EFFECTS OF BITTER MELON AND TOLL-LIKE RECEPTOR (TLR) 4 ON GLUCOSE AND LIPID PARAMETERS IN MICE FED A HIGH FAT DIET

By

MAUREEN MEISTER

Bachelor of Science in Dietetics

St. Catherine University

St. Paul, Minnesota

2011

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2013

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Thesis Approved:

Dr. Edralin A. Lucas

Thesis Adviser

Dr. Brenda J. Smith

Dr. Stephen L. Clarke

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CHAPTER I

INTRODUCTION

The prevalence of obesity in the United States is estimated to be 30% across the population and continues to rise [1,2]. Obesity is characterized as a body mass index (BMI) exceeding 30 kg/m^2. Obesity is considered to be a significant risk factor for many chronic and life threatening diseases such as hypertension, cancer, cardiovascular disease and type 2 diabetes mellitus. Obesity, which may be associated with consumption of a high fat diet, contributes to the development of insulin resistance, the primary cause of type 2 diabetes [3]. The incidence of type 2 diabetes has also increased as a high fat diet has become widely consumed [3].

Obesity is also associated with a chronic low-grade inflammation, a condition thought to be a primary factor in the development of insulin resistance in obese individuals. Inflammatory pathways are activated due to the presence of harmful stimuli in the body, with the ultimate goal of returning the body to a normal physiological state [4]. The classic inflammatory pathway leads to an increase in the secretion and circulation of pro-inflammatory cytokines, secretion of acute phase proteins, and the activation of inflammatory responses to repair maintain homeostasis [4].

The chronic low-grade inflammation associated with obesity involves an increased circulation of pro-inflammatory cytokines as well as a decrease in protective

proteins and hormones, such as adiponectin. The chronic nature of obesity-induced inflammation causes dysfunction and deregulation of metabolic functions that maintain glucose homeostasis. Inflammation leads to insulin resistance through the secretion of inflammatory cytokines, adipose tissue dysfunction, and the activation of inflammatory pathways through protein receptors such as the toll like receptor (TLR) 4 [4,5].

Insulin resistance is defined as the inability of cells to respond to circulating insulin. Insulin resistance inhibits the insulin signaling cascade and prevents the entry of glucose into cells. Insulin exerts its main effects on cells within the adipose tissue, muscle and liver. It is responsible for regulating glucose entry into these cells and is also needed for the regulation of plasma free fatty acid (FFA) concentrations by inhibiting lipolysis [6]. The action of insulin is inhibited in obese individuals due to increases in pro-inflammatory cytokines, which inhibit insulin signaling by acting on the insulin receptor substrate (IRS) 1. This interruption in insulin signaling leads to insulin resistance, altering glucose homeostasis and causing an increase in plasma FFA concentrations.

TLR 4 is a protein receptor located on the cell membrane of most cells in the body, with the expression of this receptor on adipocytes and immune cells such as monocytes and macrophages being especially high [5,7]. Activation of TLR 4 is a result of ligand binding by free fatty acids, lipopolysaccharides (LPS) and endogenous proteins. These ligands, particularly free fatty acids, are increased in obese individuals due to the expansion of adipocytes and are often elevated in association with the consumption of a high fat diet [7].

The activation of TLR 4 leads to stimulation of the innate immune system, the body's first line defense in response to pathogens, and subsequent inflammatory processes, inhibition of insulin signaling, and stimulation of pro-inflammatory cytokine production. Together, these effects impair insulin function and signaling. TLR 4 deficient mice have been shown to exhibit alterations in body composition and reduced activation of inflammatory pathways, which lead to improved insulin sensitivity [3,5]. However, it is important to note that TLR 4 is not the only mechanism through which a high fat diet contributes to insulin resistance. For example, a high fat diet has been shown to elevate the production of pro-inflammatory cytokines from the white adipose tissue which can induce insulin resistance independent of TLR 4 [4,5].

Dietary approaches to improve the inflammatory state associated with obesity are also an important approach on which much research is focused. Dietary measures such as the consumption of specific fruits and vegetables or the phytochemicals from these foods have been shown to aid in reduction of inflammation and insulin resistance [8, 9, 10]. While the mechanism(s) responsible for these effects remains uncertain, dietary phytochemicals have been linked to a reduction in adipose tissue mass, improvements in glucose tolerance, and reduced inflammation [11].

A specific food used in this manner is bitter melon (*Momordica charantia, MC)*, an Asian fruit that has been demonstrated to have hypoglycemic effects [11-14]. Various studies have shown bitter melon's insulin-like effects due to its ability to increase glucose uptake by peripheral tissues [12]. Bitter melon contains the peptides charantin, vicine, and polypeptide-p, compounds thought to be responsible for the hypoglycemic effect of bitter melon [13,14]. MC also contains significant amounts of vitamins and minerals that give its

antioxidant and anti-inflammatory effect [11]. It is hypothesized that because of the bioactive components found in MC, the addition of MC in the diet will provide protection from obesity-associated inflammation subsequently lessening tissue insulin resistance.

The **objective** of this study is to determine the effect that MC in 1% and 10% (w/w) dose in combination with a high fat diet (60% kcal from fat) on clinical parameters and body composition in both mice that easily develop obesity and insulin resistance (C57Bl/6) and mice with a null mutation in the TLR 4 receptor (C3H/HeJ).

Our **null hypotheses** are that:

1) Bitter melon added to a high fat diet will not improve the plasma lipid profile in both strains of mice.
2) Bitter melon will not affect glucose homeostasis in both strains of mice.
3) Biomarkers of inflammation will not be lower in mice fed bitter melon compared to mice consuming a high fat diet in both strains of mice.
4) Bitter melon will not affect body composition in both strains of mice.

If bitter melon is found to have a positive effect on lipid parameters, body composition and glucose homeostasis, it could potentially be used as a dietary option to improve these parameters. The findings of this study may also aid in identifying the role of TLR 4 in the development of inflammation and insulin resistance. The results of this study may also provide an understanding whether inhibition of the activation of TLR 4 is one of the mechanisms by which bitter melon exerts positive effects on glucose and lipid parameters.

CHAPTER II

REVIEW OF LITERATURE

Prevalence of Obesity and Type 2 Diabetes Mellitus

Obesity prevalence continues to rise and has significantly increased over the last decade. As reported by the Center for Disease Control (CDC), the prevalence of obesity in the United States is estimated to be over 30% in most age groups and in both genders [1]. Due to the rise in obesity, complications associated with obesity, such as heart disease diabetes mellitus, and some cancers have also shown an increase [1].

Research efforts are focused on determining the pathogenesis of obesity-associated complications [4]. Of these complications, the development of insulin resistance in response to chronic inflammation has been indicated as the direct link between obesity and type 2 diabetes mellitus [1, 2]. Parallel to the rise in obesity is an increase in the prevalence of type 2 diabetes. Regions in the United States with the highest obesity rates also have the highest incidence of type 2 diabetes [15]. The American Diabetes Association estimated that 25.8 million Americans suffer with type 2 diabetes or complete insulin resistance and that nearly 79 million are prediabetic, characterized as a fasting blood glucose between 100 mg/dL and 126 mg/dL [16]. Individuals with insulin resistance and diabetes are at an increased risk for hypertension

and dyslipidemia, which lead to an increased risk for developing cardiovascular disease and other complications [17].

Healthcare costs related to diabetes are estimated to be $218 billion in the United States annually; however this does not account for the costs associated with complications and chronic conditions developed due to type 2 diabetes and obesity [18]. High healthcare costs, as well as the complications associated with type 2 diabetes and obesity, have left clinicians and patients searching for additional prevention and treatment methods.

Pro-inflammatory State of Obesity

Obesity is associated with a chronic low-grade inflammation due to the body's response to harmful stimuli, specifically elevated levels of endogenous free fatty acids derived from adipose tissue [4]. The body's response involves activation of the innate immune system as well as systemic increases in circulating pro-inflammatory cytokines. Tumor necrosis factor (TNF) α, interlukin-6 (IL-6) and C-reactive protein (CRP) are especially elevated in obese individuals [4,19].

The accumulation of lipids in the adipocytes causes an expansion, via hypertrophy, of adipose tissue and leads to an increase in secretions from adipose tissue. Adipose tissue secretes adipokines, hormones, and cytokines that act both locally and systemically to affect various tissues [5]. The increase in these secretions leads to an elevation in the systemic amounts of pro-inflammatory cytokines and adipokines. These secretions also act locally on the endothelial cells within the adipose tissue to promote macrophage recruitment to the site of inflammation [20]. The increased presence of

macrophages within adipose tissue also elevates the inflammation locally and further contributes to the inflammation present systemically. Together, these inflammatory changes associated with obesity lead to chronic low grade inflammation which has the potential to cause complications such as insulin resistance and type 2 diabetes.

Insulin Resistance

Insulin resistance has been considered clinically relevant because of its association with several medical conditions; including hypertension, dyslipidemia, non-alcoholic fatty liver disease, and metabolic syndrome, all contributing to the development of cardiovascular disease. Insulin resistance is defined as the inability of insulin-sensitive tissues to respond to normal amounts of circulating insulin and affects several metabolic pathways including glucose transport, glycogen synthesis and inhibition of lipolysis [21]. Tissues such as skeletal muscle, liver and adipose tissue are especially affected by insulin resistance [19,22]. Insulin resistance leads to the inability of these tissues to bring glucose into the cell to be utilized for energy. The state of insulin resistance is characterized by increased plasma free fatty acid concentrations and hyperglycemia, due to the inability for insulin to modulate lipolysis, hepatic glucose output and skeletal muscle glucose utilization [21,22].

Insulin is an anabolic hormone essential for regulating metabolic processes throughout the human body. The actions of insulin are carried out through activation of intracellular signaling cascades [19]. The binding of insulin to its receptor, the insulin receptor (IR), causes the phosphorylation of tyrosine residues present on the insulin receptor substrate (IRS). This phosphorylation is important to induce the activation of

kinases such as phosphatidylinositol 3-kinase (PI3K), which in turn causes the activation of 3-phosphoinositide-dependent protein kinase (PDK) 1, and further activation of Akt/protein kinase B (PKB) [19,23]. PKB targets downstream proteins allowing for the translocation of glucose transporter type (GLUT) 4, an insulin sensitive receptor, from vesicles within the cytoplasm to the cell membrane to facilitate glucose entry into the cell, specifically within adipose tissue and skeletal muscle. Within the liver, insulin regulates gluconeogenesis and the production of glycogen to maintain glucose homeostasis.

Interruption at any point along the insulin signaling cascade results in insulin resistance. Down regulation of the insulin receptor or the insulin receptor substrate protein level is often seen in state of hyperinsulinemia and obesity leading to insulin resistance in insulin sensitive cells. Insulin induces the phosphorylation of serine and tyrosine on insulin substrates an action requiring fine tuning and a strict balance of serine and tyrosine phosphorylation at the appropriate time during insulin signaling [24]. Inhibitory sites on the IRS can be phosphorylated in response to stimuli, including free fatty acids, pro-inflammatory cytokines and various inflammatory pathways, all of which may be elevated in obesity, there by promoting insulin resistance [5,19]. Serine phosphorylation, rather than tyrosine, of the IRS is also a major point of alteration along the insulin signaling cascade. Serine phosphorylation of IRS prevents further insulin signaling from occurring [19].

Genetic, lifestyle, and environmental factors play an important role in the development of insulin resistance [22]. Risk factors for developing insulin resistance include obesity, physical inactivity, and aging [22]. Insulin resistance initially results in

increased insulin secretion by the pancreatic β cells. Pancreatic islets increase their cell mass and β cells increase insulin secretion in order to compensate for the developing resistance [25]. However, sustained inflammation and the presence of oxidative stress associated with obesity leads to pancreatic β cell dysfunction [25,26]. Pancreatic β cells are especially vulnerable to oxidative stress because of their lack of antioxidant-containing enzymes [25]. Increased inflammation, due to elevated levels of pro-inflammatory cytokines and chemokines, disrupts the circulation of blood to the β cells and contributes to dysfunction. Hyperglycemia, related to over nutrition and the progression of insulin resistance may also contribute to apoptosis of the β cells [27]. Because of the mounting dysfunction within the pancreas due to the resistant state of obesity, the pancreas is no longer able to compensate which leads to dysfunction in glucose homeostasis within the periphery and the development of type 2 diabetes [24-28].

Obesity-Associated Insulin Resistance

There is no known direct cause of insulin resistance in obese individuals; rather, there are many compounding factors that contribute to the development of insulin resistance in obese individuals. Insulin resistance due to obesity occurs as a result of many underlying mechanisms, including increased caloric intake, increased circulating free fatty acids (FFA), inflammatory cytokines, tissue inflammation and many others [28]. Described below are the most understood mechanisms associated with the development of insulin resistance in obese individuals.

Free Fatty Acids

An increase in endogenous circulating free fatty acid concentration is linked to the development of obesity associated insulin resistance. FFA concentrations in obese individuals are generally increased due to a decreased level of clearance by the liver, as well as an increased release from expanding adipose tissue and a reduced suppression of lipolysis by insulin, leading to an elevation in plasma FFA levels [21,28]. FFA are released from adipocytes during lipolysis when intracellular triglycerides (TG) are hydrolyzed to form FFA and a glycerol, a process that is regulated by peroxisome proliferator-activate receptors (PPAR) γ and insulin [27,29]. The persistent low-grade chronic inflammation present in obese individuals and specifically elevations in TNF-α, induces lipolysis and contributes to the release of FFA from adipocytes [29-32]. Development of insulin resistance contributes to increased concentrations of FFA and is caused in part by increased FFA levels, creating a vicious cycle.

FFA aid in the mounting inflammation present in obese individuals further contributing to the development of obesity-associated insulin resistance. FFA, specifically long chain fatty acids, make a significant contribution to the low-grade, chronic inflammation present in obesity by acting as a ligand for TLR 4. The activation of TLR 4 leads to the downstream activation of the inflammatory pathway nuclear factor κ B (NFκB) [5]. Activation of this pro-inflammatory pathway increases the transcription of pro-inflammatory genes which increase the expression of pro-inflammatory cytokines, contributing to obesity-associated insulin resistance. Boden *et al.* showed an increased expression of monocyte cheomoattractant (MCP) 1, TNF-α, and IL-6 in livers of insulin resistant rats when infused with lipids to increase FFA concentrations, suggesting the

regulation of these pro-inflammatory cytokines by FFA through a NFκB dependent mechanism [21,29].

