The Effects of Moringa Leaves on Hepatic Lipid Accumulation and Inflammation in a Guinea Pig Model of Hepatic Steatosis

Manal Almatrafi

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The Effects of Moringa Leaves on Hepatic Lipid Accumulation and Inflammation in a Guinea Pig Model of Hepatic Steatosis

Manal Almatrafi, PhD

University of Connecticut, 2017

Moringa leaves (ML), have been recognized for their protective effects against chronic disease. To evaluate the protective effects of ML on hepatic steatosis and systemic inflammation, we used the guinea pig model. Male Hartley guinea pigs were assigned (n=8/group) to consume either a control diet (0 g Moringa), Low Moringa (LM) (10 %) or High Moringa (HM) (15%) diets, which were supplemented with 0.25% dietary cholesterol to induce hepatic steatosis. After 6 weeks, blood, liver, and adipose tissue were collected for determination of plasma lipids and lipoproteins, tissue cholesterol concentrations, inflammatory cytokines and expression of genes regulating hepatic cholesterol and triglyceride metabolism. There were no differences in plasma total cholesterol, LDL, HDL, triglycerides, glucose or insulin among groups. However, medium HDL concentrations as measured by nuclear magnetic resonance were higher and plasma lecithin cholesterol acyl transferase activity was higher in both Moringa groups compared to controls suggesting HDL metabolism was affected. Hepatic total cholesterol and triglycerides exhibited a dose response effect with the lowest values observed in the HM group (p < 0.01). The hepatic cytokines interleukin (IL)-1β, 1L-10 and interferon γ were lowest in the HM group, intermediate in the LM and highest in the control group (p<0.05). There was a lower degree of steatosis and lipid accumulation in livers in the guinea pigs fed the HM compared to the other groups in agreement with hepatic cholesterol and triglyceride concentrations. Guinea pigs fed ML had lower gene expression of cluster of differentiation 68 and sterol regulatory element binding protein1c compared to controls (p<0.05).
Expressions of diglyceride acyl transferase and peroxisome proliferator-activated receptor γ were lowest in the HM, intermediate in LM and highest in the control group (p< 0.01). The high cholesterol diet resulted in accumulation of cholesterol and inflammation in the adipose tissue, which was not prevented by Moringa treatment. We conclude that ML protect against hepatic steatosis by affecting genes involved in synthesis and uptake of hepatic lipids resulting in lower concentrations of cholesterol and triglycerides and reduced inflammation, however, plasma lipids and adipose tissue cholesterol and inflammation were not affected significantly by ML consumption.
The Effects of Moringa Leaves on Hepatic Lipid Accumulation and Inflammation in a Guinea Pig Model of Hepatic Steatosis

Manal Mused Almatrafi

B.S Umm Al-Qura University, 2001
M.S Umm Al-Qura University, 2008

A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the

University of Connecticut

2017
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Manal Mused Almatrafi

2017
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First, praise our almighty “God” who gave us life, showed us the straight ways and invited us to look at his creation. All the praise is for God for guiding me towards the completion of this work.

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<td>Proprotein convertase subtilisin Kexin Type 9</td>
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<td>Term</td>
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<td>Psyllium</td>
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<td>P38 Mitogen Activated Protein Kinase</td>
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<td>Very low-density lipoprotein</td>
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Chapter 1

Introduction
Hypercholesterolemia is defined as a lipoprotein metabolic disorder characterized by high total and LDL cholesterol, which are considered the most important risk factors in the development and progression of atherosclerosis and cardiovascular disease (CVD) (1). Hypercholesterolemia causes major problems to society because of the close correlation between CVD and lipid abnormalities (2).

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease and is considered a hepatic manifestation of metabolic syndrome (3, 4). NAFLD is characterized by the accumulation of fat in the liver without a history of alcohol abuse (5). The spectrum of NAFLD includes simple fatty liver, and non-alcoholic steatohepatitis (NASH), which shows steatosis and inflammation and may progress to liver cirrhosis, hepatocellular carcinoma, and advanced liver disease (6). In the liver, mitochondrial dysfunction, oxidative stress, and hepatocyte apoptosis lead to hepatocellular injury. In addition, lipotoxic mediators and intracellular signals activate Kupffer cells, which initiate the inflammatory response and development of fibrosis (7).

