the difference in the content of medium-chain fatty acids (C8:0-C12:0) (MCFAs) (3.6%, and 11.8% of total caloric intake for the 12%-SF, and 24%-SF diets, respectively). These variations exist as it was not possible for the composition of the SF in each of the HFDs to be consistent while utilizing various lipid-rich ingredients and simultaneously controlling for the omega-6:omega-3 and MUFA:PUFA ratios. A previous study reported that a diet composed of 12% caprylic (C8:0) and capric acid (C10:0), both MCFAs, augments the rate of fat mass loss compared to a diet composed of 12% olive oil that contains mostly MUFAs (91). Furthermore, there is substantial evidence demonstrating that small doses of MCFAs and HFDs rich in MCFAs can effectively reduce body weight, fat mass gains, and minimize IR compared to HFDs rich in LCFAs (92-96). Additionally, MCFA-rich diets have been associated with an improved cholesterol profile (97-100) and reduced inflammation (101, 102) compared to other iso-caloric diets. The reported reduction in body weight produced by MCFAs is associated with higher energy expenditure and up-regulated FA oxidation (103-105). As a result of their shorter chain length, MCFAs can be transported from the intestines directly to the liver where they can be quickly oxidized (92, 106). LCFAs, on the other hand, are first incorporated into chylomicrons before they leave the intestine via the lymphatic system and travel through the blood to extra-hepatic tissues to be stored or metabolized. Not only do MCFAs and LCFAs differ in their digestive routes, but their
propensity to be oxidized is also dissimilar (41). Once inside a cell, MCFAs are less likely to be stored as AT as they can enter the mitochondria to be oxidized independent of carnitine palmitoyltransferase 1, unlike LCFAs.

It is important to point out that the MCFA argument may not be valid when comparing the 6%-SF and 12%-SF diets (.1% versus 3.6% of total calories from MCFAs, respectively). It is likely that the MCFA-caloric content of the diet would need to be higher to produce similar effects as generated by the 24%-SF diet. The differences in adiposity we did observe between the 6%-SF and 12%-SF-fed mice may be due to the fact that the 12%-SF diet was composed of more obesogenic LCSFAs (8.4% vs. 5.9% of total calories). However, we find this unlikely as the 24%-SF diet had the largest percentage of LCSFAs (12.2% of total calories) and resulted in a similar level of adiposity as the mice consuming the 6%-SF diet.

Another possible rationale for the differences in IR and inflammation across the diets may be the disparities in the linoleic content (C18:2), (11.4%, 9.4%, and 5.4% of total caloric intake for the 6%-SF, 12%-SF, and 24%-SF diets, respectively). Even when controlling for various ratios within an iso-caloric diet, the manipulation of one macronutrient, or sub-set of macronutrients, results in an uncontrollable alteration of another. As such, in the current study, alterations in the percentage of SF across diets also resulted in changes in the percentage of unsaturated fat. Thus, although the ratio of omega-6:omega-3 FAs was the same for each of the HFDs, the absolute quantity of linoleic acid in the 6%-SF and 12%-SF diets was greater than in the 24%-SF diet. Linoleic acid serves as a short-chain, parent omega-6 FA necessary for the synthesis of essential omega-6 LCFAs (107) that can play an important role in the promotion of
inflammatory processes through the production of various eicosanoids (43, 107, 108). In fact, others have shown that a HFD rich in omega-6 FAs can increase inflammation more so than a HFD rich in SFAs (109). Further, omega-6 FAs are prone to peroxidation leading to accumulation of 4-Hydroxy-2-nonenal (4-HNE), which has been shown to induce IR (110).

It is also likely that the differences in MCFAs, LCFAs and linoleic content across the diets can explain some of the other unexpected reported findings. For instance, the discrepancies in MCFA and linoleic content between the 6%-SF and 24%-SF diets may explain the similar adiposity but different levels of IR seen in these groups; previous work has shown that MCFA-rich diets can hinder the development of IR without influencing body weight (111). And it may be that the greater content of linoleic acid in the 6%-SF diet produced significantly more 4-HNE leading to a greater IR compared to the 24%-SF diet. Also, it is certainly possible that the relatively high content of both linoleic acid and LCSFAs in 12%-SF diet may explain the high degree of adiposity, macrophage infiltration and IR compared to the 6%-SF diet. Clearly, the disparate findings across the three HFDs do not result from variations of a single group of FAs, but instead stem from alterations in the content of several classes of FAs.

It is well established that SFAs play a role in inflammatory signaling (7, 35, 112, 113). However, the degree to which individual FAs activate pro-inflammatory pathways remains somewhat controversial as there has been at least one study to report that SFAs do not activate TLRs in vitro (114). Further, it is evident from our findings as well as from previous research that varying the composition of dietary SFAs can differentially regulate inflammatory processes in vivo as compared to in vitro. For example, Lee and
others have shown that lauric acid (C12:0), a MCFA that varies considerably with increasing percentage of SF in our diets, can serve as a potent agonist of TLR signaling *in vitro* (7, 35). On the contrary, Rivera et. al. (101) reported that a MCFA-rich diet more effectively attenuated non-alcoholic steatohepatitis and reduced hepatic TLR-4 expression versus a PUFA-rich diet. These convergent findings may be explained by the inclination of lauric acid to be oxidized *in vivo*, thus limiting its ability to serve as a ligand for TLRs (41). This highlights the importance of additional research to better understand the role of various SFAs on inflammatory-related signaling, and further, whether the effects observed *in vitro* are actually reflected *in vivo*.

Additionally, our data, and that of others, suggests that iso-caloric diets with a greater content of omega-6 PUFAs can produce greater IR, result in a poorer TC/HDL-C ratio, and may even increase inflammation (101, 109) more so than an iso-caloric diet composed of significantly more SF. Meanwhile, others have shown that high consumption of omega-6 FAs has been associated with reduced inflammation, a more favorable TC/HDL-C ratio (109) and no negative effects on inflammatory markers (115, 116). It should be noted, however, that the majority of these studies were performed in humans, where it is extremely difficult to control for the nutrient composition of the diet and the activity level of the subjects – two factors known to greatly influence metabolism and inflammation (117, 118). This affirms the need for future research utilizing various controlled diets to better understand the role that omega-6 FAs have on physiological processes.