FFA also inhibit insulin signaling in skeletal muscle and liver. In the liver, FFA impair hepatocyte insulin function which enhances the process of gluconeogenesis and TG production and limits glycogen synthesis [29, 30]. When FFA concentrations are high, FFA are the preferred fuel source over glucose in the muscle through the Randle cycle and their use for fuel results in an elevated blood glucose level [29]. Systemic increases in FFA can lead to lipid deposition in the liver and muscle, contributing to whole body insulin resistance [28, 29]. FFA also inhibit insulin signaling in the skeletal muscle and liver by activating pro-inflammatory pathways which induce serine phosphorylation of IRS-1 [19, 24].

Not only does an increased FFA concentration contribute to the development of insulin resistance, but the presence of insulin resistance also leads to increased plasma FFA levels. Insulin resistance results in a decreased ability of insulin to suppress lipolysis in the adipose tissue, resulting in an increased FFA flux. This is especially common in individuals with android, or central, obesity as visceral fat has a higher rate of lipolysis when compared with subcutaneous fat [28, 29, and 32]. Increases in plasma FFA concentrations contribute to the presence of inflammation in obese individuals and exacerbate insulin resistance progression through pathways discussed previously.

Inflammatory Pathways and Insulin Signaling

Obesity is associated with a chronic low-grade inflammation that contributes to the development of obesity-associated insulin resistance [5, 6, 22]. Obesity, often driven by

the consumption of a high fat diet and nutrient overload, triggers inflammatory pathways, which inhibit insulin-receptor signaling [33, 34]. Additionally, in the state of chronic inflammation, pro-inflammatory cytokines are elevated which can also disrupt the action of insulin and interfere with insulin signaling. The two prominent inflammatory pathways that affect insulin signaling include JUN N-terminal kinase (JNK) and NFκB [35].

Insulin signaling requires the binding of insulin to the insulin receptor located on the plasma membrane of myocytes, adipocytes and hepatocytes. The binding of insulin to the insulin receptors induces signaling cascades and allows for GLUT 4 to translocate to the cell membrane to bring glucose into the cell, specifically within myocytes and adipocytes [6, 22, and 23]. IRS-1 signaling can specifically be modified in inflammatory states through the phosphorylation of serine residues present on IRS-1. Insulin signaling requires tyrosine phosphorylation of IRS-1 in order to activate the insulin receptor. Inflammatory cytokines and certain protein kinases cause the phosphorylation of serine residues, rather than tyrosine, present on IRS-1 [6, 22]. The phosphorylation of serine residues induces the degradation of IRS-1 and prevents the binding of insulin to the insulin receptor, thus causing inhibition of insulin signaling.

Obesity is associated the activation of JNK at multiple sites such as the muscle, adipose tissue and liver. JNK can be activated by various stress signals including the presence of reactive oxygen species (ROS), inflammatory cytokines, free fatty acids and pathogens [35]. JNK phosphorylation due to the presence of these stress factors, leads to serine phosphorylation of IRS, disrupting insulin signaling. JNK activity has been shown to be elevated in obesity and JNK knockout mice have been shown to be protected from developing insulin resistance when fed a high fat diet [35]. In addition to phosphorylating

serine residues on the insulin receptor, activation of JNK also promotes elevated secretions of pro-inflammatory cytokines, exacerbating the inflammatory response and causing further activation of other inflammatory pathways such as NFκB.

The activation of NFκB also contributes to the development of insulin resistance, especially in obesity. NFκB activation stimulates the transcription of inflammatory mediators, including TNF-α and IL-6 [36]. The increase in inflammation caused by activation of this pathway inhibits insulin signaling by phosphorylating serine residues on IRS-1 and acting directly on the insulin receptor. The NFκB pathway is activated through the binding of pro-inflammatory ligands such as TNF-α and lipopolysaccharides (LPS) to their membrane bound receptors, for example the toll like receptors. This causes the phosphorylation of the IκB protein, the inhibitor of NFκB, allowing for it to disassociate from NFκB and thus removing the inhibition of this pathway [36]. NFκB is then able to translocate to the nucleus to exert its effects by inducing the transcription of pro-inflammatory proteins. Similar to JNK, the inflammatory mediators needed to activate the NFκB pathway are more readily available in an obese state, allowing for an increased NFκB activity and increasing inflammation.

The chronic inflammatory state of obesity, is characterized by an increased concentration of inflammatory cytokines as well as an increase in free fatty acids as a result of adipocyte expansion. As these molecules act as mediators for inflammatory pathways, the activation of these pathways is increased. The activation of protein kinases and inflammatory pathways that is present in obesity provides a direct link between inflammation and obesity-associated insulin resistance.

Inflammatory Cytokines

The increase in presence of inflammatory cytokines circulating systemically has long been linked to the pathogenesis and development of insulin resistance and type 2 diabetes mellitus [37-39]. TNF-α, IL- 6 and IL-1β are expressed a high levels in obese individuals both in the adipose tissue and in circulation. The presence of these cytokines induces the activation of classic pro-inflammatory pathways including NFκB and JNK within adipocytes, hepatocytes and macrophages [37-39]. Activation of these inflammatory pathways contributes to the development of insulin resistance by interrupting insulin signaling.

Inflammation at the systemic level is associated with increased adiposity, which increases the release of TNF-α, IL-6 and MCP-1 [40, 41]. Elevated MCP-1 attenuates the inflammatory response at the site by recruiting circulating monocytes, which differentiate into macrophages within the adipose tissue. TNF-α has been indicated to play a profound role in insulin resistance. Evidence from *in vitro* studies has shown that treating 3T3-L1 adipocytes with TNF-α for 96-hours results in a significant decreased expression of GLUT4 and IRS- 1 compared to control [40]. These results indicated the primary role of TNF-α in insulin resistance is in regulation of expression of genes related to insulin signaling.

IL- 6, released from both adipocytes and macrophages, is an endocrine cytokine that acts on tissues distant from the site of secretion. IL-6 affects the liver, bone marrow and endothelium. It is important in regulating the acute-phase response in the liver and the release of CRP, a plasma protein involved in the complement cascade [42, 43]. IL-6 has

also been implicated to be involved in energy balance by modulating food intake and increasing energy expenditure, possibly attempting to regulate obesity; however, it has been postulated that a state of IL-6 resistance develops in obesity.

Adipose Tissue

Obesity is associated with the expansion of adipocytes, an increase in adipose tissue mass and volume, as well as a change in the distribution of adipose tissue throughout the body [37-39]. Obesity is generally associated with higher amounts of visceral adipose tissue when compared with the amount of subcutaneous fat [38]. The increase in visceral adipose tissue and overall increase in adipose tissue volume, leads to an increase in pro-inflammatory markers. As discussed earlier, the activation of inflammatory processes inhibit insulin signaling and contribute to the development of insulin resistance.

Adipose tissue is a metabolically active tissue that is not only the storage site for extra calories in the form of triglycerides but is also an endocrine organ important in hormone secretion and its contribution to the development of obesity related insulin resistance [38]. Adipose tissue is composed of pre-adipocytes, adipocytes, endothelial tissue and immune cells such as macrophages and monocytes, all of which are increased in obesity [5, 6]. Supporting the adipose tissue is the stromal vascular fraction, which contains pre-adipocytes, smooth muscle precursors, fibroblasts, mesenchymal stem cells along with the immune cells macrophages and monocytes [5, 6]. Increases in adiposity and adipocyte hypertrophy is associated with dysregulation of metabolic and endocrine functions which lead to alterations in the body's physiological response, increasing

inflammation and diminishing insulin sensitivity [44,45]. Obesity-associated inflammation is triggered primarily in the adipose tissue due to macrophage infiltration and increased secretions from adipocytes and macrophages [46, 47]. Adipocytes have the ability to synthesize and secrete a number of bioactive molecules, known as adipokines which affect inflammatory processes and glucose metabolism [46].

Adipocytes and macrophages residing within the adipose tissue are also able to secrete pro-inflammatory cytokines such as TNF- α, IL-6 and leptin, which activate inflammatory pathways and contribute to inflammation. These inflammatory cytokines cause the development of insulin resistance in obesity by activating pro-inflammatory pathways including JNK and NFκB, inhibiting insulin signaling and preventing the entry of glucose into the cell.

Hypoxia in adipose tissue of obese individuals has also been implicated as a source of inflammation [48]. Hypoxia contributes to the activation of NFκB, regulating the gene expression of inflammatory cytokines and contributing to the recruitment of macrophages to the adipose tissue. Adipose tissue hypoxia may also contribute to the reduction of adiponectin and elevation of leptin seen in obese conditions [48]. Inhibition of adipogenesis and triglyceride synthesis that is caused by hypoxia, may also contribute to the increased concentrations of FFA seen in obesity.

Adipokines

Adipokines are bioactive compounds synthesized and released by adipocytes, and play an important role in inflammation, lipid metabolism and glucose homeostasis [47]. Several adipokines are especially important in relation to the control of insulin sensitivity

and the development of insulin resistance. Adipokines such as adiponectin and leptin have been specifically indicated as having a role insulin signaling. Adiponectin is the most widely released adipokine secreted by the adipose tissue and is expressed only in adipocytes [49].

Plasma levels of adiponectin are reduced in obese individuals and in individuals with type 2 diabetes. Adiponectin in obese individuals is often deceased due to inhibition by the pro-inflammatory cytokine, TNF-α [45]. Adiponectin has been shown to increase peripheral insulin sensitivity and improve β cell insulin secretion, resulting in an increase in glucose uptake and utilization [49, 50]. Inflammatory cytokines, specifically TNF-α secreted by adipocytes, have been shown to have a paracrine effect and act to inhibit the expression of adiponectin in obese individuals [49]. Research performed by Kern *et al.* found that individuals with the highest levels of adiponectin exhibited significantly lower TNF-α mRNA expression in adipose tissue [50]. Because of the role of TNF-α in the development of insulin resistance, adiponectin provides a protective measure from insulin resistance and the development of type 2 diabetes.

Adiponectin deficient mice have been shown to become insulin resistant when fed a high fat diet, indicating the protective nature of adiponectin from insulin resistance [49, 51]. The under expression of adiponectin and over expression of TNF-α in obesity, results in a vicious cycle of inflammation within the adipose tissue of obese individuals. TNF-α secretion also results in an increased IL-6 expression within the adipose tissue which further aggravate the inflammatory response and increasing the likelihood of the development of insulin resistance.

Similarly, leptin is a hormone derived from adipocytes that is important in regulating energy intake through its action in the hypothalamus [46]. Leptin also plays a role in improving insulin sensitivity in peripheral tissues and increasing insulin secretion by the pancreatic β cells, however, these effects are generally not observed in obese individuals due to the development of leptin resistance [47]. Leptin levels increase as adiposity grows, however obese individuals develop resistance to the controls of leptin in the hypothalamus and the catabolic effects of leptin in relation to weight maintenance. Leptin's direct effects on the immune system through the stimulation of the innate immune system cause an increase in inflammatory conditions as leptin levels increase in obesity.

IL-6, an endocrine cytokine which targets sites distant from the area of release, is also secreted from cells in the adipose tissue [45]. IL-6 is released from adipocytes and macrophages in the adipose tissue. Similar to TNF-α and leptin, the release of IL- 6 is increased with increasing adiposity. As mentioned earlier, IL-6 acts to regulate the inflammatory response of the liver by causing the release of acute phase proteins, specifically CRP which aids in the recruitment of host defense mechanisms. IL-6 also acts to increase the expression of adhesion molecules which contributes to the inflammation at the site and enables the attraction and adherence of macrophages to the endothelium.

Macrophage Infiltration

Increased infiltration by macrophages into the adipose tissue is a major source of pro-inflammatory molecules within obese individuals. Inflammation associated with the

progression of obesity causes an infiltration of immune cells, specifically macrophages, in the adipose tissue. Pre-adipocytes have a high capacity for becoming phagocytic and transforming into macrophages prior to maturity [38, 46]. In an obese state, increased expression of the cytokine, TNF-α, can induce the release of MCP-1 from pre-adipocytes. MCP-1 attracts monocytes and macrophages to adipose tissue. Endothelial cells within the adipose tissue also secrete MCP-1, further increasing infiltration of macrophages and inflammation. The overall change in adipocyte secretions, such as increasing leptin, causes the accumulation of macrophages within the adipose tissue. Adipocyte necrosis and hypoxia increases due to adipocyte hypertrophy in obesity which may also provide a mechanism for the increase in macrophages in adipose tissue [46].

Macrophage concentration within adipose tissue is strongly associated with adipocyte size and total body weight, indicating the presence of macrophage infiltration in obesity [45, 47]. The degree of macrophage accumulation in adipose tissue is directly proportional to adiposity and contributes to the inflammation [49]. Gene expression studies have shown that macrophages are primarily responsible for the elevated concentration of TNF-α in obese individuals [46]. Therefore, macrophage infiltration into the adipose tissue is at least in part responsible for metabolic changes and inflammatory responses observed in obesity.

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone superfamily of receptors which are ligand activated and act to control metabolic processes[52, 53]. Ligand-activated PPARs are important regulators of metabolic activities

which play a role in fatty acid metabolism, lipolysis, lipid storage and processes related to inflammation [52, 53]. PPARs, specifically PPARγ, play a role in improving insulin sensitivity as well as preventing dyslipidemia and fatty liver. PPARs have also been shown to play an important role in repressing inflammation and have also been implicated as being important regulators of nutrient-gene interactions through their ability to recognize fatty acids and respond with regulation of lipid and glucose metabolism [53].

PPAR activity is regulated by phosphorylation. Phosphorylation of individual PPARs, result in changes associated with ligand affinity, target genes, and co-factors needed for activation. Three different isoforms of PPARs exist, PPARα, PPARβ and PPARγ. These isotypes differ by tissue location, ligand preference, and affinity.

PPARα is found primarily in the liver and acts to prevent fat build up in the liver. PPARα has the ability to detect levels of circulating fatty acids and respond by modulating lipid metabolism and the peroxisomal fatty acid oxidizing system, which is responsible for metabolism of very long-chain fatty acids [54]. PPARα can also be activated by exogenous ligands such as fibrates, steroids or thiazolidinediones (TZDs). It has also been hypothesized that PPARα activation can occur indirectly through the enhanced synthesis of endogenous ligands, stimulated by an exogenous component [53].