Natural compounds from plant sources have been used effectively in the treatment of dyslipidemias and NAFLD. The World Health Organization (WHO) reported that 80% of the populations from Africa and Asia use traditional medicine for primary health care (8, 9). For example, diets which are high in antioxidants including polyphenols (10), carotenoids and minerals have been useful in reducing inflammation and oxidative stress (11). *Moringa oleifera* (MO) is a plant that belongs to the monogeneric family *Moringaceae*. Most recent studies have been concentrated on the medicinal uses of MO in addition to the important phytochemical and pharmacological properties of different parts of the plant. Studies have reported the beneficial effects of *MO* on chronic disease (12, 13, 14). Nearly every part of MO, including the roots,
leaves, fruit (pods), seed, and seed oil have been used for various biological activities, including anti-diabetic agents (15), hypotensive agents (16) cardiovascular action, regulation of thyroid hormone status (17), antitumor agent and protection against oxidative stress (18) and treatment of inflammation (19). However, there are still some gaps in the literature related to the effects of Moringa leaves on hepatic steatosis.

Guinea pigs are similar to humans in the different responses to dietary cholesterol as hyper- or hypo-responders (19). Accumulation of hepatic cholesterol is associated with increased plasma cholesterol concentrations when dietary cholesterol is increased. The guinea pigs handle the increased hepatic cholesterol by suppressing Hydroxyl methylglutaryl-CoA (HMG-CoA) reductase activity and decreasing the LDL receptor in hepatic membranes (20, 21). In addition, guinea pigs have been shown to respond to phytochemicals similarly to humans (22, 23, 24).

Therefore, the main objective of this study was to evaluate the protective effects of MO on hepatic steatosis in an appropriate animal model, the cholesterol-fed guinea pig (25).

We hypothesized that MO leaves would prevent hepatic steatosis in the cholesterol fed guinea pig by altering gene expression of key regulators of cholesterol and triglyceride metabolism.

There were three main objectives to this project, which were to evaluate:

Objective 1: Effects of Moringa leaves on plasma lipids, lipoprotein subfraction and size and on lipoprotein metabolism.

Hypothesis 1: Moringa leaves reduce plasma lipids, atherogenic lipoproteins and modify HDL metabolism.

Objective 2: Effects of Moringa leaves on hepatic steatosis
Hypothesis 2: Moringa leaves prevent hepatic steatosis by altering expression of genes involved in lipid accumulation and uptake.

Objective 3: Effects of Moringa leaves on adipose tissue.

Hypothesis 3: Moringa leaves decrease inflammation in adipose tissue
References


Chapter 2

Literature Review
**Cholesterol definition**

The cholesterol molecule has a steroid structure and hydrocarbon side chain. Cholesterol has numerous functions in the human body. It is a precursor for steroid hormones, vitamin D and bile acids, and a structural component of cell membranes and lipoprotein particles. The substrate for cholesterol synthesis is acetyl CoA, an intermediate of fat, carbohydrate and protein metabolism (1). Practically, all cells have the ability to synthesize their cholesterol, despite the capacity of the gastrointestinal tract to absorb large quantities. However, only hepatocytes have the ability for degrading cholesterol and eliminating it from the body (2).

**Exogenous and endogenous cholesterol**

In the body, cholesterol includes both dietary sources absorbed from the intestine and endogenous sources (produced in the liver and peripheral tissues) (3). The human diet supplies ~400 mg of cholesterol daily and the liver secretes ~1 g daily (4). About 50% of the cholesterol is absorbed in the intestine; the remainder is excreted in feces (5).

Exogenous cholesterol is absorbed by enterocytes which also contain cholesterol from endogenous sources. Endogenous cholesterol is obtained from three sources; non-dietary cholesterol absorbed from the lumen, cholesterol derived from plasma lipoproteins and cholesterol synthesized *de novo* (6, 7). Cholesterol is transported mainly as esterified cholesterol by the lymphatic system. The HDL protein particle accumulates cholesteryl esters by the esterification of cholesterol by Lecithin-cholesterol acyltransferase (LCAT). Two enzymes which are involved in the esterification of cholesterol are cholesterol esterase (8) and acyl-CoA cholesterol acyltransferase (ACAT) (9).
The major precursor of cholesterol synthesis is acetyl-CoA which is converted to hydroxyl methylglutary-CoA (HMG-CoA) and then gives rise to mevalonic acid (MVA) by HMGCoA reductase (16). The daily fecal loss of cholesterol from bile and desquamated cells is 550 mg, while 250 mg comes from unabsorbed bile salts. A total of about 900 mg must therefore be derived from the diet or synthesized each day (10).

**Cholesterol absorption**

In Western populations, cholesterol intake is around 200–600 mg/day, from which 40-80% is absorbed (11, 12). Approximately, 10–15% of dietary cholesterol is esterified, and before absorption is cleaved by a pancreatic enzyme, carboxyl ester lipase, so, most of the dietary cholesterol does not require pancreatic hydrolysis (13).