In summary, we examined the influence of three 40% HFDs differing in the percentage of total calories from SF (6%, 12% and 24%) on body composition,
macrophage behavior, inflammation, and metabolic dysfunction in mice. In general, the 12%-SF diet, most closely mimicking the standard American diet, led to the greatest adiposity, macrophage infiltration, and IR, whereas the 24%-SF diet had the lowest levels of these outcomes. In conclusion, our findings support previously published data that ad libitum, high-fat feeding can lead to an increased risk of obesity and obesity-related side effects. However, the extent of excess fat accumulation and adverse health perturbations is not necessarily proportional to the percentage of SF in the diet. Although SFAs and omega-6 FAs have been implicated as pro-inflammatory molecules, future research should examine the degree to which diets differing in SFA and omega-6 FA content impact individual TLR activation, macrophage behavior, inflammatory signaling and subsequent metabolic dysfunction as well as the effect that manipulating the absolute quantities of omega-6 and omega-3 FAs, without changing the ratio between these FAs, has on these outcomes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES


### FIGURES

**Table 2.1** Diet composition of treatment diets. SFAs, Saturated Fatty-Acids; MCSFAs, Medium-Chain Saturated Fatty Acids; LCSFAs, Long-Chain Saturated Fatty Acids; USFAs, Unsaturated Fatty Acids; MUFAs, Monounsaturated Fatty Acids; PUFAs, Polyunsaturated Fatty Acids.

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<th>12% Saturated Fat Diet</th>
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<td>10</td>
<td>10</td>
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<td>Mineral Mix (AIN-76A)</td>
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<td>35</td>
<td>35</td>
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<tr>
<td><strong>Energy (kcal/g)</strong></td>
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<td>4.57</td>
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<td><strong>Energy (% kcal)</strong></td>
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<td>Carbohydrate</td>
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<tr>
<td>Protein</td>
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<td>19.1</td>
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<td>13</td>
<td>13</td>
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<tr>
<td><strong>Fatty Acid Profile (g/kg)</strong></td>
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<td>Caprylic Acid (C8:0)</td>
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<td>0</td>
<td>.075</td>
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<td>0</td>
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<td>.45</td>
<td>13.5</td>
<td>43.3</td>
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<td>.23</td>
<td>5.5</td>
<td>17.1</td>
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<tr>
<td>Palmitic Acid (C16:0)</td>
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<td>5.5</td>
<td>22.7</td>
<td>26</td>
<td>28.3</td>
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<tr>
<td>Palmitoleic Acid (C16:1)</td>
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<td>.4</td>
<td>1.7</td>
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<td>Stearic Acid (C18:0)</td>
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<td>52.9</td>
<td>43.2</td>
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<td>α-Linolenic Acid (C18:3)</td>
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<td>Arachidic Acid (C20:0)</td>
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<td>0</td>
</tr>
<tr>
<td><strong>% of Total Calories from SFAs (%)</strong></td>
<td>1.4%</td>
<td>1.7%</td>
<td>6%</td>
<td>12%</td>
<td>24%</td>
</tr>
<tr>
<td><strong>% of Total Calories from MCFAs (C6:0-C12:0) (%)</strong></td>
<td>-</td>
<td>-</td>
<td>.1%</td>
<td>3.6%</td>
<td>11.8%</td>
</tr>
<tr>
<td><strong>% of Total Calories from LCSFAs (C14:0-C20:0) (%)</strong></td>
<td>1.4%</td>
<td>1.7%</td>
<td>5.9%</td>
<td>8.4%</td>
<td>12.2%</td>
</tr>
<tr>
<td><strong>% of Total Calories from USFAs (%)</strong></td>
<td>10.8%</td>
<td>10.5%</td>
<td>34%</td>
<td>28%</td>
<td>16%</td>
</tr>
<tr>
<td><strong>% of Total Calories from MUFAs (%)</strong></td>
<td>3.6%</td>
<td>7%</td>
<td>22.6%</td>
<td>18.6%</td>
<td>10.6%</td>
</tr>
<tr>
<td><strong>% of Total Calories from PUFAs (%)</strong></td>
<td>7.2%</td>
<td>3.5%</td>
<td>11.4%</td>
<td>9.4%</td>
<td>5.4%</td>
</tr>
<tr>
<td><strong>% of Total Calories from n-3 FAs (%)</strong></td>
<td>.15%</td>
<td>.16%</td>
<td>.53%</td>
<td>.45%</td>
<td>.25%</td>
</tr>
<tr>
<td><strong>% of Total Calories from n-6 FAs (%)</strong></td>
<td>7.0%</td>
<td>3.2%</td>
<td>10.8%</td>
<td>8.9%</td>
<td>5.1%</td>
</tr>
<tr>
<td>Cholesterol (mg/kg)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>34</td>
<td>65</td>
</tr>
<tr>
<td>Ratio: MUFAs:PUFA</td>
<td>1:2</td>
<td>2:1</td>
<td>2:1</td>
<td>2:1</td>
<td>2:1</td>
</tr>
<tr>
<td>Ratio: n-6:n-3 FA</td>
<td>45:1</td>
<td>20:1</td>
<td>20:1</td>
<td>20:1</td>
<td>20:1</td>
</tr>
</tbody>
</table>
Figure 2.1 The effect of consuming HFDs differing in saturated fat composition on (A) weekly mean body weight, (B) retroperitoneal (Rp), mesentery (Mes), epididymal (Epi), and total visceral (Total) fat pad weights, and (C) adipocyte size at sacrifice (n=8-9). Diets not sharing a common letter differ significantly from one another (P≤.05). *Significantly different from AIN-76A (week 11: 12%-SF only, weeks 12-20: 6%-SF, 12%-SF, and 24%-SF diets, P≤.05). &Significantly different from AIN-76A Mod (weeks 11-12: 12%-SF only, weeks 13-20: 6%-SF, 12%-SF, and 24%-SF diets, P≤.05). %Significantly different from 6%-SF (weeks 15, 17-20: 12%-SF only, P≤.05). $Significantly different from 24%-SF (weeks 17-20: 12%-SF only, P≤.05).
Table 2.2 Body composition including, fat mass, lean mass, and body fat % of mice assessed at baseline (4 weeks) and incrementally (weeks 8, 12, 16 and 20) throughout the course of the study (n=8-9). Values not sharing a common letter (abcd) differ significantly over time within the given diet treatment (P≤.05). Values not sharing a common symbol (&%^) differ significantly among diet treatments within the given week (P≤.05).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Week 4</th>
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<th>Week 12</th>
<th>Week 16</th>
<th>Week 20</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>1.6±.10^a</td>
<td>4.1±.18^a</td>
<td>5.0±.41^b</td>
<td>5.1±.40^b</td>
<td>5.9±.57^ab</td>
</tr>
<tr>
<td>10:1</td>
<td>1.6±.11^a</td>
<td>5.9±.43^b</td>
<td>5.7±.58^b</td>
<td>6.4±.70^b</td>
<td>6.2±.71^b</td>
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<tr>
<td>5:1</td>
<td>1.6±.10^a</td>
<td>6.5±.53^b</td>
<td>7.4±.80^ab</td>
<td>11.9±1.3^c#</td>
<td>14.8±1.3^c#</td>
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<tr>
<td>1:1</td>
<td>1.6±.08^a</td>
<td>7.9±.78^b</td>
<td>8.7±.61^c</td>
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<table>
<thead>
<tr>
<th>Lean Mass (Grams)</th>
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<th></th>
<th></th>
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<tr>
<td>AIN-76A Mod</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>13.1±.43^a</td>
<td>16.5±.34^b</td>
<td>18.3±.56^c</td>
<td>19.0±.70^d#</td>
<td>19.7±.55^d#</td>
</tr>
<tr>
<td>10:1</td>
<td>12.6±.44^a</td>
<td>18.6±.37^b</td>
<td>18.6±.56^c</td>
<td>19.1±.60^d#</td>
<td>20.1±.56^d#</td>
</tr>
<tr>
<td>5:1</td>
<td>13.2±.52^a</td>
<td>19.4±.22^b</td>
<td>19.4±.51^c</td>
<td>21.2±.50^d#</td>
<td>22.3±.65^d#</td>
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<tr>
<td>1:1</td>
<td>12.7±.45^a</td>
<td>18.5±.48^b</td>
<td>20.5±.69^c</td>
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<td>24.0±.85^d</td>
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<table>
<thead>
<tr>
<th>Body Fat %</th>
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<td>AIN-76A Mod</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>11.0±.38^a</td>
<td>19.9±.75^b</td>
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<td>21.2±1.7^b#</td>
<td>22.9±1.9^b#</td>
</tr>
<tr>
<td>10:1</td>
<td>10.9±.57^a</td>
<td>23.9±1.5^b</td>
<td>23.2±1.8^c#</td>
<td>24.7±2.1^c#</td>
<td>23.0±2.1^c#</td>
</tr>
<tr>
<td>5:1</td>
<td>11.0±.35^a</td>
<td>24.9±1.6^b</td>
<td>27.1±2.1^c#</td>
<td>35.5±2.6^d#</td>
<td>39.4±2.1^d#</td>
</tr>
<tr>
<td>1:1</td>
<td>10.9±.36^a</td>
<td>29.4±1.7^b</td>
<td>29.6±1.6^c#</td>
<td>37.8±1.3^d#</td>
<td>41.0±1.3^d#</td>
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</tbody>
</table>