PPARγ is abundant in adipose tissue and plays an important role in regulating adipocyte differentiation and adipogenesis [53, 55]. By stimulating adipocyte differentiation and adipogenesis, PPARγ indirectly reduces free fatty acid flux in the liver and muscle, therefore improving insulin sensitivity in these tissues. PPARγ also plays an important role in glucose metabolism not only through improving insulin sensitivity, but also by

increasing glucose uptake in the muscle and reducing glucose production in the liver. PPARγ synthetic ligands, TZDs, are currently being used in the treatment of type 2 diabetes mellitus by improving insulin sensitivity through promoting fatty acid storage thereby reducing lipotoxicity [51]. However, the role of PPARs in improving insulin sensitivity may also be associated with their ability to reduce the presence of chronic inflammation.

PPARs also have an important role in repressing inflammation. PPARα and PPARγ have been shown to prevent NFκB activation by forming a complex with pro-inflammatory transcription factors of this inflammatory pathway [55]. PPARs may also prevent NFκB activation by up regulating transcription of genes which act to prevent activation of the NFκB pathway, such as IKK and IκBα [55]. Due to the multiple roles of PPARs in improving insulin sensitivity and reducing inflammation, dietary and natural agonists for PPARs are being investigated to improve and prevent the development of various metabolic conditions, including type 2 diabetes.

Toll Like Receptors

Toll like receptors (TLR) are type 1 integral membrane spanning glycoproteins [56, 57]. TLRs are protein receptors, present on either the cell surface or the endosome and are expressed on nearly all cells of the body, including adipocytes and immune cells such as macrophages and monocytes. TLRs are classified as pattern recognition receptors (PRR) that play a role in the activation of the innate immune system by stimulating pro-inflammatory pathways and transcription factors in response to the presence of pathogens [56,57]. PRRs have the ability to recognize pathogen-associated molecular patterns

(PAMPs), molecules produced only by bacteria but not eukaryotic cells [57]. This ability allows PRRs to distinguish between self and non-self-molecules and stimulate the innate immune system.

The majority of TLR ligands are PAMPs and are diverse in structure and origin [56]. Currently, there are eleven and thirteen types of TLRs that have been identified in humans and mice, respectively. The different types of TLRs have multiple ligands and may require an additional adapter protein for activation. Some ligands and adapter proteins for TLRs remain unidentified [57, 58]. Following the binding of ligand, TLRs heterodimerize and undergo conformational change allowing them to initiate downstream signaling cascades [56]. The signaling cascades are induced either through the activation of the adapter protein myeloid-differentiating factor (MyD) 88 causing the production of inflammatory proteins, or independent of MyD88 as is the case of TLR 3 and TLR 4. The use of the adaptor protein MyD88 is specifically important in the relationship between TLRs and inflammation, as this adapter protein is responsible for the activation of inflammatory pathways such NFκB and the subsequent production of the cytokines TNF-α, IL-6 and MCP-1. Among the many TLRs, TLR 4 has been especially recognized for its role in contributing to inflammation through the mentioned pathways.

TLR 4 and its Role in Obesity-Associated Insulin Resistance

TLR 4, the most widely characterized TLR, is a protein receptor located on the cell membrane of several cell types including adipocytes, hepatocytes, macrophages and monocytes [58-60]. Ligands that activate TLR 4 include saturated fatty acids (SFA), endogenous free fatty acids and LPS, produced by the death of gram-negative bacteria

within the gut and present on the cell wall of gram-negative bacteria [4,61,62]. Activation of TLR 4 through these ligands requires several adapter proteins such as the LPS-binding protein, CD14 and MyD88 as described earlier. The binding of ligands to TLR 4, results in the stimulation of the body's innate immune system and initiates the inflammatory response.

TLR 4 activation can be independent or dependent of the adapter protein MyD88. TLR 4 is the only toll like receptor with the ability to be activated independent of MyD88. First evidence of the MyD88-independent pathway was revealed by Kawai and colleagues [63]. Their work demonstrated the activation of NFκB following administration of LPS in MyD88 knockout mice indicating activation of TLR 4 independent of MyD88 [63]. TLR 4 activation independent of MyD88, is secondary to the presence of interferon (IFN) –β, a protein produced in response to pathogens, and leads to the production of inflammatory cytokines. The ability of TLR 4 to be activated dependent or independent of MyD88, indicates that the expression of inflammatory genes is regulated by several molecules during TLR 4 signaling.

TLR 4 activation dependent on MyD88 is the classical pathway of TLR 4 activation. Upon activation, interleukin-1 receptor-associated kinase (IRAK) is recruited by MyD88, leading to the activation of the NFκB inflammatory pathway, resulting in an increased transcription of inflammatory proteins [5]. Downstream signaling through MyD88 causes the phosphorylation of IκB, the protein inhibitor of the NFκB pathway, causing its degradation and allowing for NFκB translocation to the nucleus of the cell [56].

Activation of NFκB results in the transcription of inflammatory genes and the induction of other inflammatory pathways.

The activation of TLR 4 results in the increased presence of pro-inflammatory cytokines, chemokines, and ROS, all of which play a role in the innate immune system [57]. TLR 4 activation increases inflammatory molecules by stimulating transcription via the NFκB pathway. This increase in inflammatory markers contributes to the development of insulin resistance by enhancing inflammation. Due to its contribution to the inflammatory state, TLR 4 has been implicated as a potential mechanism through which obesity contributes in the development of insulin resistance.

TLR 4 is recognized as an important link between a dysregulated immune system and the development of insulin resistance, especially when associated with obesity [60-62]. It is well established that TLR 4 contributes to insulin resistance through activation of the NFκB pathway; however TLR 4 can also induce the inflammatory response independently of NFκB [57]. For example, TLR 4 is involved in regulating the activation of JNK, an important protein kinase in the modulation of insulin resistance. The role of TLR 4 in inducing inflammation, which alters insulin signaling, makes it a key player in the development of obesity-associated insulin resistance.

TLR 4 activation is increased in obesity, especially when coupled with the consumption of a high fat diet, due to an increase in the presence of ligands for the receptor and an increase in number and size of cells containing the receptor. Obesity is associated with an increase in adipocyte number and size as well as macrophage infiltration into the adipose tissue. Both macrophages and adipocytes readily express TLR

4, resulting in an increase in TLR 4 expression in obese individuals. Nutrient excess, a diet rich in saturated fat, and an increase in circulating endogenous FFA due to lipolysis, all provide ligands for TLR 4 activation [61, 65]. The increase in ligand availability as well as the increased expression of TLR 4, leads to a higher amount of TLR 4 activation and the chronic inflammation seen in obesity.

C3H/HeJ Animal Model

The use of the C3H/HeJ animal model has been important in determining the role of TLR 4 in the development of obesity-associated insulin resistance through regulation of the innate immune system. C3H/HeJ mice contain a defect in their response to LPS through a mutation in the cytoplasmic domain of TLR 4 His^{712} of the TLR 4 protein whereas wild-type or LPS-responsive animals would possess a proline [61, 62]. Due to this mutation, C3H/HeJ mice have been shown to have an altered response to a high fat diet, such as improved glucose tolerance and body composition when fed a high fat diet [4, 66-68]. These results demonstrate that TLR 4 plays an important role in the development of obesity-associated insulin resistance.

Suganami *et al.*(2007) compared the effects of a high fat diet (60% kcal from fat) versus a standard chow diet (5.4% kcal from fat) in wild-type (C3H/HeN) and TLR 4 deficient mice (C3H/HeJ) [66]. After feeding for 16 weeks, C3H/HeJ mice showed significantly lower glucose and insulin levels, indicating improved insulin sensitivity compared to wild-type mice. C3H/HeJ mice also showed higher adiponectin concentrations and reduced FFA levels when fed a high fat diet compared to standard chow diet. Lower FFA concentrations, indicates an improved insulin response due to

insulin's role in regulating lipolysis. Their findings demonstrate that TLR 4 plays a critical role in modulating obesity-associated insulin resistance.

Suganami *et al.* was also able to demonstrate an improved inflammatory response within the adipose tissue of C3H/HeJ mice compared to the wild-type mice when fed a high fat diet [66]. Similar results were observed by Poggi *et al.* who showed a reduction of the inflammatory response in adipocytes and macrophages of cells from C3H/HeJ mice fed a high fat diet [4]. These results indicate that TLR 4 plays a role in controlling obesity-associated insulin resistance both at the systemic and tissue level.

A similar study by Tsukumo *et al.* (2007) showed that TLR 4 deficient mice (C3H/HeJ) had a significantly lower body weight and epididymal fat pad weight compared to wild-type (C3H/HeN) mice when fed a high fat diet (55% kcal from fat) [68]. Adiponectin levels were also significantly decreased in C3H/HeN mice but not in C3H/HeJ mice [66, 68]. C3H/HeJ on the high fat diet also showed an improved glucose tolerance following an intraperitoneal glucose tolerance test (IGTT) compared wild type mice fed a high fat diet. Not only were improvements seen in weight and glucose control, improvements in the inflammatory response was also observed. C3H/HeJ mice exhibited reduced activation of the inflammatory pathways NFκB and JNK, which are commonly induced following high fat diet feeding [68]. These findings indicate that TLR 4 deficiency prevents the development of obesity-associated insulin resistance and induction of inflammation when mice are fed a high fat diet.

Overall, studies using the C3H/HeJ model, a model of TLR 4 deficiency, have shown improved glucose tolerance, insulin sensitivity and an improved body composition

as well as a reduced inflammatory response. These results indicate the important role of TLR 4 in obesity-associated insulin resistance. Based on these findings, we chose to use the C3H/HeJ model in the present study to investigate whether bitter melon could prevent or delay the development of insulin resistance by preventing the activation of TLR 4.

Phytochemicals

Phytochemicals are defined as non-nutrient or secondary metabolites that provide color and taste in fruits and vegetables but play no role in growth or development of plants [69]. Unlike vitamins and minerals, there are no current recommendations for the consumption of phytochemicals; however their use in preventative medicine has recently been a subject of extensive research [70]. Phytochemicals are hypothesized as a significant factor in preventing or delaying the development of disease [70]. While specific data regarding individual phytochemicals is limited, the role of phytochemicals as anti-inflammatory agent and their role in the prevention of chronic diseases such as type 2 diabetes and cardiovascular disease is extensive [71]. Phytochemicals have various mechanisms of action to exert their health benefits. Besides their action as an antioxidant to diminish oxidative damage, phytochemicals, may also act as hormones or to stimulate enzymatic action [71].

Polyphenols are the most relevant group of phytochemicals in regards to their role in providing extensive health benefits [72]. Polyphenols are made up of several different classes, lignans, flavonoids, phenolic acids, stilbene, and xanthones, all of which are found in various fruits, vegetables, beverages and spices [73]. While the use of polyphenols in the prevention of chronic disease is widespread, the use of polyphenols in relation to obesity is especially prevalent [71].

Polyphenols: Role in Inflammation, Obesity and Insulin Resistance

Diet with a higher nutrient density has been reported to reduce body weight and improve obesity-related conditions [73]. This indicates that intake of certain food components play a role in preventing obesity-related inflammatory diseases. Polyphenols are used as a multi-targeted remedy, due to their various mechanisms of action, against the development and progression of insulin resistance and type 2 diabetes mellitus [70]. Polyphenols act as antioxidants, anti-inflammatory agents as well as being key players in regulating glucose and lipid metabolism [71].

Bioactive food components exert their health benefits through several different mechanisms. These mechanisms include modulation of the NFκB, JNK and PPAR signaling pathways. Some polyphenols, such as resveratrol, have been implicated as ligands for PPARγ, interfering with the activation of the pro-inflammatory pathway NFκB and preventing the transcription of inflammatory genes [73]. Polyphenols such as resveratrol and quercetin have been shown to reduce NFκB and JNK activation, both of which contribute to the development of insulin resistance [74]. Polyphenols like genistein and amentoflavone have also been noted to enhance the gene expression of PPAR's [73]. In addition to attenuating the inflammatory response, polyphenols act as antioxidants by chelating metals, scavenging free radicals, and inhibiting free radical generation. Inhibition of oxidative stress and inflammation by phenolic compounds delays or prevents the development of obesity-related insulin resistance due to the prevention of cell damage [75].

Certain polyphenols have been shown to directly influence insulin sensitivity and glucose uptake. Quercetin, from bitter melon juice, for example enhances glucose uptake

in muscle cells [73]. Common spices such as cinnamon and cloves have exhibited insulin-like qualities [73]. There are many on-going studies examining the use of phytochemicals and bioactive food components in reducing insulin resistance. One functional food that has shown promise in improving insulin sensitivity is bitter melon. Therefore we investigated the use of bitter melon in modulating insulin resistance in a model of diet-induced obesity.

Bitter melon (*Momordica charantia, MC)*

Bitter melon (*Momordica charantia, MC)* also known as bitter gourd or balsam pear, is a tropical vegetable belonging to the cucurbitaceous family, including cucumbers, melons and gourds. MC is known for its bitter taste, which is enhanced as it ripens. MC is popular in Asian countries and has been widely used in traditional medicine as an anti-diabetic remedy throughout the world. Specifically, MC has been used by the indigenous populations in areas such as South America, India and Asia as a hypoglycemic agent [76]. MC has been demonstrated to not only provide hypoglycemic effects, but has also been used as an anti-cancer, anti-viral and anti-inflammatory agent [77].

Composition

Bitter melon contains significant amounts of micronutrients such as vitamin C, folate, calcium and magnesium, as well as being a good source of dietary fiber [78, 79]. MC also contains phytochemicals such as polypeptide-p, charantin, and vicine, all of which have been demonstrated to have insulin-like properties and aid in controlling blood glucose levels in diabetics [79]. Nutrient composition of the bitter melon variety used in this study can be found in Table 1.

A variety of phytochemicals have been isolated from the fruit, seeds and the whole MC plant. Charantin, containing sitosterol and stigmastadienol glucosides, has been implicated in being responsible for the hypoglycemic effects of MC, with the exact mechanism of action remaining unknown [77]. Vicine, isolated from the seeds of the plant, has also been shown to provide a hypoglycemic response when administered orally [77]. The isolated fraction from bitter melon, polypeptide-p, also referred to as p-insulin or v-insulin, has been most widely indicated as an insulin analog in both animal models and clinical trials of diabetes [80]. Other bioactive compounds have been isolated and contribute to the health benefits of bitter melon consumption, for example the component momordin, an oleanolic acid glycoside has been shown to contribute to the anti-tumor effects of bitter melon [81].