The intestine has important functions in regulating cholesterol absorption in the body. Intestinal cholesterol is derived from sloughed cells of the intestinal lining, dietary sources and direct secretion via bile. The absorption of cholesterol is complex, involving a balance between transport into the enterocytes [by the Niemann-Pick C1 -like 1 (NPC1L1) protein], and transport out of the enterocytes by ATP-binding cassette (ABC) G5/8 and possibly by transintestinal cholesterol efflux (14).

Cholesterol absorption is dependent on the velocity of intestinal transit, as it increases with a slow transit time and decreases with rapid transit under physiological conditions or after pharmacological stimulation of intestinal movement (15). In addition, detergent capacity of the bile acid species prevailing in the enterohepatic circulation defines the cholesterol-solubilizing capacity of bile acid micelles in both bile and intestinal contents (16).
A diet-related factor which influences cholesterol absorption is dietary fiber due to physical interactions within the intestinal lumen. Viscous or gel-forming soluble fiber binds bile acids in the intestine, promoting their fecal elimination and thereby stimulating the hepatic catabolism of cholesterol to bile acids (17). Also, soluble fiber could inhibit cholesterol absorption through its effect of increasing the thickness of the unstirred water layer overlaying the intestinal mucosa (18).

Plant sterols inhibit cholesterol absorption by competition with cholesterol due to their hydrophobicity and poor absorbability, which remain in intestinal micelles and interfere with the micellar solubility of cholesterol (19).

**Lipoproteins**

The majority of lipids (cholesterol, phospholipids and triglyceride) are carried in plasma by large complex particles called lipoproteins. The densities of these lipoproteins are related to the relative amounts of lipids to proteins in the complex. The higher the protein content, the higher the density of the lipoprotein. According to their density, lipoproteins are classified into; chylomicrons (lowest density lipoproteins) followed by chylomicron remnants, very low-density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoproteins (LDLs), and high-density lipoproteins (HDLs).

**Chylomicrons**

Dietary cholesterol is transported from the intestine to inside the body by chylomicrons (CM), a triglyceride rich lipoprotein characterized by the presence of ApoB-48 (20). CM passes through the lymphatic system and reaches the bloodstream (21). In the lymph and blood, CM acquires other apolipoproteins such as apoCI, apoCII, apoCIII and apoE. ApoCII activates the
endothelial cell-associated lipoprotein lipase (LPL), which hydrolyzes the triglycerides from the core of CM and the resulting free fatty acids (FFA) are transported to the extra hepatic tissues for energetic needs or storage. CM remnants are taken up by the liver via the hepatic LDL receptor (LDL-R) and LDLR-related protein-1 (LRP1) through the interaction with ApoE.

**Very low density lipoprotein (VLDL)**

Very low-density lipoprotein (VLDL) another class of triglyceride-rich lipoprotein is synthesized and secreted by liver and contains ApoB100, apoE, apoCII and apoCIII and is also packed up with endogenous cholesterol, lipid soluble vitamins and carotenoids (22). In addition, VLDL triglyceride content is hydrolyzed resulting in cholesterol-rich remnant particle and ultimately LDL (23).

Production of VLDL by the liver has been demonstrated in different studies utilizing the rat (24, 25, 26) and monkey (27) liver and hepatocytes grown in short-term culture (28). In addition, studies of subcellular fractionation of the liver (29) reported that precursors of plasma VLDL (300-1000 Å, diameter particles) are present in the Golgi region of the hepatocyte. Although the majority of plasma VLDL is synthesized in and secreted from the liver, the intestine also produces lipoproteins similar in size and lipid composition to plasma VLDL which contain a low molecular weight form of apolipoprotein B (30).

LDL is composed of a hydrophilic surface layer of phospholipids (PL), free cholesterol and hepatically-derived apo B100. The core of the particle contains esterified cholesterol and triglyceride together with the fatty acid tails of the PL (31). LDL is sub-divided according to
size into a pattern A and a pattern B. Pattern B, termed small dense LDL has been associated with an increased risk for atherosclerosis (32).

LDL is the best-known lipoprotein not only due to the receptor for LDL which was discovered by Goldstein and Brown (33) and the importance of the receptor in maintaining cholesterol homeostasis but also because statins, which inhibit HMG-CoA reductase, were found to upregulates the LDL receptor, lower cholesterol in the blood stream and reduced CVD (34). Also, LDL was reported to supply cholesterol all over the body to maintain cell viability and to provide cholesterol for the synthesis of the steroid hormones (35).