Body Fat %

53
Figure 2.2 Epididymal adipose tissue gene expression of (A) F4/80, (B) CD11c, (C) CD206, and (D) TLR-2 after 16 weeks of diet treatment (n=8-9). Diets not sharing a common letter differ significantly from one another (P≤.05) (n=8-9). Representative images of (E) H&E and (F) F4/80 staining of epididymal adipose tissue at a magnification of 20x for each of the treatment diets.
Figure 2.3  Representative western blots of (A) phosphorylated p38 MAPK (Tyr182)/total p38 MAPK, (B) phosphorylated JNK (Thr183/Tyr185)/total JNK, and (C) phosphorylated p65 NFκB (Ser536)/total p65 NFκB presented relative to control diets in epididymal adipose tissue following 16 weeks of diet treatment (n=5-6). Diets not sharing a common letter differ significantly from one another (P≤.05).
**Figure 2.4** Epididymal adipose tissue gene expression of (A) MCP-1, (B) TNF-α, (C) IL-6, (D) IL-10, (E) SOCS1, and (F) IFN-γ after 16 weeks of diet treatment (n=8-9). Diets not sharing a common letter differ significantly from one another (P≤.05).
Figure 2.5 Plasma concentrations of (A) IL-6, (B) MCP-1, (C) Adiponectin and (D) Leptin collected at sacrifice (n=8-9). Diets not sharing a common letter differ significantly from one another (P≤.05).
Table 2.3 Fasting metabolic panel assessed incrementally (weeks 8, 12, 16 and 20) throughout the course of the study (n=8-9). Values not sharing a common letter (abc) differ significantly over time within the group (P≤.05). Values not sharing a common symbol (&&##%) differ significantly among groups within the given week (P≤.05).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 16</th>
<th>Week 20</th>
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<td>9.1±0.7a</td>
<td>7.3±0.5b</td>
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<td>10.3±1.5</td>
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<td>10.4±0.7b#</td>
<td>12.0±1.1b#</td>
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<tr>
<td>AIN-76A Mod</td>
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<tr>
<td>6% SF</td>
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<td>12% SF</td>
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<td>AIN-76A Mod</td>
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<tr>
<td>6% SF</td>
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<tr>
<td>12% SF</td>
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<td>24% SF</td>
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<table>
<thead>
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<tr>
<td>AIN-76A Mod</td>
</tr>
<tr>
<td>6% SF</td>
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<tr>
<td>12% SF</td>
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<tr>
<td>24% SF</td>
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<table>
<thead>
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<tr>
<td>AIN-76A Mod</td>
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<td>6% SF</td>
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<table>
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<th>HDL-C (mmol/l)</th>
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<td>AIN-76A Mod</td>
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<td>6% SF</td>
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<tr>
<td>12% SF</td>
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<td>24% SF</td>
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<td>AIN-76A Mod</td>
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<tr>
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<td>24% SF</td>
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<table>
<thead>
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<td>AIN-76A Mod</td>
</tr>
<tr>
<td>6% SF</td>
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<tr>
<td>12% SF</td>
</tr>
<tr>
<td>24% SF</td>
</tr>
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</table>
Chapter III

Impact of Dietary Saturated Fat on Non-Alcoholic Fatty-Liver Disease

\textsuperscript{2} Enos, R. T., J. M. Davis, K. T. Velazquez, and E. A. Murphy. To be submitted to \textit{PLOS One}. 
ABSTRACT

We examined the effects of three high-fat diets differing in the percentage of total calories from saturated fat (SF) (6%, 12%, and 24%), but identical in total fat (40%), on the progression of Non-Alcoholic Fatty-Liver Disease. Diets were administered for 16 weeks. Hepatic lipid accumulation was assessed gravimetrically and by Oil Red O staining. Hepatic expression of marker genes for inflammatory mediators (F4/80, TLR-2, TLR-4, MCP-1, TNF-α, IL-6) was measured along with activation of NFκB, JNK and p38-MAPK. It was found that a diet’s SF content, independent of total fat, can greatly impact NAFLD progression. The 6%-SF and 12%-SF diets led to the greatest accretion of hepatic lipids, whereas the 24%-SF diet resulted in similar hepatic lipid accumulation as control diets. With regard to inflammation, in general, the 12%-SF diet led to the greatest degree of inflammation as evidenced by an increase in JNK activation and F4/80 and TLR-4 gene expression.