Studies on Health Benefits of Bitter Melon

Bitter melon has not only been used in Ayurvedic and traditional medicine, but has also been indicated as being potentially important in "westernized" medicine. Bitter melon has been investigated in association with a variety of health benefits, including its role in reducing inflammation, improving lipid parameters and most notably reducing the severity and preventing the development of insulin resistance [77, 81]. The various health benefits of MC have been demonstrated in models of human disease as well as in *in vivo* models.

The anti-inflammatory effects of MC extract in macrophage-like cells were investigated by *Kobori et al*. [82]. Raw 264.7macrophage-like cells were treated with LPS to induce inflammation in combination with a butanol extract of MC [82]. The MC butanol

fraction reduced the production of TNF-α and the activity of NFκB and JNK, common inflammatory intermediaries induced by LPS [82]. Bitter melon juice was shown to reduce inflammation by increasing anti-inflammatory cytokine secretion when administered to peritoneal macrophages concurrently with LPS [83]. However, a high dose of bitter melon, 500 µg/mL was needed to exert the anti-inflammatory effect.

The effect of MC in animal models of chronic conditions have also been investigated. For example, the effect of supplementation with a 3% (w/w) bitter melon seed diet for 100 days were investigated in female Zucker rats, a commonly used model of obesity [84]. This study showed the ability of MC to down-regulate the expression of NFκB within adipose tissue of obese mice. Bitter melon seed supplementation also reduced total cholesterol and low-density lipoprotein cholesterol. This work exemplifies the anti-inflammatory properties of bitter melon seeds and the possible cardio protective role of bitter melon.

Bitter melon has also been shown to improve lipid metabolism in animal models [85, 86]. Senanayake and colleagues investigated the effects of different dried MC fruit dosages and methanol extracts of various MC varieties in male Sprague-Dawley rats [86]. The methanol extract of the Koimidori variety was most effective in lowering hepatic triglyceride concentrations and serum triglyceride concentrations [86]. In a study performed by the same research group, male Sprauge -Dawley rats that were given the same methanol extract of the Koimidori variety in various amounts (0.5, 1 and 3% w/w) showed a dose-dependent improvement in liver triglyceride and cholesterol levels [86]. These results highlight the role of MC in attenuating the development of atherosclerosis and non-alcoholic fatty liver disease.

MC has also been demonstrated to control weight gain and reduce visceral fat mass accumulation in rats [87, 88]. This study was performed using female Sprague-Dawley rats that were either fed a high fat or low-fat diet for 6 weeks. At week 7, the low-fat group was switched to high fat, however, those on high fat diet were switched to a high fat diet containing different amounts of freeze-dried bitter melon powder (0.375, 0.75 or 1.5% w/w). Rats fed a high fat diet with bitter melon (0.75 and 1.5% w/w) exhibited an improved glucose tolerance following an oral glucose tolerance test, however no significant changes in body weight or fat mass was observed [87]. A separate experiment was conducted using male Sprague-Dawley rats that were randomly assigned to a high fat or low-fat diet and at 5 weeks the high fat fed animals were assigned to three different levels of MC (0.375%, 0.75%, or 1.5% w/w) [87]. The results of this study showed the ability of MC to significantly reduce fat mass, which contributed to the reduced free fatty acid concentrations as well as lower serum insulin and leptin concentrations. The reduction in fat mass also contributed to the ability of MC to improve glucose tolerance in this animal model [87].

Bitter Melon and Glucose Homeostasis

While research in humans is limited, overwhelming amounts of research verify the hypoglycemic effect of bitter melon in animal models [81, 88-90]. However, the mechanism through which the active components of MC act is unclear. Suggested hypoglycemic mechanisms of action include inhibition of glucose absorption, improved glucose utilization by the liver, adipose tissue and skeletal muscle as well as a possible improvement in insulin action [89]. MC also affects the regulation of PPARα and PPARγ,

which play a role in glucose metabolism and lipid homeostasis [83]. Alterations in the PPARα and PPARγ pathways are linked to the pathogenesis of insulin resistance [83].

Several animal studies have shown the role of MC in regulating glucose homeostasis through a PPAR mediated pathway [84, 88]. A study performed by Gadang *et al.* using Zucker rats showed that MC seeds increased the expression of PPARγ in white adipose tissue [84]. Bitter melon also had the ability to down-regulate several targets of PPARγ, including reducing the expression of NFκB [84].

An animal study using male C57BL/6J mice, gave evidence to the role of MC in regulating the PPAR mediated pathway [89]. This study analyzed the effectiveness of MC compared to rosiglitazone, a type of TZD prescribed in the treatment of type 2 diabetes, in improving glucose homeostasis in mice fed a high fat diet. The researchers observed that bitter melon, increased the expression of PPARγ in adipose tissue, leading to the increased uptake of glucose and lowered expression of leptin. This study demonstrated the role of MC in improving glucose homeostasis and regulating adipocyte differentiation similar to the action of TZDs [83]. Similar research using a fructose-induced diabetes model in male Sprague-Dawley rats, attributed the role of MC in improving insulin resistance to increased mRNA expression of PPARγ in white adipose tissue. Sprague-Dawley rats were fed a control or high-fructose (60% fructose) diet for 8 weeks and were then subdivided into six different treatment groups where they were fed varying doses of MC or rosiglitazone [90]. Addition of MC to a high-fructose diet, increased protein expression of GLUT 4 in the skeletal muscle and prevented fructose-induced leptin abnormalities, both of which contributed to improved insulin sensitivity [90].

Other signaling pathways involved in glucose homeostasis have also been shown to be altered by bitter melon. Tan *et al.* showed that MC activated the AMPK pathway which stimulated GLUT 4 translocation from the cytosol to the cell membrane to improve glucose uptake and enhance insulin sensitivity in skeletal muscle [91]. MC has also been shown to reverse the inhibition of tyrosine phosphorylation of the insulin receptor substrate (IRS) 1, a common dysregulation on insulin signaling present in insulin resistant individuals [92]. The various modes of action in which MC is able to act indicate its multiple roles in attenuating insulin resistance and the deregulation of glucose homeostasis.

The role of bitter melon in controlling glucose homeostasis and improving insulin sensitivity has been compared with the use of prescribed medications commonly used in patients with type 2 diabetes mellitus. When compared to Metformin in a clinical trial, bitter melon improved fructosamine concentrations in patients, however no effect was exhibited on fasting plasma glucose levels or the ability of the body to clear glucose in an oral glucose tolerance test [93]. Therefore, it is important to note that further research is needed to determine dosages and methods of supplementation of bitter melon appropriate in humans to obtain similar results seen in animal studies.

CHAPTER III

METHODOLOGY

This study was designed to determine the dose-dependent effects of bitter melon on glucose homeostasis, body composition, and lipid parameters in mice fed a high fat diet. This study also examined the effects of TLR 4 on high fat diet induced obesity, inflammation and insulin resistance, therefore the C3H/HeJ strain was used.

Animal Care and Dietary Treatment

The Institutional Animal Care and Use Committee (IACUC) at Oklahoma State University approved the protocol for this animal study. Eight-week old male C3H/HeJ mice (n=48) were purchased from Jackson Laboratories (Bar Harbor, ME) and C57BL/6 mice (n=49), were purchased from Charles River Laboratory (Kingston, NY). Mice were housed in an Oklahoma State University (OSU) animal research facility in a climate-controlled room with a 12hr light/dark cycle. After 7 days of acclimation, mice from each strain were randomly divided into four dietary treatment groups (n=12-13/group): control with 10% kcal from fat, high fat (HF) with 60% kcal from fat, HF +1% (w/w) freeze dried bitter melon (MC) and HF + 10% (w/w) freeze dried MC.

Bitter melon was purchased from a local ethnic grocery store, washed, dried, seeds were removed, cut into smaller pieces, freeze-dried and ground before being incorporated into the diet. The control diet was based on the AIN-93M diet (Reeves,

1993). The macronutrient, calcium, and phosphorus content of MC were analyzed by Nestle Purina Analytical Laboratories (St. Louis, MO). Contributions of MC were adjusted to assure that all HF diets had similar macronutrient, calcium, and phosphorus content. Composition of MC and experimental diets used in this study and of MC can be found in Tables 1 and 2, respectively. Mice had access to food and deionized water *ad libitum*. Food intake was monitored three times per week and body weights were measured on a weekly basis. Dietary treatment lasted for nine weeks.

Glucose Tolerance Test

After 8 weeks of dietary treatment, a glucose tolerance test was performed. Following a 6 hr fast, mice were administered an intraperitoneal injection of 20% glucose solution (2 g glucose / kg of body weight). Blood samples were obtained from the tail vein and glucose concentrations were measured at 0, 5, 15, 30, 60 and 120 minutes post glucose injection using an AlphaTrak blood glucose monitoring system (Abbot Laboratories, Chicago, IL).

Necropsy, Tissue Collection and Storage

After 9 weeks of dietary treatment, following a 6 hr fast, mice were anesthetized with ketamine/xylazine cocktail (10 mg/ml ketamine and 1 mg/mL xylazine) with a dosage of 0.006 mL/gram of body weight. Body composition and bone density were measured using dual energy x-ray absorptiometry (GE Lunar Piximus, Fitchburg, WI). Blood was collected from the carotid artery and placed in a 1.5 mL microcentrifuge tube coated with an anticoagulant, ethylenediamine tetraacetic-acid (EDTA). Liver, visceral and peri-renal white adipose tissue, thymus, spleen, and kidneys were collected and

weighed. Portions of the tissues were stored in 10% neutral buffered formalin (NBF) and the remainder was snap frozen in liquid nitrogen and stored at -80ºC for further analyses. Plasma was obtained by centrifugation of whole blood for 20 min at 4 x g, aliquot and stored at -80°C for later use.

Clinical Parameters of Inflammation and Diabetes

Plasma triglycerides, total cholesterol, fructosamine, glucose and high-density lipoprotein (HDL) concentrations were determined using the BioLis24i clinical analyzer (Carolina Liquid Chemistries Corporation™, Brea, CA). Reagents were purchased from Carolina Liquid Chemistries Corporation™ (Brea, CA) with the exception of NEFA which was purchased from Wako Diagnostics (Richmond, VA).

Measurement of triglyceride concentrations were determined by mimicking the hydrolysis of fatty acids to glycerol in the liver. Plasma triglycerides were hydrolyzed to form free fatty acids and a glycerol. Glycerol was then phosphorylated by glycerol-1-phosphate which produced hydrogen peroxide (H_2O_2) which reacts with *p*-chlorophenol and 4-aminoantipyrine to yield a red color quinoneimine. Change in absorbance was measured as 505/692 nm, which represented the concentration of triglycerides in the sample (Carolina Liquid Chemistries Corporation™, Brea, CA).

To determine total cholesterol, cholesterol was hydrolyzed by cholesterol esterase to free cholesterol and free fatty acids, forming H_2O_2 . Total cholesterol is further esterified by cholesterol esterase; in this reaction H_2O_2 was formed. Hydrogen peroxide then reacts with 4-aminoantipyrine and p-hydroxyenzoate to form red color quinoneimine. The red color formed absorbs at 500 nm and is directly proportional to the

cholesterol concentration in the sample. Glucose concentrations were determined by the use of the enzymatic hexokinase and glucose-6-phosphate dehydrogenase reaction in the presence of magnesium where glucose-6 phosphate and adenosine diphosphate (ADP) are produced. Glucose-6-phosphate dehydrogenase acts to oxidize glucose-6-phosphate and NAD^+ to form 6-phosphogluconate and NADH. The amount of NADH produced is proportional to the amount of glucose present in the plasma, NADH absorbs at 340 nm which is proportional to the amount of glucose in the sample (Carolina Liquid Chemistries Corporation™, Brea, CA)

To determine HDL concentrations, HDLD detergent is added to plasma samples, solubilizing the HDL fraction and allowing HDL to be released. Concentrations of HDL are determined by the interaction of HDL with cholesterol esterase, cholesterol oxidase and chromogens to from color. The color produced, which absorbs at 600 nm, is proportional to HDL cholesterol in the sample (Carolina Liquid Chemistries Corporation™, Brea, CA).

Plasma non-esterified free fatty acids (NEFA) concentrations were measured using a colorimetric assay kit following the manufacturer's instructions. In the presence of ATP and CoA, NEFA are converted to thiol esters of CoA by acyl-CoA synthetase. Acyl-CoA is then oxidized and H_2O_2 is produced as a byproduct which allows for condensation and the formation of purple color. The color produced absorbs at 550 nm which is proportional to NEFA concentrations (Wako Diagnostics, Richmond, VA).

Fructosamine levels were analyzed to indicate glycemic control over the prior 2-3 weeks. Fructosamine concentrations were determined by a colorimetric procedure based

on the ability of ketoamines to form formazan dye. Nitroblue tetrazolium (NBT) is reduced to a formazan dye when reagent is added to the plasma sample; the rate of formazan dye formation is proportional to fructosamine concentration based on the absorbance at 505 nm (Carolina Liquid Chemistries Corporation™, Brea, CA).

Hemoglobin A1c (HbA1c) levels were analyzed in whole blood samples collected with EDTA anticoagulant utilizing an antigen and antibody reaction using a commercial kit (Carolina Liquid Chemistries Corp., Brea CA). Over time, hemoglobin A1C is formed by the addition of glucose to the end of the hemoglobin chain allowing this measure to indicate the control of blood glucose levels in diabetic individuals. The reagents, mouse antihuman HbA1c monoclonal antibody and goat anti-mouse IgG polyclonal antibody are added allowing for agglutination. Agglutination is proportional to the amount of HbA1c absorbed on latex particles which is then measured as absorbance at 660 nm.

Plasma leptin and adiponectin levels were measured using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The principle of this assay involves the capture and concurrent binding of the given molecule, leptin or adiponectin in this case and the absorbance is measured, which is directly proportional to the amount of bound analyte. Concentrations of TNF-α were measured by a Quantikine immunoassay kit, a solid phase ELISA (R&D Systems, Minneapolis, MN). Briefly, this kit contains a plate pre-coated with a monoclonal antibody specific for the mouse TNF-α. When samples are added, mouse TNF-α is bound by the antibody. After washing, and adding a stop solution, the intensity of the color is measured, by absorbance, which is proportional to the amount of TNF-α present in the sample (R&D. Minneapolis, MN).