The correlation between high LDL cholesterol (LDL-C) and increased risk for CHD has been well documented (36). A predominance of small, dense LDL particles, which are considered to be more atherogenic than the larger more buoyant cholesteryl ester (CE) enriched fraction (Pattern A subclass), would be representative of the Pattern B subclass (37). Small LDL subclass was showing to be associated with a 3-fold increase in CHD risk due to the easy entrance of the smaller particles into the arterial wall and binding to the proteoglycans and their increased susceptibility to oxidation (38, 39, 40). This enhanced susceptibility may be due to lowered tocopherol content or increased polyunsaturated fat concentration in the denser LDL subclass (41, 42).

**High density lipoprotein (HDL)**

HDL is the smallest and densest plasma lipoproteins. They comprise a heterogeneous mixture of sub-populations containing particles of differing size (43). They contain a number of apolipoproteins, with apoA-I accounting for about 70%, apoA-II about 20%, with smaller amounts of several others, including apoA-IV, apoE, the Capoli- poproteins, apoD, apoL, and
apoM. HDL subclasses can be grouped as large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm), and small HDL particles (7.3–8.2 nm) according to the nuclear magnetic resonance (NMR) spectroscopy method. Large HDL has been negatively associated with CVD (44, 45) while, low concentrations of large HDL have been associated positively with coronary artery disease in patients with Type 1 diabetes (46). HDL possesses several functions to protect against CVD including promotion of cholesterol efflux from cells, antioxidant properties, inhibition of vascular inflammation, anti-thrombotic properties, an ability to improve endothelial function as well as promotion of endothelial repair, promotion of angiogenesis, stimulation of pancreatic β cells to synthesize and secrete insulin, enhancement of insulin sensitivity and improvement in diabetic control and may also be involved in the innate immune system (47).

The antiatherogenic function of HDL is its ability to boost the efflux of cholesterol from cells. Also, HDL has antioxidant, anti-inflammatory, and antithrombotic properties The anti-inflammatory functions of HDL include limiting lipid peroxidation, influencing expression of cytokines, modulating recruitment/adhesion of monocytes, and altering other aspects of endothelial function (48). Among other functions, HDL reverses endothelial cell dysfunction, stimulates prostacyclin production (which is both vasodilatory and antithrombotic), inhibits endothelial cell apoptosis, decreases platelet aggregability, and inhibits LDL oxidation (48). The major function of HDL may be reverse cholesterol transport (RCT); however, an important function relates to its ability to protect LDL from oxidation. Thus, HDL can reduce the atherogenicity of LDL (49).

In addition to the role of favoring RCT, HDL serves several antiatherogenic functions, such as limitation of the oxidation of PL within LDL (50). By limiting LDL oxidation, HDL plays a key anti-inflammatory role in slowing atherogenesis. HDL inhibits the oxidation of LDL via
antioxidant enzymes and apo- lipoprotein (apo) A-I (51). Also, HDL normally attenuates cytokine-induced adhesion molecule expression on endothelial cells and promotes efflux of cholesterol out of vascular macrophages and the arterial wall itself, limiting the formation of inflammatory foam cells (52).

HDL appears to have evolved as part of the innate immune system, which in part uses an enhanced oxidative state as a non-specific means of protecting against many pathogens. In the absence of acute or chronic inflammation, HDL is anti-inflammatory in mice, rabbits, and humans (53). The major apolipoprotein of HDL is apoA-I, which may be altered by oxidative processes in patients with atherosclerosis. The ability of HDL to inhibit the inflammatory properties of oxidized PL and LDL also had been reported (53).

**Dietary cholesterol**

Several studies reported that there is an individual variation in regard to responses to dietary cholesterol (54). Elevated plasma cholesterol is considered a risk factor for heart disease (55). Cholesterol challenges above the usual intake; disturb cholesterol homeostasis resulting in metabolic disorders such as dyslipidemia, endothelial dysfunction, hepatic steatosis, CVD and systemic inflammation (56). One study in humans reported that, dietary cholesterol increased plasma TC and LDL-C without a consequent increase in HDL-C which is a major risk factor for atherosclerosis and CVD (57). However clinical studies from our laboratory have shown that, dietary cholesterol increased both LDL and HDL (58, 59) or on some occasions only increased HDL (60).

Eggs are the richest sources of cholesterol in the diet. Numerous studies failed to find any correlation between the intake of eggs and CVD risk (61). This lack of correlation might be
due to the other factors found in eggs that may influence CVD risk, such as antioxidant carotenoids (lutein and zeaxanthin) (62). Also, eggs are a rich source of PL, which are a potential source of bioactive lipids and affect inflammation, cholesterol metabolism, and HDL function (63). Egg PL, such as phosphatidylcholine (PC) and sphingomyelin (SM) are reported to reduce cholesterol and fatty acids (FA) absorption by possibly interfering with lipid mobilization from mixed micelles (64).