Keywords: Saturated Fat, High-Fat Diet, Non-Alcoholic Fatty-Liver Disease, Inflammation, Obesity
INTRODUCTION

Non-Alcoholic Fatty-Liver Disease (NAFLD) is characterized by evidence of hepatic steatosis with “no causes of secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication, or hereditary disorders” (1). The disease presents itself in 70-80% of diabetic and obese patients and is the principal cause of hepatological clinical referrals in the United States (1, 2). NAFLD is a single term describing two pathological conditions. The first condition, known as hepatic steatosis, is characterized by the accumulation of excess fat in the liver. Hepatic steatosis precedes the second condition, non-alcoholic steatohepatitis (NASH), which is typified not only by hepatic steatosis, but also by hepatic inflammation and hepatocellular damage.

Hepatic steatosis is a product of a long-term imbalance between caloric intake and energy expenditure. When caloric intake exceeds energy expenditure, excess energy is stored in the form of triglycerides in adipocytes. Although adipocytes have a significant capacity to store energy, a prolonged overindulgence in energy intake without a sufficient matching energy expenditure can result in adipose tissue dysfunction (3). Adipose tissue dysfunction is characterized by many factors including, an increase in adipocyte size and number, adipocyte secretion of pro-inflammatory cytokines, as well as the promotion of a pro-inflammatory environment and ectopic fat deposition, including the liver (3, 4). As lipids continue to accumulate in the liver, the liver eventually becomes insulin resistant, inflamed, and fibrotic (NASH), leading to an increased risk of mortality (1).

Mounting evidence suggests that HFDs promote NAFLD (5, 6). Further, it seems the type of fat consumed in the diet can greatly affect the development of NAFLD (2);
both *in vitro* and *in vivo* models provide evidence that diets higher in saturated fat induce endoplasmic reticulum (ER) stress, modulate mitochondrial metabolism, promote an inflammatory environment, and invoke liver damage (2, 7, 8). However, there have been no controlled studies, which have examined the effects of HFDs different in the percentage of saturated fat on NAFLD.

The purpose of this study was to examine the effects of three HFDs differing in the percentage of total calories from SF (6%, 12%, and 24% of total caloric intake), but identical in total fat (40%), on the development of NAFLD. We hypothesized that high dietary fat intake would lead to the development of NAFLD and this effect would be augmented as the percentage of SF increased.

**METHODS**

**Animals**

Male *C57BL/6* mice were bred and cared for in the animal facility at the University of South Carolina. They were housed, four-five per cage, maintained on a 12:12-h light-dark cycle in a low stress environment (22°C, 50% humidity, low noise) and given food and water *ad libitum*. Principles of laboratory animal care were followed, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

**Diets**

At four weeks of age, mice were randomly assigned to 1 of 5 treatment diets (n=8-9/group): two control diets (AIN-76A, AIN-76A Mod) and three HFDs (6%-SF,
12%-SF, and 24%-SF) (BioServ, Frenchtown, NJ) (Table 1). The percentage of calories provided by each of the three macronutrients, the ratio of polyunsaturated:monounsaturated FAs (PUFA:MUFA), and the ratio of omega-6:omega-3 FAs were identical for the HFDs and were designed to be similar to the standard American diet (9, 10). The second control diet (AIN-76A Mod) was used in order to match the PUFA:MUFA and omega-6:omega-3 ratios of the HFDs.

Tissue collection

At 20 weeks of age, mice were sacrificed for tissue collection. The liver was removed and immediately snap-frozen in liquid nitrogen and stored at -80°C.

Oil Red O Staining and Tissue Lipid Accumulation

Frozen 10 µm liver sections were cut using a cryostat (Leica Biosystems, Germany) set at -20°C. Hematoxylin and eosin (H&E) and oil red o (Sigma, St. Louis, MO) staining of the liver was performed as previously described (11). Lipids were isolated from the liver utilizing the Folch extraction method and were quantified gravimetrically (12).

Western Blots

The liver was homogenized in radioimmunoprecipitation buffer (Sigma, St. Louis, MO), which included a protease inhibitor cocktail (Sigma, St. Louis, MO), and 1% glycerophosphate (100x), 0.5% sodium orthovanadate (1 mM), and 1% sodium fluoride (5 mM). The protein concentration was determined by the Bradford method. Western
blots were performed as previously described using primary antibodies for phosphorylated (Ser536) and total NFκB p65, phosphorylated (Thr183/Tyr185) and total JNK (Cell Signaling, Danvers, MA), and phosphorylated (Tyr182) and total p-38 MAPK (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (13). As there were no differences in the activation of any of the measured proteins between the two control diets (AIN-76A and AIN-76A Mod), these samples were combined to represent the “control” diets for these analyses.

**Gene expression**

The liver was homogenized under liquid nitrogen using a polytron and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantification of hepatic gene expression for F4/80, MCP-1 (monocyte chemotactic protein-1), IL-6 (interleukin 6), TLR-4, TLR-2, and TNF-α (tumor necrosis factor-alpha) was performed as previously described (14) using Applied Biosystems reagents (Foster City, CA).

**Statistical analysis**

All data were analyzed using a one-way ANOVA using commercial software (SigmaStat, SPSS, Chicago, IL). Student-Newman-Keuls test was used for all post-hoc analyses. Statistical significance was set with an alpha value of P≤0.05. Data are presented as mean (±SEM).

**RESULTS**
Excess lipid accumulation is evident in hepatic tissue of 6%-SF and 12%-SF-fed mice

Although the mechanisms at the root of ectopic fat storage are not completely understood it is believed that in a chronic positive energy state, the capacity for subcutaneous adipose tissue to store triglycerides reaches a limit after which excess energy is allocated to ectopic fat depots and stored as triglycerides. Accrual of ectopic fat has been linked to an array of molecular dysfunctions (3, 15, 16).

We show a significant accumulation of excess lipids in the liver as a result of HFD-consumption. Interestingly, only the 6%-SF and 12%-SF diets produced this effect; the 24%-SF diet resulted in a similar level of hepatic lipid accumulation as the control diets (P<.05) (Figure 3.1A-B).