Total RNA Extraction

Total RNA was extracted from liver and visceral white adipose tissue samples (n=6/group) of mice using RNA STAT-60 reagent (Tel-test Inc., Friendswood, TX) and protocol (AMS Biotechnology, Lake Forest, CA). Briefly, samples were homogenized in 1 mL of STAT-60 reagent/100 mg of tissue. Following homogenization, samples were allowed to sit for 5 min on ice, and then chloroform (200 µL/1 mL of STAT-60) was added and allowed to sit for 3 min to aid in phase separation. Samples were centrifuged for 15 min at 12 x g at 4° C, and the colorless aqueous phase was transferred to a new centrifuge tube. Isopropanol (0.5 mL/1 mL STAT-60) was added and samples were allowed to precipitate on ice for 30 min. Samples were centrifuged again for 10 min at 12 x g at 4° C , isopropanol was decanted and 75% ethanol (1 mL/1 mL STAT-60) was used to wash and remove remaining contaminants. Samples were centrifuged for 5 min at 12 x g at 4° C, ethanol was decanted and RNA was dissolved in diethylpyrocarbonate (DEPC) treated water.

The integrity of the total RNA was analyzed using 1% agarose gel electrophoresis to assure the presence of 18S and 28S bands with ethinium bromide staining. The RNA concentration (ng/µL) was determined by analysis of optical density at 260/280 nm using a NanoDrop Analysis machine (Thermo Scientific, Wilmington, DE).

Real-Time Polymerase Chain Reaction (PCR)

Following extraction of total RNA from the liver and WAT, complementary DNA (cDNA) was synthesized. This cDNA was then used for real-time PCR. Real-time PCR

was performed using two pathway focused RT^2 Profiler PCR arrays purchased from Qiagen (Qiagen, Valencia, CA). The manufacturer's instructions were followed. The following arrays were used to determine gene expression of particular interest; PPAR and toll-like receptor signaling in the liver and white adipose tissue respectively. Liver samples of C57BL/6 mice from each dietary treatment group (n=4/group) were used for the PPAR array. White adipose tissue samples of HF and HF + 10% BM groups (n=4/group) of both strains (C57BL/6 and C3H/HeJ) were used for the toll-like receptor signaling array.

Liver Histology

Portions of liver were stored in 10% neutral buffered formalin for histology. Liver portions (n=6/group) were dehydrated through a graded ethanol series using a Thermo Scientific Shandon-Citadel 1000 tissue processor (Austin, TX) and then embedded in paraffin wax using the Leica EG1160 paraffin embedder (Buffalo Grove, IL). Samples were sectioned into 7 µm thicknesses using the high performance Leica RM 2165 microtome (Buffalo Grove, IL) and stained using a hematoxylin and eosin (H&E) stain. Stained liver samples were scored based on degree of steatosis and inflammation. Liver samples were analyzed and scored by a pathologist (Dr. Stan Lightfoot, Department of Pathology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK). The degree of steatosis was graded on a scale of 0-4, 0 indicating no steatosis and 4 indicating excessive steatosis.

Statistical Analyses

Data was analyzed using SAS version 9.2 (SAS Institute Inc., Cary, NC). Data was analyzed using a 2X4 factorial; results were reported as mean ± standard error of the mean and considered statistically significant if the *p*-value was less than 0.05. Categorical data derived from the pathology scoring were compared using a chi-square test followed by Fisher's Exact Test to confirm the significance. Results of the PCR array were analyzed using online software (RT^2 Profiler PCR Array Data Analysis version 3.5) from SABiosciences (Qiagen, Valencia, CA); results were compared to the set control with a *p*-value less than 0.05 considered statistically significant.

CHAPTER IV

FINDINGS

Effects of Bitter Melon and TLR 4 on Body Weight and Food Intake (Table 3 and Figure 1)

Prior to initiation of dietary treatment, there were no significant differences in body weights between strain or dietary treatment groups. Significant differences in body weights were observed starting at week two of dietary treatment (Figure 1). After 90 days of dietary treatment, C3H/HeJ mice had a significantly higher body weight compared to C57BL/6 mice. Mice that received the high fat (HF) and HF + 1% MC had similar final body weights and were higher than the control group and HF + 10% MC group. The control group has the lowest final body weight, while the HF + 10% MC had a final body weight in between the HF and control groups (Table 3).

While there was a difference in final body weight between the two strains, no significant difference in caloric intake was observed (Table 3). Therefore, the difference in body weight between the two strains cannot be explained by the differences in food intake. The HF + 1% MC had the highest caloric intake, followed by the HF + 10% MC

which was similar to the HF group. As expected, the control group had the lowest caloric intake.

Effects of Bitter Melon and TLR 4 on Relative Tissue Weights (Table 4)

Tissue weights were analyzed relative to body weights to normalize the changes in body weight. Peri-renal white adipose tissue (WAT) relative weights were significantly lower in C3H/HeJ mice, however no difference was observed in visceral WAT relative weights (Table 4). In both strains, HF + 10% MC significantly reduced the accumulation of fat in the visceral and peri-renal regions compared to mice in the HF and HF + 1% MC groups, though not to the level of control animals. Relative thymus and kidney weights also differed among strains; C3H/HeJ mice had higher kidney weights and lower thymus weights compared to C57BL/6 mice (Table 4). No significant differences in relative spleen and liver weights were observed between strains. HF + 10% MC reduced relative liver and thymus weights and had no effect on relative kidney weights in both strains of mice.

A significant interaction effect was observed between diet and strain in the relative weight of peri-renal WAT (Table 4). Bitter melon, in both 1% and 10% increments, had lower peri-renal WAT weights in C3H/HeJ mice compared to C57BL/6 mice. Also, the high fat diet had less of an effect on C3H/HeJ mice compared to C57BL/6 mice in regards to lipid accumulation and adipocyte expansion in peri-renal WAT regions, indicating a diet effect as well as a strain effect.

Effects of Bitter Melon and TLR 4 on Body Composition (Table 5)

Body composition was measured prior to sacrifice to determine effect of diet and strain. C3H/HeJ mice had a significantly higher body fat percentage and a lower lean body mass (LBM) percentage compared to C57BL/6 mice, which is consistent with the findings of Poggi and colleagues [4]. However, C3H/HeJ mice also exhibited an increased bone mineral density (BMD) and content (BMC), and area (BMA) compared to C57BL/6 mice (Table 5).

An effect of dietary treatment was also observed in regards to body composition. Mice fed a diet with HF + 10% MC but not HF + 1% MC, were shown to have a lower body fat percentage and a higher LBM percentage when compared to other high fat groups (Table 5). Mice fed a HF + 10% MC were also shown to have higher BMC and total bone area in both strains of mice, compared to the HF + 1% MC. There was no difference in BMD between dietary treatments.

Effects of Bitter Melon and TLR 4 on Plasma Lipid Parameters (Table 6)

Clinical lipid parameters were analyzed to determine the role of TLR 4 and bitter melon in lipid metabolism. C3H/HeJ mice were shown overall to have significantly higher lipid levels when compared to C57BL/6 mice. Specifically, total cholesterol and triglyceride levels were elevated in the TLR 4 deficient strain (Table 6). However, C3H/HeJ also had elevated HDL cholesterol levels. The levels of non-esterified fatty

acids (NEFA) levels in C3H/HeJ mice were significantly lower compared to C57BL/6 mice, which may be related to improved insulin sensitivity.

When comparing the effects of dietary treatment, mice fed the HF + 10% MC diet showed improved lipid profiles. Cholesterol and triglyceride levels of mice fed the HF + 10% MC diet were significantly lower compared to HF and HF + 1% MC groups (Table 6). However, the protective cholesterol, HDL, was also significantly lower which contributed to the reduced cholesterol levels. Interestingly, triglyceride levels of mice fed the HF + 10% MC were significantly lower than the mice fed the control diet. These results indicate the beneficial effect on lipid parameters of supplementing a high fat diet with bitter melon in 10% dose.

Effects of Bitter Melon and TLR 4 on Parameters Related to Glucose Metabolism (Tables 7 and Figure 2)

Clinical parameters related to glucose metabolism were analyzed to determine the effects of bitter melon and TLR 4 on glucose metabolism and utilization. Hemoglobin A1C (HbA1C) was analyzed to determine the effects of bitter melon and TLR 4 on overall glucose control (Table 7). C3H/HeJ mice were shown to have a significantly lower HbAIC level compared to C57BL/6 mice, indicating improved glucose control in the long-term and a possible role of TLR 4 activation playing a role in glucose utilization. Fructosamine was also analyzed to determine a similar affect; however fructosamine is more indicative of short-term glucose control in mice. Fructosamine was significantly

lower in C3H/HeJ mice than in C57BL/6 mice, again indicating improved glucose control in these mice (Table 7). There was no effect of diet on HbA1C or fructosamine levels, indicating no effect of bitter melon on glucose metabolism; however, the experimental period may not have been long enough to induce a dietary effect as the turnover of red blood cells which contributes to HbA1C levels is every 3 months.

A dietary treatment effect was seen on fasting blood glucose levels while no difference was observed between the two strains. Mice fed the HF + 10% MC had the lowest blood glucose concentrations and were comparable to the control group; indicating a positive effect of bitter melon on glucose metabolism.

A glucose tolerance test was performed at 8 weeks of dietary treatment and total area under the curve (AUC) was calculated to compare the overall glucose response due to dietary treatment and strains. There was a significant difference in glucose at all-time points of the glucose tolerance test except at 15 minutes post-glucose injection (Figure 2). Mice fed the HF + 10% MC diet had significantly lower total AUC compared to HF and HF + 1% MC and similar to mice fed the control diet. A lower total AUC indicates the body's ability to clear circulating glucose at a faster rate compared to other groups. C3H/HeJ mice had a significantly lower total AUC compared to C57BL/6 mice, again indicating an improved response in the periphery to circulating glucose (Table 7). An interaction between diet and strain was also observed in regards to total AUC. Bitter melon, specifically in a 10% dosage, was able to significantly improve the blood glucose

response of C3H/HeJ mice to the level mice fed the control diet. Also, C3H/HeJ mice fed a high fat diet alone, had a significantly lower total AUC compared to C57BL/6 mice fed a high fat diet. These results indicate not only an effect of diet on the glucose response but also an effect of strain and more specifically, TLR 4.

Effects of Bitter Melon and TLR 4 on Liver Steatosis and Inflammation (Table 8)

Liver and white adipose tissue histology samples were analyzed by a pathologist, Dr. Stan Lightfoot. There was not a significant amount of inflammation observed within the liver of the animals in either strain or diet. There was also no difference between strain on degree of liver steatosis. However, significant differences were observed between diets in the degree of liver steatosis. Mice fed the HF + 10% MC diet had lower degree of steatosis compared to mice fed the HF or HF + 1% MC diet, confirmed by a Fisher's Exact Test (p = 0.0054). Interestingly, mice that received the HF +10% MC diet had lower degree of liver steatosis compared to those that received the control diet.

Effects of Bitter Melon and TLR 4 on Gene Expression

The PPAR PCR array was analyzed in the liver of C57BL/6 animals and results were compared to the group fed a high fat diet without bitter melon. Bcl2l1, a member of the BCL-2 family, was up regulated in the HF + 10% MC group. The family of BCL-2 proteins play a role in regulation of apoptosis, or programmed cell death. Our results might indicate an increase in apoptosis in the liver of these animals [89]. Significant results

were also found in relation to insulin signaling. PDPK1, also known as PDK-1 or 3-phosphoinositide-dependent protein kinase-1, was significantly increased in the HF + 10% MC group compared to the group fed a high fat diet alone. Phosphoinositide-dependent kinase 1 is a serine/threonine protein kinase important in regulating insulin signaling through activation of Akt/protein kinase B (PKB) [19,23]. An increased expression of PDK-1 in these animals may suggest improved insulin signaling compared to mice fed a high fat diet. There were no significant differences between the groups in genes involved in fatty acid oxidation (Acox1), fatty acid synthesis (Acaca), gluconeogenesis (Fbp1 and G6pc), glycogenolysis (Gck), or hormones secreted from the adipose tissue (Lep & Retn). There was also no significant difference between groups in the gene that encodes for IRS-1, which is important as this is a major point of insulin signaling regulation or in the inflammatory kinase JNK pathway.

A second PCR array was completed to determine the effects of both bitter melon and TLR 4 on genes related to the toll-like receptor signaling pathways. Results indicated an increase in C3H/HeJ mice of genes related to the innate immune response (CD14, Hspd1, Csf3, Fos, Irak1, and Ly86) indicating an increase in the inflammatory response in C3H/HeJ compared to C57BL/6 mice when fed a high fat diet. Results also indicated an increase in Chuk, a gene which encodes for the IKK α, the inhibitor of NFκB possibly demonstrating a decreased activation of NFκB. However, there were no changes in TLR 4 which would have been expected in C3H/HeJ mice since they only contain a mutation related to functionality of the receptor. Additionally, there were no significant differences

in the MyD88, an adapter protein for TLR 4, or downstream proteins of TLR 4 activation (JUN). Additional results from this PCR array were more in line with expected outcomes. Wild-type mice (C57BL/6) fed the HF +10% MC diet had significantly lower TNF levels when compared with mice fed the high fat diet alone. These results implicated an anti-inflammatory effect of bitter melon.

Table 1. Composition of Bitter Melon Used in the Study*

Analyte	Amount (g/100g)
Carbohydrate	**75.3**
Protein	**10.2**
Fat	**2.69**
Crude Fiber	**14.2**
Ash	**8.91**
Calcium	**0.231**
Phosphorus	**0.603**
Calories (kcal/100g)	**366**

***** Analyzed by Nestle Purina Analytical Laboratories (St. Louis, MO).

Table 2. Diet Composition [a]

	Control (AIN-93M)[b]	High Fat (HF)[c]	HF +1% Bitter Melon[c]	HF + 10% Bitter Melon[c]
Ingredients	*(g/kg of diet)*			
Freeze dried MC			**10**	**100**
Total Carbohydrate	**600.0**	**295.0**	**295.0**	**295.0**
Cornstarch	495.5	44.5	37.2	
Maltodextrin	40.0	160.0	160.0	131.6
Sucrose	100.0	90.0	90.0	90.0
Contribution of freeze dried MC[d]			7.53	75.3
Total Protein	**210.0**	**265.0**	**265.0**	**265.0**
Casein	210.0	265.0	264.0	254.8
Contribution of freeze dried MC[d]			1.02	10.2
Total Fat	**40.0**	**340.0**	**340.0**	**340.0**
Soybean oil	40.0	30.0	29.7	27.3
Lard		310.0	310.0	310.0
Contribution of freeze dried MC[d]			0.269	2.69
Total Fiber	**50.0**	**50.0**	**50.0**	**50.0**
Cellulose	50.0	50.0	48.6	35.8
Contribution of freeze dried MC[d]			1.42	14.2
Vitamin mix (AIN -93VX)[a]	10.0	10.0	10.0	10.0
Mineral mix (AIN-93G-MX)[a]	35.0	35.0	35.0	35.0
Choline Bitartrate	2.5	2.5	2.5	2.5
L-cysteine	3.0	3.0	3.0	3.0
Tert-butylhydroquinone	0.04	0.06	0.06	0.06

[a] All diets were prepared by Harlan Teklad Diets (Madison, WI)

[b] Based on AIN-93M formulations, caloric contribution for carbohydrate, fat and protein is 72%, 4% and 14%, respectively (Reeves *et al*., 1997)

[c] All high fat diets have similar carbohydrate, fat, protein, calcium and phosphorus content. The caloric contribution for carbohydrate, fat and protein is 23.0%, 59.1%, and 17.9%, respectively.