Egg PL appears to influence hepatic lipid metabolism through effects on cholesterol and bile acid synthesis, fatty acid (FA) oxidation, and lipoprotein secretion (64). Hepatic lipid levels showed to be decreased by dietary PL in animals by inhibiting intestinal lipid absorption and affecting hepatic nuclear receptors that regulate lipid metabolism (65). SM and PC are reported to regulate cholesterol absorption and inflammation and the incorporation of egg PL into HDL appears to be a major factor in the cholesterol-accepting capacity of this lipoprotein (66). Ovotransferrin, a protein present in egg, has antibacterial properties, antiviral activity, as well as antioxidant and anti-inflammatory properties (67). Also, egg yolk proteins including vitellogenin, lipovitellin and phosvitin have been shown to participate in the immune defense system, capable of killing bacteria and viruses as well as promoting phagocytosis activity (68).

Several experimental studies confirmed that atherosclerosis was directly alleviated by HDL (69) and improved CVD outcomes by its ability to remove cholesterol from the arteries via RCT. Regular consumption of egg yolk has been shown to increase plasma HDL-C and increase mean size of HDL particles in healthy (70), overweight (71), and metabolic syndrome (MetS) individuals which may be due to the high intake of egg PL (72). Also, in addition to increasing HDL cholesterol, egg yolks also increase plasma lecithin-cholesterol acyltransferase (LCAT) activity and serum cholesterol efflux capacity (73, 74).
Dietary intake of egg may also reduce inflammation. Consumption of eggs resulted in a reduction in plasma C-reactive protein (CRP) and an increase in adiponectin in overweight men (75). Also, egg consumption improved plasma inflammatory markers in adults with MetS. In combination with carbohydrate restriction, egg intake led to decreases in plasma tumor necrosis factor (TNFα) and serum amyloid A in men and women with MetS (76). Egg yolk contains a good amount of choline as PC which has been shown to be inversely correlated with serum inflammation markers in healthy adults (77).

Because Increased LDL-C has been suggested as a major risk factor for CVD, dietary guidelines to prevent CVD were established as 300 mg/d (78). Egg is a major source of dietary cholesterol (200 mg cholesterol). On the other hand, eggs contain other compounds such as minerals, folate, vitamins B, proteins, and mono-unsaturated fatty acids which reduce the risk of CVD (79). However, the new dietary guidelines (80) have removed the upper limits for dietary cholesterol (81) confirming that eggs do not increase the risk for heart disease.

Dietary cholesterol has been shown to increase the formation of larger LDL (82) and HDL (83), increase LCAT (84) and CETP (85) activities, and boost cholesterol efflux from macrophages (86) indicating an enhancement of RCT. This information confirms the lack of correlation between dietary cholesterol and CVD as indicated in multiple epidemiological studies (87).

In individuals classified with MetS, cholesterol intake has not shown any detrimental effect on lipoprotein profiles, contrary to the belief that eggs and dietary cholesterol should not be provided to patients at risk for CVD (88). In addition, studies conducted in individuals with
MetS have reported that consuming an additional 550 mg of dietary cholesterol (via eggs) for 12 weeks resulted in a significant increase in HDL with no changes in LDL (83).

Small LDL possess a number of features that make them more atherogenic, including their ability to penetrate the arterial wall and become easily oxidized, making them a perfect target for macrophages, which initiate the whole process of atherosclerosis (89). In the situation of a low carbohydrate diet, a challenge of additional 640 mg of cholesterol per day resulted in higher concentrations of the large LDL particle and reduced number of the small atherogenic LDL, which is considered a less atherogenic lipoprotein profile (82).

However, in animal models, it is a different situation because they are fed with very high amounts of dietary cholesterol in order to induce atherosclerosis, systemic inflammation or liver injury. For example, in guinea pigs, it has been shown that a challenge of 0.25% of dietary cholesterol (equivalent to 2500 mg/day in the human situation) for six weeks results in hypercholesterolemia when compared to a control group, associated with high plasma activity of liver enzymes, alanine amino transferase (ALT) and aspartate amino transferase (AST), which are biochemical indicators of hepatic damage. In addition, high cholesterol feeding resulted in increased hepatic accumulation of TG along with total, free, and esterified cholesterol, resulting in modest hepatic steatosis. At the same time, high cholesterol was capable of causing hepatic inflammation and injury in liver tissue as cell necrosis (90).

**Cholesterol balance and homeostasis**

Cholesterol balance involves consideration of both bile salts and cholesterol because bile salt molecules are formed by enzymatic modification of cholesterol. Hepatocytes clear bile salts from the portal blood and re-secrete them into bile. The conversion of cholesterol to bile acids
occurs at a rate that exactly balances the loss into the feces. This is achieved through sensing
the concentrations of bile salts in both the liver and intestine by nuclear hormone receptor
(FXR). The net synthesis of daily cholesterol is equal to the amount of cholesterol lost in the
feces minus the dietary cholesterol (~ 1.2 g/day). This takes into consideration that the amount
of cholesterol absorbed was equal to the amount lost (91).