Upregulation of JNK activation in the liver of the 12%-SF diet

Inflammation has been closely linked with metabolic dysfunction, including insulin resistance (17). Hepatic phosphorylated-JNK (p-JNK) was the only inflammatory signaling molecule found to be upregulated in the liver; it was found to be significantly elevated in the 12%-SF diet compared to the control and 6%-SF-fed mice (P≤.05) (Figure 3.2).

Inflammation minimal to non-existent in the hepatic tissue of HFD-fed mice

With regard to inflammatory markers, the 12%-SF diet increased hepatic F4/80 and TLR-4 expression compared to both control diets and the AIN-76A diet, respectively (P≤.05) (Figure 3.3). Regarding the 6%-SF and 24%-SF diets, only the 24%-SF diet
elicited any increase in any of the inflammatory markers measured as the 24%-SF-fed mice displayed an increase in hepatic TNF-α expression compared to the AIN-76A-fed mice (P≤.05) (Figure 3.3).

DISCUSSION

Obesity is characterized by the accumulation of excess body fat and an increased risk of disease resulting from a complex network of interrelated molecular perturbations, including ectopic lipid accumulation in the liver leading to the development of NAFLD. Large lipid depositions in the liver (hepatic steatosis) have been shown to play a prominent role in the development of metabolic dysfunction (16, 18). However, the degree to which NAFLD is influenced by lipid composition has not been completely resolved. We have recently reported that adiposity, macrophage behavior, inflammation, and insulin resistance can be greatly affected by dietary SF content (19). However, it was found that these outcomes are not necessarily proportional to the percentage of SF in the diet; a diet most closely mimicking the standard American diet (12% and 40% of overall calories from saturated fat and total fat, respectively) led to the greatest adiposity, macrophage infiltration into adipose tissue, and insulin resistance, whereas diets composed of 6% (6%-SF) and 24% (24%-SF) of total calories from SF, but an equivalent level of overall calories from fat (40%), produced lower levels of these variables with the 24%-SF diet resulting in the least degree of insulin resistance. We felt that these results warranted a more thorough examination into the influence that HFDs, of varying SF content, have on NAFLD. It was discovered that a diet’s SF content, independent of total fat, can greatly impact NAFLD progression.
We found that consumption of the 6%-SF and 12%-SF diets resulted in elevated levels of lipid accumulation compared to the control or 24%-SF diets. We next examined inflammatory signaling molecules as well as markers of inflammation. We have previously reported that adipose tissue concentration of phosphorylated JNK was significantly increased in all HFDs, whereas only the 12%-SF-fed mice exhibited a significantly higher degree of NFκB p65 activation compared to control-fed mice (19). Of the three inflammatory pathways examined, only hepatic phosphorylated JNK was significantly elevated in 12%-SF-fed mice compared to control and 6%-SF-fed mice. Upon activation, JNK can produce insulin resistance via serine phosphorylation of IRS-1 (20). The 12%-SF mice also exhibited increased expression of the macrophage marker, F4/80, and TLR-4. It is well known that non-alcoholic fatty liver disease is a progressive disorder in which hepatic steatosis precedes non-alcoholic steatohepatitis. Based upon the results of this study, it seems that JNK activation, and accumulation of macrophages precede the full progression into steatohepatitis. Thus, it is likely that a longer period of HF feeding would be necessary before the development of a substantial hepatic pro-inflammatory environment. The finding that the consumption of the 24%-SF diet resulted in elevated hepatic TNF-α gene expression without any significant change in phosphorylated JNK, p38 MAPK, NFκB-p65 indicates that other signaling pathways are likely involved in influencing the expression of this inflammatory marker.

It is evident that the hepatic tissue of the 12%-SF-fed mice exhibited more characteristics of deregulatory processes than the hepatic tissue of the 6%-SF-fed mice despite having similar levels of hepatic lipid accumulation. The difference in cellular disturbances may be due to the concentration of SFAs in the 6%-SF and 12%-SF diets.
and/or the FA composition of the hepatic lipid depositions. SFAs are precursors for intracellular lipid intermediates, such as diacylglycerols and ceramides, which can function as secondary messengers within the cell and have been linked to metabolic dysfunction (16). In support of this, Wang et. al., through the utilization of two HFDs, one composed largely of SFAs and the other composed primarily of PUFAs, showed that, despite a similar degree of hepatic steatosis, the SFA-laden diet resulted in a greater concentration of hepatic SFA accumulation producing ER stress and apoptosis – two perturbations not induced by the PUFA-rich diet (7). The result that the 24%-SF diet did not produce hepatic steatosis is likely due to the increased MCFA content of the diet (12% versus .1% and 4% of total caloric intake for the 24%-SF, 6%-SF, and 12%-SF diets, respectively). As a result of their shorter chain length, MCFAs are more likely to be oxidized (21, 22). For a more elaborate explanation on why differences in SF composition existed among the HFDs and a more thorough background on MCFAs influencing metabolic processes, please see Enos et. al. (19).

In conclusion, this is the first study to examine the influence of three HFDs differing in the percentage of total calories from saturated fat (6%, 12%, 24%), but identical in total fat (40%), on NAFLD development. Findings from this study paired with those from our previous study (19) show that the SFA composition of a HFD can greatly influence the processes responsible for obesity-related diseases, as well as provide further evidence that the mechanisms at the root of these diseases are diet and tissue-sensitive. In the future, time-line and dietary manipulation studies should be employed to better understand the tissue-specific pathology of dysfunctional cellular processes and the
influence that dietary manipulation has on these outcomes so that therapeutic modalities may be conceived to more efficiently combat the obesity epidemic.