[d] Bitter melon composition was analyzed by Nestle Purina Analytical Laboratories (St. Louis, MO). Please see Table 1.

Table 3. Initial and final body weights and food intake

Dietary Treatment	Strain	n	Initial body weight (g)	Final body weight (g)	Food intake (kcal/mice/day)
AIN	C3H/HeJ	11	18.2 ± 0.4	29.5 ± 0.9	14.6 ± 0.3^{c}
AIN	C57BL/6	12	18.0 ± 0.4	27.1 ± 0.9	13.3 ± 0.3^{d}
HF	C3H/HeJ	12	18.4 ± 0.4	35.6 ± 0.9	15.7 ± 0.3^{b}
HF	C57BL/6	12	18.0 ± 0.4	34.9 ± 0.9	15.4 ± 0.3^{bc}
HF+1% MC	C3H/HeJ	12	18.0 ± 0.4	36.0 ± 0.9	16.0 ± 0.3^{b}
HF+1% MC	C57BL/6	12	18.0 ± 0.4	35.3 ± 0.9	17.0 ± 0.3^{a}
HF+10% MC	C3H/HeJ	12	18.4 ± 0.4	32.3 ± 0.9	16.0 ± 0.3^{b}
HF+10% MC	C57BL/6	13	18.0 ± 0.4	30.3 ± 0.8	15.5 ± 0.3^{b}
Dietary Treatments					
AIN		23	18.1 ± 0.3	28.3 ± 0.6^{C}	14.0 ± 0.2^{C}
HF		24	18.2 ± 0.3	35.3 ± 0.6^{A}	15.6 ± 0.2^{B}
HF+1% MC		24	18.0 ± 0.3	35.6 ± 0.6^{A}	16.5 ± 0.2^{A}
HF+10%MC		25	18.2 ± 0.3	31.3 ± 0.6^{B}	15.8 ± 0.2^{B}
Strain					
C3H/HeJ		47	18.3 ± 0.2	33.3 ± 0.4	15.6 ± 0.2
C57BL/6		49	18.0 ± 0.2	31.9 ± 0.43	15.3 ± 0.2
P values					
Diet			0.9472	< .0001	< .0001
Strain			0.3993	0.0210	0.3300
Diet*Strain			0.9316	0.6711	0.0005

Values represent mean ± standard error of the mean. $p < 0.05$ is considered statistically significant. Values that do not share the same letter are considered statistically different. Lower case letters indicate significant differences related to a diet and strain interaction, capital letters indicate significant differences between dietary treatment group.

Table 4. Relative tissue weights (gram of tissue/gram body weight)

Dietary Treatment	Strain	n	Liver (%)	Peri-renal WAT (%)	Visceral WAT (%)	Spleen (%)	Kidney (%)	Thymus (%)
AIN	C3H/HeJ	11	3.95 ± 0.10	0.63 ± 0.10^{d}	2.19 ± 0.25	0.35 ± 0.07	1.83 ± 0.05	0.13 ± 0.01
AIN	C57BL/6	12	4.05 ± 0.10	0.72 ± 0.09^{d}	2.06 ± 0.24	0.32 ± 0.07	1.35 ± 0.05	0.21 ± 0.01
HF	C3H/HeJ	12	3.50 ± 0.10	1.16 ± 0.09^{bc}	4.59 ± 0.24	0.31 ± 0.07	1.56 ± 0.05	0.12 ± 0.01
HF	C57BL/6	12	3.64 ± 0.10	2.06 ± 0.09^{a}	4.56 ± 0.24	0.27 ± 0.07	1.17 ± 0.05	0.21 ± 0.01
HF+1% MC	C3H/HeJ	12	3.41 ± 0.10	1.11 ± 0.09^{c}	4.49 ± 0.24	0.27 ± 0.07	1.53 ± 0.05	0.11 ± 0.01
HF+1% MC	C57BL/6	12	3.52 ± 0.10	2.01 ± 0.09^{a}	4.78 ± 0.24	0.25 ± 0.07	1.22 ± 0.05	0.19 ± 0.01
HF+10% MC	C3H/HeJ	12	3.49 ± 0.10	1.06 ± 0.09^{c}	4.19 ± 0.24	0.51 ± 0.07	1.65 ± 0.05	0.08 ± 0.01
HF+10% MC	C57BL/6	13	3.55 ± 0.09	1.39 ± 0.09^{b}	3.35 ± 0.23	0.27 ± 0.06	1.30 ± 0.05	0.20 ± 0.01
Dietary Treatments								
AIN		23	4.00 ± 0.07^{A}	0.67 ± 0.07^{C}	2.13 ± 0.17^{C}	0.34 ± 0.05	1.59 ± 0.04	0.17 ± 0.01^{A}
HF		24	3.57 ± 0.07^{B}	1.61 ± 0.07^{A}	4.58 ± 0.17^{A}	0.29 ± 0.05	1.36 ± 0.03	0.16 ± 0.01^{AB}
HF+1% MC		24	3.47 ± 0.07^{B}	1.56 ± 0.07^{A}	4.63 ± 0.17^{A}	0.26 ± 0.05	1.38 ± 0.03	0.15 ± 0.01^{AB}
HF+10% MC		25	3.52 ± 0.07^{B}	1.23 ± 0.06^{B}	3.77 ± 0.17^{B}	0.39 ± 0.05	1.48 ± 0.03	0.14 ± 0.01^{B}
Strain								
C3H/HeJ		47	3.59 ± 0.05	0.99 ± 0.05	3.86 ± 0.12	0.36 ± 0.03	1.64 ± 0.02	0.11 ± 0.01
C57BL/6		49	3.69 ± 0.05	1.55 ± 0.05	3.69 ± 0.12	0.28 ± 0.03	1.26 ± 0.02	0.20 ± 0.01
P values								
Diet			< .0001	< .0001	< .0001	0.2487	< .0001	0.1298
Strain			0.1361	< .0001	0.3106	0.0767	< .0001	< .0001
Diet*Strain			0.9802	< .0001	0.1197	0.2518	0.2945	0.2949

Values represent mean ± standard error of the mean. $p < 0.05$ is considered statistically significant. Values that do not share the same letter are considered statistically different. Lower case letters indicate significant differences related to a diet and strain interaction, capital letters indicate significant differences between dietary treatment groups.

Table 5. Body composition

Dietary Treatment	Strain	n	Lean Mass (g)	Fat Mass (g)	% Fat	% Lean	BMD (mg/cm^2)	BMC (mg)	BMA (cm^2)
AIN	C3H/HeJ	11	21.4 ± 0.5	7.90 ± 0.6	26.8 ± 1.0	73.2 ± 1.0	59.7 ± 0.6	655.9 ± 13.5	11.0 ± 0.2
AIN	C57BL/6	12	20.2 ± 0.4	6.68 ± 0.5	24.9 ± 0.9	75.1 ± 0.9	49.2 ± 0.6	497.2 ± 12.9	10.1 ± 0.2
HF	C3H/HeJ	12	22.9 ± 0.4	12.9 ± 0.5	35.9 ± 0.9	64.1 ± 0.9	58.1 ± 0.6	591.8 ± 12.9	10.2 ± 0.2
HF	C57BL/6	12	22.9 ± 0.4	12.1 ± 0.5	34.2 ± 0.9	65.8 ± 0.9	49.4 ± 0.6	462.8 ± 12.9	9.37 ± 0.2
HF+1% MC	C3H/HeJ	12	22.8 ± 0.4	13.5 ± 0.5	37.0 ± 0.9	63.0 ± 0.9	57.5 ± 0.6	573.8 ± 12.9	9.97 ± 0.2
HF+1% MC	C57BL/6	12	23.2 ± 0.4	12.4 ± 0.5	34.7 ± 0.9	65.3 ± 0.9	49.3 ± 0.6	461.2 ± 12.9	9.35 ± 0.2
HF+10% MC	C3H/HeJ	12	21.6 ± 0.4	10.7 ± 0.5	33.0 ± 0.9	67.0 ± 0.9	59.2 ± 0.6	618.0 ± 12.9	10.4 ± 0.2
HF+10% MC	C57BL/6	12	20.9 ± 0.4	8.83 ± 0.5	29.6 ± 0.9	70.4 ± 0.9	48.9 ± 0.6	470.8 ± 12.9	9.61 ± 0.2
Dietary Treatments									
AIN		23	20.8 ± 0.3^{B}	7.30 ± 0.4^{C}	25.8 ± 0.7^{C}	74.2 ± 0.7^{A}	54.4 ± 0.4	576.5 ± 9.3^{A}	10.5 ± 0.1^{A}
HF		24	22.9 ± 0.3^{A}	12.5 ± 0.4^{A}	35.0 ± 0.7^{A}	65.0 ± 0.6^{C}	53.7 ± 0.4	527.3 ± 9.1^{BC}	9.77 ± 0.1^{BC}
HF+1% MC		23	23.0 ± 0.3^{A}	12.9 ± 0.4^{A}	35.9 ± 0.7^{A}	64.1 ± 0.6^{C}	53.4 ± 0.4	517.5 ± 9.1^{C}	9.66 ± 0.1^{C}
HF+10% MC		25	21.2 ± 0.3^{B}	9.80 ± 0.4^{B}	31.3 ± 0.7^{B}	68.7 ± 0.7^{B}	54.0 ± 0.4	544.4 ± 9.1^{B}	10.0 ± 0.1^{B}
Strain									
C3H/HeJ		47	22.2 ± 0.2	11.3 ± 0.3	33.2 ± 0.5	66.8 ± 0.5	58.6 ± 0.3	609.9 ± 6.5	10.4 ± 0.1
C57BL/6		48	21.8 ± 0.2	10.0 ± 0.3	30.9 ± 0.5	69.2 ± 0.5	49.2 ± 0.3	473.0 ± 6.5	9.61 ± 0.1
P values									
Diet			< .0001	< .0001	< .0001	< .0001	0.3588	0.0001	< .0001
Strain			0.2017	0.0011	0.0005	0.0005	< .0001	< .0001	< .0001
Diet*Strain			0.2535	0.7475	0.7924	0.7924	0.1469	0.3084	0.8708

Values represent mean ± standard error of the mean. $p < 0.05$ is considered statistically significant. Values that do not share the same letter are considered statistically different. Lower case letters indicate significant differences related to a diet and strain interaction, capital letters indicate significant differences between dietary treatment groups. BMC = bone mineral content, BMD = bone mineral density, BMA = bone mineral area.

Table 6. Plasma lipid parameters

Dietary Treatment	Strain	n	Cholesterol (mg/dl)	Triglycerides (mg/dL)	HDL (mg/dL)	NEFA (mEq/L)
AIN	C3H/HeJ	11	138.7 ± 7.9	118.7 ± 8.2	68.0 ± 3.1	0.66 ± 0.04^{bc}
AIN	C57BL/6	12	108.3 ± 8.7	42.1 ± 9.1	63.6 ± 3.4	0.94 ± 0.04^{a}
HF	C3H/HeJ	12	199.7 ± 7.5	101.3 ± 7.9	90.3 ± 2.9	0.71 ± 0.04^{bc}
HF	C57BL/6	12	152.6 ± 7.5	44.4 ± 8.2	77.3 ± 2.9	0.74 ± 0.04^{bc}
HF+1% MC	C3H/HeJ	12	209.1 ± 7.9	109.2 ± 8.2	89.7 ± 3.1	0.70 ± 0.04^{bc}
HF+1% MC	C57BL/6	12	163.6 ± 7.5	40.6 ± 7.9	78.0 ± 2.9	0.71 ± 0.04^{bc}
HF+10% MC	C3H/HeJ	12	177.9 ± 7.9	73.7 ± 8.2	81.9 ± 3.1	0.65 ± 0.04^{c}
HF+10% MC	C57BL/6	13	137.0 ± 7.2	30.1 ± 7.6	71.6 ± 2.8	0.77 ± 0.04^{b}
Dietary Treatments						
AIN		23	123.5 ± 5.9^{C}	80.4 ± 6.1^{A}	65.8 ± 2.3^{C}	0.80 ± 0.03
HF		24	176.1 ± 5.3^{A}	72.8 ± 5.7^{A}	83.8 ± 2.1^{A}	0.73 ± 0.03
HF+1% MC		24	186.3 ± 5.4^{A}	74.9 ± 5.7^{A}	83.8 ± 2.1^{A}	0.71 ± 0.03
HF+10% MC		25	157.5 ± 5.3^{B}	51.9 ± 5.6^{B}	76.8 ± 2.1^{B}	0.71 ± 0.03
Strain						
C3H/HeJ		47	181.3 ± 3.89	100.7 ± 4.07	82.5 ± 1.51	0.68 ± 0.02
C57BL/6		49	140.4 ± 3.88	39.3 ± 4.11	72.6 ± 1.50	0.79 ± 0.02
P values						
Diet			< .0001	0.0041	< .0001	0.1296
Strain			< .0001	< .0001	< .0001	0.0003
Diet*Strain			0.7268	0.2117	0.5340	0.0102

Values represent mean ± standard error of the mean. $p < 0.05$ is considered statistically significant. Values that do not share the same letter are considered statistically different. Lower case letters indicate significant differences related to a diet and strain interaction, capital letters indicate significant differences between dietary treatment groups. HDL = high density lipoprotein, NEFA = non-esterified fatty acids.