Cholesterol levels in the liver play an important role in the body since the liver is the organ
involved in the maintenance of whole body cholesterol homeostasis. When cholesterol enters
by LDL endocytosis to the liver, it becomes part of the free cholesterol pool which is
metabolically active and can be used as a substrate for different enzymes. The liver is important
for regulating cholesterol homeostasis in the body through several mechanisms (92). The most
important one is the regulation of HMG-CoA reductase, the rate-limiting enzyme in cholesterol
synthesis (93). HMG-CoA reductase is found in all tissues, but the highest level of this enzyme
and the feedback regulation by dietary cholesterol occurs in liver. Another enzyme used by the
liver is Acyl CoA cholesteryl acyltransferase (ACAT) and together with HMG-CoA reductase
it is involved in controlling the intracellular and extracellular levels of cholesterol (94). ACAT
esterifies free cholesterol with fatty acyl CoA, leading to the reduction of toxic effects of
excessive cholesterol in cell membranes. The liver also has the highest concentrations of the
LDL receptor (LDL-R), which is used to remove LDL cholesterol from circulation (8). In
addition, cholesterol 7α-hydroxylase (CYP7A1), the initial enzyme in bile acid biosynthesis
uses free cholesterol as a substrate for the synthesis of bile acids. Cholesterol homeostasis is
maintained when these enzymes respond to changes in the hepatic bile acid pool (95, 96).

Cholesterol in the liver is determined by a balance between LDL uptake from plasma and
intracellular de novo synthesis in addition to cholesterol secretion via VLDL and dietary
cholesterol coming from the diet via chylomicron remnant. Liver cells have LDL receptors that are responsible for feedback inhibition. The subsequent liberation of free cholesterol into the cytosol has three main effects: (1) inhibition of HMG-CoA reductase activity thereby decreasing the rate of *de novo* cholesterol synthesis; (2) increase of the activity of ACAT, the enzyme that esterifies intracellular cholesterol, yielding a more non-polar molecule that coalesces into intracellular lipid droplets; and (3) inhibition of LDL receptor synthesis, resulting in a decreased uptake rate of LDL from plasma (97).

**HMG-CoA Reductase**

HMG-CoA reductase is the rate limiting and regulatory enzyme of the metabolic pathway of cholesterol synthesis. It is a transmembrane protein, containing 8 domains, which are anchored in the membrane of the endoplasmic reticulum. It is the major target of statins, cholesterol lowering drugs, which are the best-selling pharmaceutical drugs in the world (98). HMG-CoA reductase can be present in active and inactive forms. The inactive form of the enzyme appears to be converted into the active one by dephosphorylation of the enzyme (99, 100).

Regulation of HMG-CoA reductase occurs by feedback at 4 different levels; 1) regulation of transcription of the reductase gene when levels of cholesterol decrease; 2) translation of the HMG-CoA reductase mRNA, which is inhibited by farnesylpyrophosphatase; 3) degradation of intact reductase by endoplasmic reticulum (ER)-associated degradation when the level of sterol increases. A final regulation is phosphorylation and inactivation by an AMP-activated protein kinase (101, 102, 103).
Acyl CoA-cholesterol acyltransferase (ACAT)

ACAT is a membrane-bound protein that utilizes long-chain fatty acyl-CoA and cholesterol as substrates to form cholesteryl esters and plays an important role in cellular cholesterol homeostasis in various tissues. ACAT is the enzyme primarily responsible for cholesterol esterification in the liver and intestine. To maintain cholesterol balance, ACAT esterifies excess free cholesterol into cholesteryl esters. The cholesteryl esters produced become part of the components of apoB-containing lipoproteins synthesized in the liver. ApoB-containing lipoproteins enriched in cholesteryl esters increases by increased activity of ACAT, which is regulated by the amount of free cholesterol present in the cell, which usually is not enough to saturate the enzyme. Another function of ACAT is its involvement in cholesterol absorption in the intestines which can be packaged into chylomicrons (104).