REFERENCES


Figure 3.1. Hepatic lipid accumulation. Relative (A) hepatic lipid accumulation (n=5-9/group) representative images of H&E and (B) oil red o staining of liver shot at a magnification of 10x.
Figure 3.2. Inflammatory signaling. Representative western blots of p-JNK, p-p38 MAPK, and p-NFkB p65 in liver. Diets not sharing a common letter differ significantly from one another (P ≤ .05). Western blots presented as relative intensity of densitometry (IOD).
Figure 3.3. Inflammation. Relative mRNA expression of TNF-α, IL-6, MCP-1, F4/80, TLR-4, and TLR-2 in liver (n=8-9/group). Diets not sharing a common letter differ significantly from one another (P≤.05).
Chapter IV

A High-Fat Diet Supplemented with Quercetin Has No Effect at Attenuating Adipose

Tissue Inflammation or Hepatic Steatosis\textsuperscript{3}

\textsuperscript{3}Enos, R. T., J. M. Davis, K. T. Velazquez, J. L. McClellan, and E. A. Murphy. To be submitted to a journal TBD.
ABSTRACT

We investigated the effects of a high-fat diet (HFD) supplemented with quercetin (.02%) on body composition, adipose and hepatic tissue inflammatory signaling and inflammation, and glucose metabolism in mice. Diets were administered for 16 weeks. Body composition and fasting blood glucose were examined intermittently. Hepatic lipid accumulation as well as adipose tissue (AT) and liver expression of inflammatory mediators (F4/80, CD206 (AT only), TLR-2 (AT only), TLR-4 (AT only), MCP-1, TNF-α, IL-6 (AT only), IL-10 (At only)) were measured along with activation of NFκB, and JNK. Contrary to the findings of others, quercetin supplementation had no effect at mitigating adipose tissue inflammation, hepatic steatosis, or improving body composition or fasting blood glucose levels.
INTRODUCTION

Obesity has quickly become a major health concern not only in the United States, but throughout the world. Moreover, it seems that the rate of obesity is on the rise. In 1980, as little as thirty years ago, the rate of obesity in the adult population was half (15%) of what it is now (30%). Even more worrisome, is that WHO projects that by 2015 there will be approximately 2.3 billion and 700 million overweight and obese individuals, respectively.

The health risks associated with obesity not only affect the individual suffering from obesity, but they also negatively impact the economy. It is estimated that obesity costs the economy of the United States more than 120 billion dollars annually (1). The 120 billion dollar stress on the economy is due many factors, including but not limited to: an increase in health care services that are needed as a direct result of the escalating rate of obesity and decreased productivity resulting from missed work time and premature death. Additionally, taxpayers are burdened with high health care costs, which impacts the price of goods and employee raises (2).

Obesity is problematic as the condition is characterized by a chronic state of low-grade adipocyte inflammation linked to adipose tissue dysfunction. Whether the adipose tissue dysfunction precedes the low-grade inflammatory state, or vice versa, is debatable. Nonetheless, adipose tissue dysfunction and the associated low-grade inflammation have been shown to increase the rate of mortality and are characterized by a circular cascade of interconnected events including, but not limited to, an increase in adipocyte size and number, endoplasmic reticulum stress, adipose tissue hypoxia, increased infiltration of pro-inflammatory macrophages into adipose tissue, an increase in adipocyte, pre-adipocyte, and macrophage TLR-4 and TLR-2 protein content, an increase in circulating
pro-inflammatory cytokines, insulin resistance, and a decrease in mitochondrial function (3-9).

Due to the fact that western countries are plagued by diseases resulting from obesity, it has become the goal of scientists to come up with relatively inexpensive therapeutic interventions to help mitigate HFD-enhanced chronic inflammation in order to improve quality of life and decrease obesity-related deaths. Recently, scientists have utilized nutraceutical interventions to examine the effects that plant-derived metabolites have on obesity outcomes. There exists promising research that the naturally occurring flavonoid, quercetin, possesses anti-inflammatory properties and an anti-obesogenic effect when supplemented along with a HFD (10-16). However, the mechanism(s) by which quercetin elicits its positive effects and the influence that quercetin has on macrophage behavior is still poorly understood.

The purpose of this study was to examine the potential anti-inflammatory and anti-obesogenic benefits of .02% quercetin supplementation to a HFD. We hypothesized that quercetin supplementation would decrease adiposity, adipose and hepatic tissue inflammation, and would improve fasting blood glucose compared to consumption of a HFD alone.

METHODS

Animals

Male C57BL/6 mice were bred and cared for in the animal facility at the University of South Carolina. They were housed, four-five per cage, maintained on a 12:12-h light-dark cycle in a low stress environment (22°C, 50% humidity, low noise)
and given food and water ad libitum. Principles of laboratory animal care were followed, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Diets

At four weeks of age, mice were randomly assigned to 1 of 3 treatment diets (n=10/group): a control diet (AIN-76A), a HFD, and the HFD supplemented with .02% quercetin (HFD + Quer) (BioServ, Frenchtown, NJ). The percentage of calories provided by each of the three macronutrients, the ratio of polyunsaturated:monounsaturated FAs (PUFA:MUFA), and the ratio of omega-6:omega-3 FAs were identical for the HFDs and were designed to be similar to the standard American diet (17, 18).

Body weights, food intake, and body composition

Body weight and food intake were monitored weekly. Body composition was assessed every four weeks (weeks 4, 8, 12, 16, and 20). For this procedure, mice were placed under brief anesthesia (isoflurane inhalation) and were assessed for lean mass, fat mass, and body fat percentage via dual-energy x-ray absorptiometry (DEXA) (Lunar PIXIImus, Madison, WI).

Fasting blood glucose

Plasma was assessed for fasting concentrations of glucose at 16 and 20 weeks of age. Blood samples were collected from the tip of the tail after a five-hour fast. Blood
glucose concentrations were determined in whole blood using a glucometer (Bayer Contour, Michawaka, IN).

**Tissue collection**

At 20 weeks of age, mice were sacrificed for tissue collection. Epididymal, mesentery, and retroperitoneal fat pads, as well as the liver, were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin until analysis. Blood was collected from the inferior vena cava using heparinized syringes, and centrifuged at 4,000 rpm for 10 min at 4°C. Plasma was aliquoted and stored at −80°C.

**Hepatic lipid accumulation**

At sacrifice, the liver was excised from each mouse and immediately snap-frozen in liquid nitrogen and stored at -80°C. Lipids were isolated from the liver utilizing a modified Folch extraction method and were quantified gravimetrically (19).

**Western blots**

Epididymal AT and liver were homogenized in radioimmunoprecipitation buffer (Sigma, St. Louis, MO), which included a protease inhibitor cocktail (Sigma, St. Louis, MO), and 1% glycerophosphate (100x), 0.5% sodium orthovanadate (1 mM), and 1% sodium fluoride (5 mM). The protein concentration was determined by the Bradford method (20). Western blots were performed as previously described using primary
antibodies for phosphorylated (Ser536) and total NFkB p65 and phosphorylated (Thr183/Tyr185) and total JNK (Cell Signaling, Danvers, MA) (21).