Table 7. Clinical parameters related to glucose metabolism and insulin resistance

Dietary Treatment	Strain	n	Hemoglobin AIC (%)	Glucose (mg/dL)	Fructosamine (µmol/L)	Leptin (mg/mL)	Adiponectin (mg/mL)	Total AUC (min*mg/dL)
AIN	C3H/HeJ	11	4.52 ± 0.2	164.2 ± 8.5^{abc}	212.3 ± 13.7	2.77 ± 1.6	6.75 ± 0.6	31169.8 ± 1874.2^{bc}
AIN	C57BL/6	12	4.63 ± 0.2	143.5 ± 8.5^{cd}	241.3 ± 14.4	1.64 ± 1.6	11.0 ± 0.7	27320.4 ± 1874.2^{c}
HF	C3H/HeJ	12	4.25 ± 0.2	153.3 ± 8.5^{bcd}	233.2 ± 13.1	13.9 ± 1.5	7.44 ±0.7	32838.1 ± 1874.2^{b}
HF	C57BL/6	12	4.72 ± 0.2	187.6 ± 8.5^{a}	255.4 ± 13.1	16.2 ± 1.5	9.80 ± 0.6	42035.2 ± 1874.2^{a}
HF+1% MC	C3H/HeJ	12	4.30 ± 0.2	161.2 ± 8.5^{bcd}	217.0 ± 13.1	14.1 ± 1.5	6.85 ± 0.6	35404.2 ± 1874.2^{b}
HF+1% MC	C57BL/6	12	4.73 ± 0.2	172.3 ± 8.5^{ab}	255.7 ± 12.5	16.3 ± 1.5	11.9 ± 0.6	40659.4 ± 1874.2^{a}
HF+10% MC	C3H/HeJ	12	4.25 ± 0.2	138.6 ± 8.5^{d}	202.2 ± 13.1	6.26 ± 1.5	6.51 ± 0.6	30527.1 ± 1800.7^{bc}
HF+10% MC	C57BL/6	13	4.78 ± 0.2	148.6 ± 8.2^{cd}	271.4 ± 12.5	5.12 ± 1.6	9.98 ± 0.7	33893.3 ± 1874.2^{b}
Dietary Treatments								
AIN		23	4.57 ± 0.2	153.8 ± 6.0^{AB}	226.8 ± 10.0	2.20 ± 1.1^{C}	8.89 ± 0.5	29245.1 ± 1325.3^{B}
HF		24	4.48 ± 0.2	170.4 ± 6.0^{A}	244.3 ± 9.2	15.1 ± 1.1^{A}	8.62 ± 0.5	37436.7 ± 1325.3^{A}
HF+1% MC		24	4.52 ± 0.2	166.7 ± 6.0^{A}	236.3 ± 9.0	15.2 ± 1.1^{A}	9.39 ± 0.5	38031.8 ± 1325.3^{A}
HF+10% MC		25	4.52 ± 0.2	143.6 ± 5.9^{B}	236.8 ± 9.0	5.69 ± 1.1^{B}	8.24 ± 0.5	32210.2 ± 1299.5^{B}
Strain								
C3H/HeJ		47	4.33 ± 0.1	154.3 ± 4.3	216.2 ± 6.6	9.28 ± 0.8	6.89 ± 0.3	32484.8 ± 937.1
C57BL/6		49	4.71 ± 0.1	163.0 ± 4.2	255.9 ± 6.6	9.82 ± 0.8	10.7 ± 0.3	35977.1 ± 928.0
P values								
Diet			0.9835	0.0073	0.6484	< .0001	0.3417	< .0001
Strain			< .0001	0.1495	< .0001	0.6281	< .0001	0.0096
Diet*Strain			0.7778	0.0182	0.2867	0.5039	0.1831	0.0074

Values represent mean ± standard error of the mean. $p < 0.05$ is considered statistically significant. Values that do not share the same letter are considered statistically different. Lower case letters indicate significant differences related to a diet and strain interaction effect, capital letters indicate significant differences between dietary treatment groups, and * indicates significant differences between strains.

Table 8. Effects of Bitter Melon and TLR 4 on Liver Steatosis

Diet	Steatosis Frequency					Chi-Square
	0	**1**	**2**	**3**	**4**	
Control	6	2	1	3	0	
HF	1	2	4	2	0	**0.0173**
HF + 1% MC	1	1	5	3	0	
HF + 10% MC	10	1	1	0	0	
Strain						
	0	**1**	**2**	**3**	**4**	
C3H/HeJ	8	3	6	3	0	**0.8949**
C57BL/6	10	3	5	5	0	

Steatosis was graded on a 0-4 basis depending on how many hepatocytes were involved and the degree of severity in these cells, 0 indicates no steatosis and 4 indicates severe steatosis. A Chi-Square value < 0.05 is considered statistically significant.

Table 9. Relative mRNA expression of genes related to PPAR signaling in the liver of C57BL/6 mice

Gene Name	Gene Symbol	Control: AIN-93M	HF + 1% MC	HF + 10% MC	Avg. CT
Acetyl-Coenzyme A carboxylase alpha	Acaca	-30.1	-1.14	+2.93	22.2
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	+1.48	-1.15	+2.94	21.5
Bcl2-like 1	Bcl2l1	+2.65	+5.05	+21.3*	31.9
Fructose bisphosphatase 1	Fbp1	+2.06	-1.38	+2.78	27.9
Glucose-6-phosphatase, catalytic	G6pc	+1.42	+1.05	+3.14	25.4
Glucokinase	Gck	+1.21	-2.89	+1.63	28.5
Insulin receptor substrate 1	Irs1	+1.90	-1.08	-1.61	24.8
Jun oncogene	Jun	+1.16	-1.21	+2.48	17.7
Leptin	Lep	-4.11	-4.38	-2.78	25.0
Low density lipoprotein receptor	Ldlr	+1.38	-1.23	+3.02	24.6
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	Pck2	+9.35	-1.08	+4.03	29.3
3-phosphoinositide dependent protein kinase 1	PdPk1	+1.68	-3.13	+2.96*	27.3
Resistin	Retn	+1.24	-1.10	+2.57	26.1
Ribosomal protein S6 kinase polypeptide 1	Rps6ka1	-11.1*	-1.83	-2.77	17.2
Uncoupling protein 1 (mitochondrial, proton carrier)	Ucp1	+1.50	+1.02	+1.95	22.8

Values represent fold regulation which is determined using relative fold change. * indicates a significant fold regulation compared to the high fat diet group; $p < 0.05$ is considered statistically significant.

Table 10. Relative mRNA expression of genes related to toll-like receptor signaling in adipose tissue of C57BL/6 and C3H/HeJ mice receiving a high fat (HF) and 10% bitter melon (HF+10% MC) diet

Gene Name	Gene Symbol	HF + 10% MC C57BL/6	HF C3H/HeJ	HF + 10% MC C3H/HeJ	Avg. CT
CD14 antigen	Cd14	+1.11	+20.8*	+5.98	23.8
Conserved helix-loop-helix ubiquitous kinase	Chuk	+1.17	+15.6*	+6.67	25.1
Colony stimulating factor 3 (granulocyte)	Csf3	+8.08	+29.5*	+2.21	31.1
FBJ osteosarcoma oncogene	Fos	-1.36	+19.2*	+6.97	22.5
Heat shock protein 1 (chaperonin)	Hspd1	+1.32	+7.83*	+2.83	21.4
Interleukin 6	Il6	-1.70	+5.97	+1.38	29.3
Interleukin-1 receptor-associated kinase 1	Irak1	+1.36	+5.74*	+2.39	23.0
Jun oncogene	Jun	+1.37	+4.51	-21.2	23.5
Lymphocyte antigen 86	Ly86	-1.15	+103.3*	+27.9	25.6
Myeloid differentiation primary response gene 88	Myd88	-1.52	+3.94	-2.35	26.0
Toll-like receptor 4	Tlr4	+1.13	+3.70	+1.37	22.7
Tumor necrosis factor	Tnf	-2.11*	+3.47	+1.07	27.0

Values represent fold regulation which is determined using relative fold change. * indicates a significant fold regulation compared to C57BL/6 mice fed a high fat diet; $p < 0.05$ is considered statistically significant.

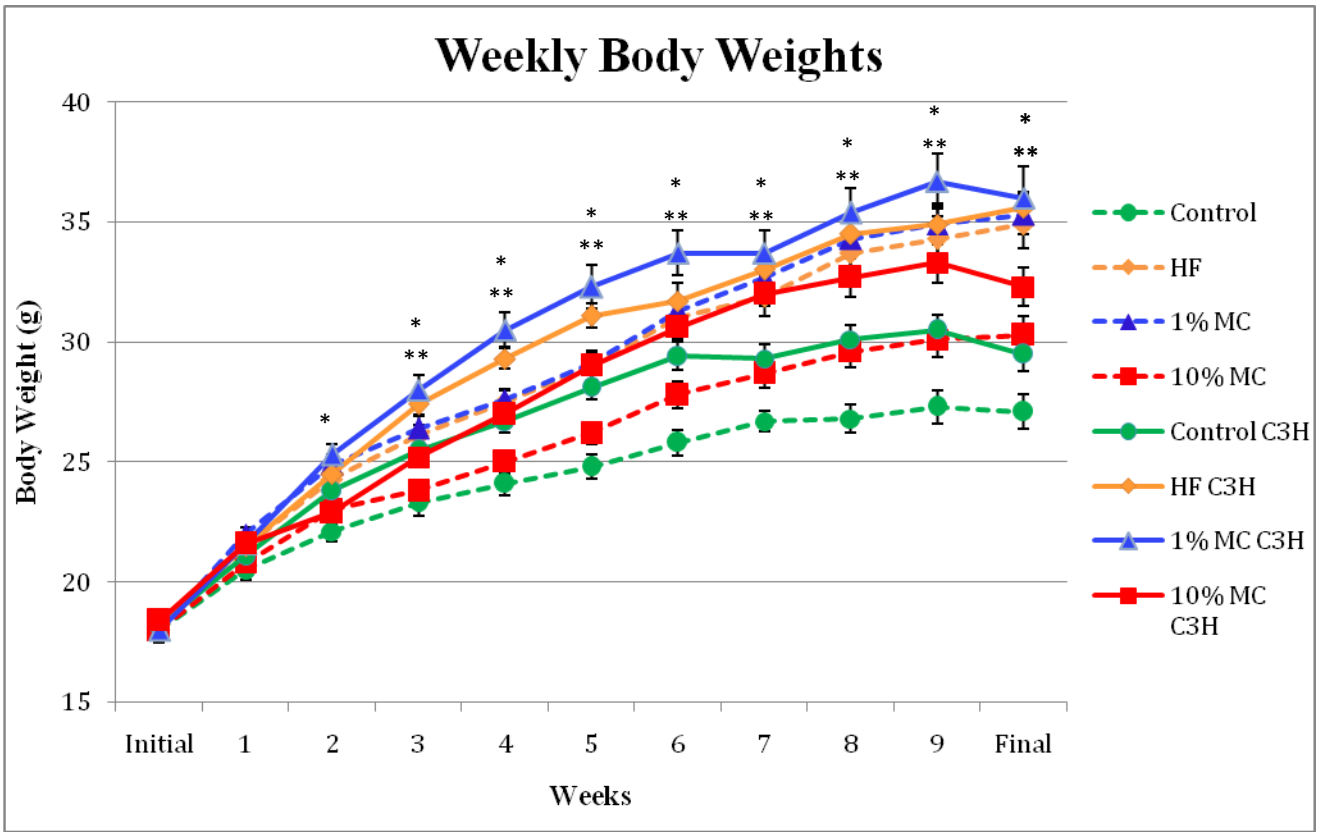

Figure 1. Weekly body weights; dashed line represents C57Bl/6 mice; solid line represents C3H/HeJ mice. * indicate significant differences between dietary treatment groups. ** indicates significant difference seen between strains.

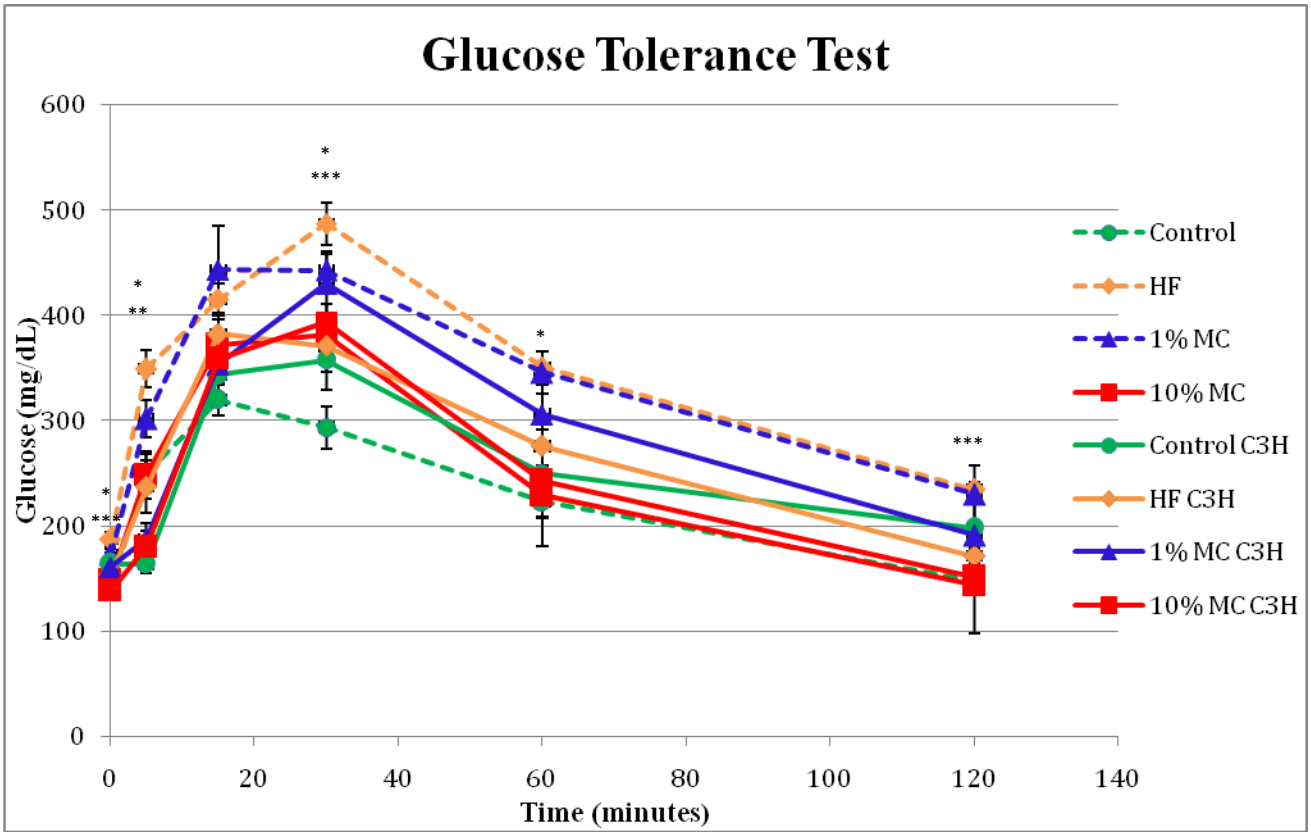

Figure 2. Results of a glucose tolerance test. * indicate significant differences between dietary treatment groups. ** indicates significant difference seen between strains. *** indicates significant diet and strain interaction.