The family of ACAT consists of ACAT1, ACAT2 and acyl-CoA: diacylglycerol acyltransferase 1 (DGAT1) (105, 106, 107, 108). These enzymes are founding members of the membrane-bound O-acyltransferase (MBOAT) enzyme family. MBOATs are multispan membrane enzymes that utilize long-chain or medium-chain fatty acyl-CoA and a hydrophobic substance as their substrates. ACAT1 is expressed in many different tissues and cell types, including hepatocytes and Kupffer cells of the liver, adrenal glands, neurons, and macrophages (109). Parini et al. (110) demonstrated the presence of both ACAT1 and ACAT2 in adult human hepatocytes. In mice and in monkeys, the ACAT1/ACAT2 distribution in various tissues is similar to that in humans except for at least one important difference, that ACAT2 is highly expressed in the livers of mice and monkeys under normal conditions (111).

ACAT is regulated by different factors including; 1) dietary cholesterol which increases in the enzyme activity in guinea pig, rats and rabbits (112, 113, 114); 2) by 25 hydroxycholesterol
and mevalonate, both of which increase the activity (104); 3) by both LDL and free cholesterol and reduced by a decrease in cholesterol availability (115).

The inhibition of this enzyme causes less cholesteryl ester formation leading to a decreased secretion of VLDL. While less VLDL is being secreted from the liver, less VLDL is converted to LDL resulting in decreases of LDL cholesterol in plasma (116). Also, ACAT inhibitors play an important role as antiatherogenic compounds, decreasing cholesterol content of the arteries and decreasing fatty streak formation (117).

**Cholesterol 7α-hydroxylase (CYP7)**

CYP7 is another important enzyme involved in the maintenance of cholesterol homeostasis in the liver. Free cholesterol is used by CYP7 as a substrate for the synthesis of bile acids. It is a protein localized in the membrane of the endoplasmic reticulum in hepatic parenchymal cells. CYP7 is considered a rate limiting enzyme in bile acid synthesis (118). It catalyzes the hydroxylation of cholesterol on carbon7. This enzyme is similar to HMG-CoA reductase, which exhibits diurnal variation. It presents maximum activity at midnight and a minimum at midday. This rhythm is present in rats and rabbits, where it is clearly determined by the levels of mRNA (119).

CYP7 has negative feedback regulation by bile acids, which appears to be at the level of transcription. In the liver, bile acids activate a nuclear receptor, farnesoid X receptor (FXR), which induces an atypical nuclear receptor small heterodimer partner, resulted in inhibits nuclear receptors, liver-related homolog-1, and hepatocyte nuclear factor 4α and results in the inhibition of transcription of the critical regulatory gene in bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1). The activity of CYP7 is increased by the lack of reabsorption of bile
acids in the hamster (120). These results support the negative feedback regulation of bile acids. Also, CYP7 activity and mRNA levels are decreased by hydrophobic bile acids, which do not undergo rapid metabolism to more hydrophilic ones. CYP7 activity is increased after cholestyramine treatment due to the increased demand for bile acids, while diminished in a state of vitamin C deficiency. Dietary cholesterol and thyroid hormone treatment cause an increase in the activity of the enzyme, as well as increasing the level of mRNA (121).

**LDL receptors**

The LDL receptor (LDL-R) plays an important role in the lowering of plasma cholesterol. The LDL-R is up-regulated to maintain hepatic cholesterol homeostasis when there is an excess of LDL cholesterol leading to the lowering of plasma LDL-C (122). The concentration of plasma LDL-C is dependent on the number of hepatic LDL-R because increased numbers of LDL-R lead to higher uptake of LDL particles and lower concentration of plasma LDL-C.

The liver compensates for increases in cholesterol intake by reducing the rate of *de novo* cholesterol synthesis and in some cases, the rate of receptor-dependent LDL-C uptake. Since the liver is the principal site of LDL catabolism, changes in hepatic LDL-R expression usually result in reciprocal changes in plasma LDL concentrations (118). The two mechanisms most commonly implicated in resistance to dietary cholesterol are 1) a diminished efficiency of cholesterol absorption and 2) an enhanced capacity to convert cholesterol to bile acids. Both mechanisms would reduce liver cholesterol and potentially lead to up-regulation of the LDL-R pathway (123).
**Proprotein convertase subtilisin Kexin Type 9 (PCSK9)**

PCSK9 is a key regulator in cholesterol metabolism, an enzyme encoded by the PCSK9 gene in humans, which binds to the LDL-R thereby reducing the number of available hepatic LDL-R leading to increased plasma levels of LDL-C. Treatment with estrogen, glucagon and a cholesterol-enriched diet reduces PCSK9 expression, while it is increased by insulin. The transcription factor SREBP-2 is partly involved in the hormonal and dietary regulation of PCSK9 as induction of PCSK9. Circulating PCSK9 has a pronounced diurnal variation and is strongly reduced during fasting in humans (124). These changes are presumably related to oscillations in hepatic intracellular cholesterol levels mediated by SREBP-2. Growth hormone treatment reduces circulating PCSK9 in men, whereas a ketogenic diet does not alter circulating PCSK9 levels (124).