Gene expression

Epididymal AT and liver were homogenized under liquid nitrogen using a polytron and total RNA will be extracted using TRizol reagent (Invitrogen, Carlsbad, CA) and RNeasy mini-spin columns (adipose tissue only) (Qiagen, Valencia, CA). Quantification of epididymal AT gene expression for F4/80, CD11c, CD206, MCP-1, IL-6, TLR-4, TLR-2, and TNF-α and liver gene expression of F4/80, TNF-α, and MCP-1 (Applied Biosystems, Foster City, CA) were performed as previously described (22).

Statistical analysis

All data were analyzed using commercial software (SigmaStat, SPSS, Chicago, IL). Body weight, body composition outcomes, and fasting blood glucose were analyzed using a repeated measures two-way ANOVA. All other data were analyzed using a one-way ANOVA. Student-Newman-Keuls test was used for all post-hoc analyses. Statistical significance was set with an alpha value of \( P \leq 0.05 \). Data are presented as mean (±SEM).

RESULTS

HFD and HFD + Quer produce a similar increase in body weight, but quercetin supplementation increases overall and visceral fat mass more so than HFD alone.
Body weights and fat pad weights are presented in Figure 4.1. The mice consuming the HFD and HFD + Quer diet had significantly elevated body weights compared to AIN-76A-fed mice starting at 11 weeks of age (P≤.05).

For the fat-pad depots, both the HFD mice and the HFD + Quer mice had significantly more fat mass stored in the mesentary and epididymal fat pads compared to AIN-76A mice (P≤.05). However, only consumption of the HFD + Quer increased the retroperitoneal fat pad weight in comparison to the AIN-76A mice (P≤.05). Furthermore, although both HFDs increased total visceral adipose tissue compared to the AIN-76A diet, the HFD + Quer led to a greater accumulation of visceral adipose tissue than the HFD-fed mice (P≤.05).

Body composition analysis revealed a significant difference among groups (P≤.05) (Table 4.1). Specifically, the HFD-fed mice, with or without quercetin supplementation, had a greater fat mass compared to AIN-76A-fed mice starting at 12 weeks (P≤.05). However, the HFD + Quer led to a significant increase in fat mass at 16 weeks (P≤.05) and a trend (P<.1) for a greater fat mass at 20 weeks compared to the HFD alone. Regarding body fat percentage, the HFD + Quer diet elicited a significant increase in body fat percentage compared to AIN-76A-fed mice starting at 8 weeks and in comparison to HFD-fed mice at week 8 only (P≤.05). On the other hand, the HFD-fed mice did not increase body fat percentage relative to the AIN-76A-fed mice until 12 weeks (P≤.05). In general, lean mass increased over time for all groups, and by week 16, the HFD and HFD + Quer mice exhibited greater lean mass compared to AIN-76A fed mice (P≤.05).
It was not possible to calculate individual food intake as mice were housed 4-5/cage. However, in general, we did not observe any differences, among the HFD-fed mice, in weekly food intake (food consumed by mice in each cage/number of mice in cage) over the course of the study.

**Quercetin supplementation does not attenuate adipose tissue inflammation despite no significant increasing in adipose tissue NFκB activation**

Both HFDs increased adipose tissue JNK activation ($P \leq 0.05$) (Figure 4.2B), however, only the HFD-fed mice without quercetin supplementation significantly increased adipose tissue NFκB activation compared to AIN-76A-fed mice ($P \leq 0.05$). Similar to JNK activation, both HFDs increased gene expression of all inflammatory markers in the adipose tissue in comparison to AIN-76A-fed mice ($P \leq 0.05$) (Figure 4.3); the only exception being TLR2, which was found to be significantly different among the three diets, with the HFD + Quer eliciting the greatest increase in TLR2 expression followed by the HFD and AIN-76A diet, respectively ($P \leq 0.05$).

**HFDs increase liver weight as a percentage of body weight as well as hepatic lipid accumulation**

Both the HFD and the HFD + Quer led to an increase in liver weight relative to body weight as well as an increase in hepatic lipid accretion (Figure 4.4A,B) ($P \leq 0.05$).

**Hepatic inflammation is augmented in HFD and HFD + Quer mice**
Consumption of a HFD regardless of quercetin supplementation increased JNK activation and MCP-1 gene expression, but did not significantly increase NFκB activation or TNF-α expression (Figures 4.5A,B and 4.6) (P<.05). Interestingly, the addition of quercetin increased F4/80 gene expression above both the AIN-76A-fed mice and the HFD-fed mice (Figure 4.6) (P<.05).

**Both HFDs increase fasting blood glucose**

Compared to the AIN-76A-fed mice, both the HFD and HFD + Quer-fed mice exhibited an increase in fasting blood glucose levels at 16 and 20 weeks (Table 4.2).

**DISCUSSION**

Obesity has become a global health concern as obese individuals are at a greater risk for developing life-threatening diseases. For example, obesity, linked to the consumption of a high-fat, Western diet, has been established as significant risk factors for cancer development and cancer-related mortality (9, 23, 24). With the rising cost of health insurance and healthcare costs it is imperative that relatively inexpensive, effective therapeutic interventions are utilized in order to improve quality of life, decrease obesity-related diseases, as well as minimize the economic burden created by obesity. Therefore, we examined if a HFD supplemented with the anti-inflammatory phytochemical, quercetin, could help mitigate the physiological perturbations linked to HFD-induced obesity. Contrary to the findings of others, quercetin supplementation had no effect at mitigating adipose tissue inflammation, hepatic steatosis, or improving body composition or fasting blood glucose levels.
Previously, a study performed by Jung et. al. found that, in mice, a 32% HFD supplemented with .025% quercetin for 9 weeks reduced body weight, liver weight, and white adipose tissue weight compared to mice consuming a HFD without quercetin (15). Along a similar note, in mice, Kobori et. al. also found that a HFD supplemented with .05% quercetin for 20 weeks hindered the development of hepatic steatosis and reduced visceral adipose tissue accumulation (11). In our study, however, quercetin did not exhibit any anti-obesogenic properties; in fact, in some analyses quercetin was actually found to be obesogenic. Although, in general, we did not see any significant changes in body weight between the HFD and the HFD + Quer, quercetin supplementation did result in an increase in visceral fat mass compared to the HFD alone. Furthermore, there was no difference between the HFD-fed mice and the HFD + Quer mice with regard to hepatic lipid accumulation.