CHAPTER V

DISCUSSION

The purpose of this study was two-fold: (1) to determine whether freeze-dried bitter melon powder supplementation, in 1 and 10% doses, could prevent the development of high fat diet induced obesity and insulin resistance, which further contributes to dyslipidemia and alterations in body composition, and (2) to determine the role of TLR 4 in the development of obesity-associated inflammation and insulin resistance and whether the deletion of TLR 4 would play a protective role. To address the objectives of this study, two strains of mice were used, C57BL/6 and C3H/HeJ, mice with a loss of function mutation of TLR 4.

Differences in strains were apparent from the results of this study. Mice with a deficiency in TLR 4 (C3H/HeJ) exhibited an increased body weight and body fat percentage compared to the C57BL/6 mice; however, the caloric intake did not differ between strains, so this could not be deemed as the primary cause in the observed differences in body fat. Our findings were similar to that of Poggi and colleagues, in which C3H/HeJ mice exhibited elevated body weights compared to wild-type mice when

fed a high fat diet [4]. They contributed the weight gain to increases in body fat mass and hypothesized that TLR 4 may play a role in regulating the balance between energy intake and expenditure [4].

Surprisingly, C3H/HeJ mice also had significantly elevated blood lipid levels, including total cholesterol and plasma triglycerides. However, their NEFA levels, a known ligand for TLR 4 activation, were significantly lower compared to C57BL/6 mice. Adiponectin, an adipokine with an anti-diabetic effect, was also significantly lower in C3H/HeJ mice. Our findings are different from what has been reported by Sugananami and colleagues [62]. Their results showed that C3H/HeJ mice had a higher serum adiponectin level as well as an increased adiponectin mRNA expression in epididymal WAT, indicating a possible role of adiponectin in improving insulin resistance [62]. However, our results do not support this hypothesis and we cannot offer an explanation at this time.

Even with significantly elevated % body fat and blood lipids, and lower adiponectin levels, C3H/HeJ mice exhibited an improved glucose tolerance compared to C57BL/6 mice. A lower total area under the curve after a glucose tolerance test indicated improved glucose control in C3H/HeJ mice compared to wild-type mice. Additionally, C3H/HeJ mice had lower fructosamine and hemoglobin A1C levels. Blood lipid levels, lower adiponectin levels and increased body fat would generally be associated with insulin resistance, however in this study C3H/HeJ mice did not appear to be insulin resistant. We therefore speculated that other factors, rather than body fat, blood lipids,

and reduced adiponectin may be an important contributing factor to glucose intolerance and insulin resistance in this strain of mice.

To assess the possible role of inflammation as a contributing factor to glucose intolerance and insulin resistance, plasma TNF-α levels were assayed as a marker of inflammation. However, the assay kit that was used was not sensitive enough and additional inflammatory markers could not be assayed due to limited amounts of plasma samples. However, C3H/HeJ mice had significantly lower NEFA concentrations, which have been shown to contribute to inflammation and insulin resistance [28]. Tsukumo and colleagues attributed the protection from glucose intolerance in C3H/HeJ mice to be due to the attenuated levels not just of the inflammatory cytokines, TNF-α and IL-6, but also to circulating NEFA [63]. We speculate that the positive effect of TLR 4 deficiency on glucose tolerance observed in our study is also due to attenuated levels of a combination of NEFA and pro-inflammatory markers. Future studies should assess these pro-inflammatory markers.

C3H/HeJ mice exhibited increased expression of several genes related to the innate immune response. Several proteins required for toll-like receptor signaling were up- regulated in C3H/HeJ mice including interleukin-1 receptor-associated kinase (IRAK) and CD14, a serine/threonine kinase which is involved in toll-like receptor signaling and an adapter protein required for TLR 4 signaling, respectively [96,97]. Other genes related to inflammation such as Csf3, which encodes proteins that function as granulocytes, as well as Hspd1, related to the functioning of heat shock proteins, were elevated in C3H/HeJ mice when compared to C57Bl/6 mice when both were fed a high

fat diet [96,97]. The role of these proteins in regulating inflammation indicates an increase in inflammation in C3H/HeJ mice compared to C57BL/6 mice. These results contradict our speculation that inflammation played a significant role in the development of insulin resistance in C3H/HeJ mice. Further research is needed to determine the mechanism through which glucose intolerance and insulin resistance develops in the absence of TLR 4.

Based on the previously reported phenotype of C3H/HeJ mice and the improved glucose tolerance observed in this study, the increased expression of inflammatory genes was not expected. However, the small sample size used in this array (n=3/group) might have contributed to this discrepancy. Also, our results showed an increased body fat percentage as well as an increased body weight which is also not normally seen in C3H/HeJ mice. These results may have contributed to the increase in inflammation in the C3H/HeJ mice. A high fat diet with 60% of calories from fat was used in this study, while previous studies have utilized either a 45 or 55% high fat diet [4, 68]. This difference in dietary treatment may have also contributed to the results observed in this study.

The other objective of this study was to determine the effects of bitter melon, in 1% and 10% doses, on the development of high fat diet induced obesity, inflammation and insulin resistance. We found that bitter melon, in a 10% dose, played a protective role in the development of insulin resistance and attenuated weight gain, specifically body fat accumulation. Mice fed a HF + 10% MC diet, had significantly lower body weights compared to the other high fat diet groups. While the caloric intake of the HF +10% MC group was lower compared to the HF + 1% MC group, it was similar to mice fed a high

fat diet alone, indicating caloric intake was likely not a major factor in modulating weight gain. We also observed that mice on the HF + 10% MC diet had significantly lower relative peri-renal and visceral fat pad weights, as well as body fat percentage and total fat mass compared to the other high fat groups. Associated with body fat percentages, leptin levels were significantly lower in mice supplemented with 10% MC compared to other high fat groups, however no differences in adiponectin were observed. Bitter melon's effect in controlling weight gain, particularly the 10% dose, was likely due to its regulation of body fat accumulation. Previous research by Chen and colleagues showed the ability of MC, in only a 1.5% dose, to attenuate visceral adiposity in rats fed a high fat diet [87]. They attributed that bitter melon prevented the accumulation of body fat in rats through up regulation of thermogenesis and an increase in expression of genes typically found in brown adipose tissue, suggesting an increase in energy expenditure [87]. They contributed to the lower amount of visceral adiposity to the reduced insulin in these animals which may have led to an increase in lipolysis. We have not investigated the mechanism of action of MC in attenuating weight gain and whether up-regulation of thermogenesis and energy expenditure occurred in our mice is not known at this time. Future research is needed to determine the role of bitter melon in thermogenesis and energy expenditure.

In line with previous research, bitter melon was able to prevent dyslipidemia associated with consumption of a high fat diet [85,86]. Total cholesterol and triglycerides were significantly lower in the HF + 10% MC group compared to other high fat diet groups. Similar results were shown by Senanayake and colleagues where the methanol extract of MC was effective in lowering hepatic and serum triglyceride levels in Sprague-

Dawley rats [86]. Another study by the same research group indicated the ability of MC to improve cholesterol levels in a dose dependent manner [85]. Senanayake and colleagues attributed the ability of bitter melon to attenuate serum lipids by modulating the synthesis of triglycerides in the liver [85].

The hypoglycemic effect of bitter melon has been extensively investigated and documented in animal models [82-84]. In the present study, bitter melon in a 10% dose, was able to normalize the glucose intolerance that is commonly induced with a high fat diet. Fasting glucose levels of mice supplemented with 10% MC were equivalent to levels observed in mice fed the control diet. Similarly, results of a glucose tolerance test following 8 weeks of feeding, showed an improved glucose tolerance in mice fed the HF + 10% MC diet. Interestingly, bitter melon had a stronger effect on C3H/HeJ mice compared to the wild-type mice. In an attempt to understand the hypoglycemic action of bitter melon, a gene expression analysis was performed. We have observed that bitter melon was able to increase PdPk1 and RPS6Ka1, indicating improved insulin signaling. Other investigators have reported several mechanisms by which bitter melon, or its active components such as polypeptide-p, vicine or charantin, modulate glucose homeostasis. These mechanisms include the activation of the AMPK pathway which acts to increase GLUT 4 translocation to the cell membrane [91]. *In vitro* and *in vivo* studies have shown that MC extracts were able to increase uptake of glucose in skeletal muscle and adipose tissue [98-100]. MC has also been shown to increase hepatic glucose utilization and glycogen synthesis as well as decrease hepatic gluconeogenesis [101-102].

Bitter melon, in a 10% dosage, was also able to attenuate liver gene expression of TNF-α compared to mice fed a high fat diet alone in wild-type mice. This suggests a role of bitter melon in attenuating inflammation. Gadang and colleagues showed that bitter melon seed (3% w/w) was able to down-regulate the expression of NFκB and increase the expression of PPARγ in Zucker rats [84]. Bitter melon's ability to attenuate inflammation may also explain the positive effect of MC on glucose tolerance. Other research has indicated bitter melon may act to regulate the PPARγ mediated pathway to prevent inflammation and insulin resistance [87,88]. Further research is required to solidify the mechanism of action of bitter melon.

The results of our study provided evidence to reject three of our four null hypotheses:

1. *Bitter melon added to a high fat diet will not improve the plasma lipid profile in both strains of mice.* Our results have shown that bitter melon, in a 10% dose, was able to attenuate total cholesterol and triglycerides. However, bitter melon had different effects on NEFA levels in C3H/HeJ and C57BL/6 mice. C3H/HeJ mice fed the HF + 10% MC diet had significantly lower NEFA levels compared to C57BL/6 mice fed the same diet.
2. *Bitter melon will not affect glucose homeostasis in both strains of mice.* In our study, 10% bitter melon was able to significantly improve the glucose tolerance of both strains of mice; however, it was more effective in C3H/HeJ mice. The total AUC in C3H/HeJ mice fed the HF + 10% MC diet was similar to mice fed the control diet.

3. *Bitter melon will not affect body composition in both strains of mice.* Bitter melon in a 10% dose was able to prevent accumulation of fat mass induced by a high fat diet, this was similar in both strains of mice. The body fat percentage of the HF + 10% MC group was significantly lower compared to mice fed a high fat diet alone.

However, we were not able to reject our null hypothesis that: *Biomarkers of inflammation will not be lower in mice fed bitter melon compared to mice consuming a high fat diet in both strains of mice*. Our PCR results have shown that gene expression of TNF was significantly lower in C57BL/6 mice fed a HF + 10% MC diet but the same effect was not observed in C3H/HeJ mice. As stated previously, additional research is needed to determine other markers of inflammation that may have been affected by our dietary treatment.

Future research is needed to determine the hypoglycemic, hypolipidemic, and body composition modulating mechanism of action of bitter melon. Additionally, studies in humans are necessary to see if these positive effects will be observed. However, the dose suitable for human consumption as well as safety needs to be examined. Another limitation of our study was the inability to quantify the inflammation present in our animals. We are currently assessing adipose tissue histology and degree of inflammation. Due to limited plasma samples, we were not able to assess plasma pro-inflammatory markers as well as insulin. This would have been helpful to determine the insulin/glucose ratio which would have helped us determine the action of bitter melon in regards to its role in insulin resistance.

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VITA

Maureen Lumena Meister

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF BITTER MELON AND TOLL-LIKE RECEPTOR (TLR) 4 ON GLUCOSE AND LIPID PARAMETERS IN MICE FED A HIGH FAT DIET

Major Field: Nutritional Sciences

Biographical: Born in St. Paul, Minnesota in 1989.

Education:

Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2013.

Completed the requirements for the Bachelor of Science in Dietetics at St. Catherine University, St. Paul, Minnesota in May, 2011.

Experience:

Graduate Research Assistant at Oklahoma State University, Stillwater, OK July 2011- Dec. 2012.
Performed experimental procedures on laboratory animals and tissues such as RNA extraction and ELISA assays. Assisted in professional journal reviewing and book chapter writing. Conducted data collection for clinical research.

Dietary Aid & Cook at Golden Oaks Village, Stillwater, OK Jan 2012-present
Prepared and served meals for the residents of an assisted living facility.

Kitchen Supervisor at Boutwell's Landing, Stillwater, MN Oct. 2009-Jun 2011.
Supervised and assisted in the preparation and service of meals.
Trained new servers and dishwashers.

Professional Memberships: American Dietetic Association / Oklahoma Dietetic Association; The Honor Society of Phi Kappa Phi.

Name: Maureen Meister Date of Degree: May 2013

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF BITTER MELON AND TOLL-LIKE RECEPTOR (TLR) 4 ON GLUCOSE AND LIPID PARAMETERS IN MICE FED A HIGH FAT DIET

Pages in Study: 70 Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: Bitter Melon (*Momordica charantia,* MC) has been investigated for its hypoglycemic and hypolipidemic effects and its ability to reduce adiposity in animal models and clinical trials. This study examined the effect of MC on glucose and lipid homeostasis in an animal model of diet-induced obesity (DIO). Additionally, fatty acids can activate toll-like receptor (TLR) 4 signaling pathway resulting in an inflammatory state which may contribute to insulin resistance associated with DIO. It has been reported that the loss of TLR 4 protected against dietary fat-induced inflammation and insulin resistance. TLR 4 mutant (C3H/HeJ) and wild-type mice (C57BL/6) were randomly assigned to four dietary treatment groups for eight weeks; control (10% calories from fat), high fat (HF, 60% calories from fat), HF + 1% MC, and HF + 10% MC.

Findings and Conclusions: C3H/HeJ strain exhibited significantly higher body weight, body fat, and lipid levels in response to a high fat diet when compared to the C57BL/6 strain. However, C3H/HeJ strain had significantly lower area under the curve after a glucose tolerance test, plasma fructosamine and free fatty acid in comparison to the C57BL/6 strain. Animals fed the HF + 10% MC also exhibited improved blood glucose control.

ADVISER'S APPROVAL: Dr. Edralin A. Lucas