Because quercetin is known to possess anti-inflammatory properties, which are believed to be modulated by quercetin's ability to modulate NFκB expression (16), we examined NFκB activation in the adipose tissue along with JNK activation, another pro-inflammatory signaling protein, which we have previously shown to be upregulated in the adipose tissue of mice consuming the HFD utilized in this study (25). It was found that quercetin supplementation hampered an increase in NFκB activation, but did not have any effect on mitigating JNK activation. Interestingly, however, consumption of both the HFD and the HFD + Quer resulted in similar degrees of adipose tissue inflammation as determined by RT-PCR despite no significant increase in NFκB activation in the HFD + Quer group. These results suggest that: (1) quercetin may be directly or indirectly modulating NFκB activation and (2) the NFκB signaling cascade may not be the primary
pro-inflammatory pathway that is involved in adipose tissue inflammation given that there was no change in adipose tissue inflammatory mediators between the HFD and HFD + Quer groups.

The liver was subsequently investigated in order to assess hepatic inflammation. Similar to the adipose tissue, we examined NFκB and JNK activation as well as F4/80, MCP-1, and TNF-α gene expression. We found no increase in NFκB activation, but activation of JNK was increased in both the HFD and HFD + Quer groups as well as MCP-1 mRNA expression. An interesting observation was the fact that quercetin supplementation increased F4/80 gene expression in the liver more so than either the AIN-76A or HFD-fed mice. However, a more thorough analysis is needed to determine the reason for the significant increase in hepatic F4/80 expression resulting from quercetin supplementation.

In order to assess how quercetin supplementation influenced glucose metabolism, fasting blood glucose was analyzed at 16 and 20 weeks. Glucose levels were found to be elevated for both HFDs at 16 and 20 weeks, however, there was no difference in glucose levels among HFD treatments. This supports, although not definitively, as fasting insulin concentrations were not evaluated, the finding of Stewart et. al. who showed that quercetin supplementation may not improve insulin sensitivity (26).

In summary, quercetin supplemented to a HFD at .02% for a period of 16 weeks did not provide any anti-inflammatory or anti-obesogenic effects in mice. In fact, quercetin supplementation was found to be obesogenic and elicited an increase in hepatic F4/80 gene expression. These findings are in great contrast to the findings of others. The discrepancies in findings between studies is likely due to the length of HFD
administration, the concentration of quercetin used, as well as the type of HFD utilized.

This study is limited by the fact that lipid metabolism and various other pro-inflammatory markers were not measured, as it is possible that quercetin may be influencing other processes not directly analyzed in this investigation. However, it is evident that future studies are needed to better understand how the interaction between high-fat feeding and quercetin supplementation influences physiological processes.

REFERENCES


Figure 4.1 The effect of consuming a HFD with, or without, .02% quercetin supplementation on (A) weekly mean body weight and (B) retroperitoneal (Rp), mesentery (Mes), epididymal (Epi), and total visceral (Total) fat pad weights at sacrifice (n=10). Diets not sharing a common letter differ significantly from one another (P≤0.05). *Significantly different from AIN-76A (week 10-20: HFD & HFD + Quer, P≤0.05). $Significantly different from HFD & AIN-76A (week 10: HFD + Quer, P≤0.05).
Table 4.1  Body composition including, fat mass, lean mass, and body fat % of mice assessed at baseline (4 weeks) and incrementally (weeks 8, 12, 16 and 20) throughout the course of the study (n=10). Values not sharing a common letter (abcde) differ significantly over time within the given diet treatment (P≤.05). Values not sharing a common symbol (&#^) differ significantly among diet treatments within the given week (P≤.05).

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<th>Lean Mass (Grams)</th>
<th>Body Fat %</th>
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<tr>
<td></td>
<td>Week 4</td>
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<tr>
<td>AIN-76A</td>
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<td>5.3±20^b</td>
<td>8.0±.42^d</td>
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Figure 4.2 Representative western blots of (A) phosphorylated p65 NFκB (Ser536) and (B) phosphorylated JNK (Thr183/Tyr185)/total JNK, presented relative to control diets in epididymal adipose tissue following 16 weeks of diet treatment (n=8). Diets not sharing a common letter differ significantly from one another (P≤.05).
Figure 4.3 Epididymal adipose tissue gene expression of F4/80, CD11c, CD206, MCP-1, TNF-α, IL-10, TLR2, and TLR4 after 16 weeks of diet treatment (n=10). Diets not sharing a common letter differ significantly from one another (P≤.05).
Figure 4.4  (A) Liver weight as a percentage of body weight at sacrifice and (B) relative hepatic lipid accumulation (n=10). Diets not sharing a common letter differ significantly from one another (P≤.05).
Figure 4.5 Representative western blots of (A) phosphorylated p65 NFκB (Ser536)/total NFκB and (B) phosphorylated JNK (Thr183/Tyr185)/total JNK, presented relative to control diets in liver following 16 weeks of diet treatment (n=8). Diets not sharing a common letter differ significantly from one another (P≤.05).
Figure 4.6 Hepatic gene expression of F4/80, MCP-1, and TNF-α after 16 weeks of diet treatment (n=10). Diets not sharing a common letter differ significantly from one another (P≤.05).
Table 4.2 Fasting blood glucose (mg/dL) collected at 16 and 20 weeks (n=10). Values not sharing a common symbol (&#) differ significantly among diet treatments within the given week (P≤.05).

<table>
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<tr>
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<th>Week 20</th>
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<td>175±9.6  &amp;</td>
<td>182±.20  &amp;</td>
</tr>
<tr>
<td>HFD</td>
<td>218±6.8  #</td>
<td>206±7.9  #</td>
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<tr>
<td>HFD + Quer</td>
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<td>208±7.7  #</td>
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Overall Conclusion

Overall, results from these studies suggest that adiposity, macrophage behavior, inflammation, insulin resistance, and NAFLD progression can be greatly affected by dietary SF content. However, it was found that these outcomes are not necessarily proportional to the percentage of SF in the diet; a diet most closely mimicking the standard American diet (12% and 40% of overall calories from saturated fat and total fat, respectively) led to the greatest adiposity, macrophage infiltration into adipose tissue, and insulin resistance, whereas diets composed of 6% (6%-SF) and 24% (24%-SF) of total calories from SF, but an equivalent level of overall calories from fat (40%), produced lower levels of these variables with the 24%-SF diet resulting in the least degree of insulin resistance and hepatic lipid accumulation. Further, contrary to the findings of others, the anti-inflammatory flavonoid, quercetin, had no effect at mitigating adipose tissue inflammation, hepatic steatosis, or improving body composition or fasting blood glucose levels in a HFD-induced obesity model. Future studies are needed to better understand how the interaction between high-fat feeding and quercetin supplementation influences physiological processes.
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development in LDLr-KO mice, in spite of displaying a pro-inflammatory profile similar to trans fatty acids. *Atherosclerosis.*