**Guinea pigs as animal models for cholesterol metabolism**

Guinea pigs have been demonstrated to be good models for cholesterol and lipoprotein metabolism due to similarities to humans in the regulation of hepatic cholesterol homeostasis and in the changes in lipoproteins induced by diet (125) or by drug treatment (126). The most striking similarity between guinea pigs and humans is that the majority of circulating cholesterol is transported in LDL (127). Similar to humans, guinea pigs experience different responses to dietary cholesterol by which we could classify them as hyper- or hypo-responders (127). Increasing dietary cholesterol results in accumulation of hepatic cholesterol and increased plasma cholesterol concentrations (128). One of the first mechanisms by which guinea pigs handle the excess of hepatic cholesterol is by suppressing HMG-CoA reductase activity. Decreases in LDL receptor in hepatic membranes have also been observed (129).
Another important feature in guinea pigs is that most of the cholesterol in liver is in the free form similar to humans and increases proportionally with dietary cholesterol (130). Hepatic CYP7 activity is not regulated by high cholesterol diets in guinea pigs. The inability of guinea pigs to respond to high dietary cholesterol by increasing hepatic cholesterol catabolism may be one of the reasons why this animal model is more responsive to dietary cholesterol. Thus guinea pigs responses to dietary cholesterol are more consistent with human responses (131).

Guinea pigs have been found to have higher ACAT activity with higher levels of dietary cholesterol by the increased substrate (free hepatic cholesterol) for this enzyme. Suppression of hepatic apo B/E receptors in guinea pigs by high intake of dietary cholesterol leads to increased hepatic cholesterol concentrations in addition to increased plasma LDL cholesterol due to suppression of LDL receptors. Higher concentrations of cholesterol in the liver of guinea pigs decrease HMG-CoA reductase activity and increase ACAT activity, because of increases in substrate availability. The major mechanism responsible for the elevated plasma LDL cholesterol concentrations is the suppression of LDL receptors by dietary cholesterol (131,132).

Regulation of cholesterol homeostasis in liver varies between male and female guinea pigs (133, 134, 135, 136). When male guinea pigs were fed high dietary cholesterol, the activities of HMG-CoA reductase and CYP7 increased while ACAT activity decreased with dietary soluble fiber (pectin, guar gum or psyllium) (133,134). Compared to female guinea pigs fed high dietary cholesterol, soluble fiber only marginally increased activities of HMG-CoA reductase and CYP7 and marginally decreased activity of ACAT (135).
**Oxidative stress**

Any chemical species containing unpaired electrons produce highly reactive free radicals (137). Free radicals are formed as by-products of cellular function such as oxidative phosphorylation which occurs in the mitochondria (138). These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) (139).

Numerous studies reported that free radicals damaged cellular proteins, membrane lipids and nucleic acids, and finally caused cell death. In contrast, the human body counteracts this damage by the use of antioxidants, which are either endogenous, or supplied through foods and/or supplements (exogenous) (140). Antioxidants could be categorized as enzymatic when they break down and remove free radicals through enzymatic reactions such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx). Non-enzymatic antioxidants worked by interrupting free radical chain reactions such as vitamin C, vitamin E, plant polyphenols, carotenoids, and glutathione (141).

Oxidative stress has a major role in the development of chronic and degenerative diseases such as cardiovascular, cancer, autoimmune disorders, cataract, and neuro-degenerative diseases (139). When the organism's capacity could not counteract the production of pro oxidant free radicals action by antioxidant systems, the organism is exposed to oxidative stress (142,143).

**Chronic inflammation**

In obesity, the adipose tissue is associated with many of the inflammatory responses. Adipose tissue mass induces the secretion of a number of inflammatory mediators including tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1). This production of inflammatory adipokines has been shown to be involved in the
development of insulin resistance and MetS (144,145). Chronic inflammation is characterized by the up-regulation of biomarkers such as leukocyte count, serum and plasma concentrations of acute-phase proteins, pro-inflammatory cytokines, chemokines, soluble adhesion molecules and prothrombotic mediators (146).

Cholesterol metabolism plays a role in inflammation. The interaction of LDL with macrophages in atherosclerotic plaques leads to an increase in inflammatory gene expression (147). Numerous studies on animals showed that high dietary cholesterol led to an increase in macrophage accumulation in the adipose tissue, increasing insulin resistance and chronic systemic inflammation (9). Accumulation of cholesterol in the adipocytes has been shown to be cytotoxic and is associated with recruitment of pro-inflammatory cells, and aggravated systemic inflammation (148, 149).

**Relation between oxidative stress and inflammation**

Oxidative stress and inflammation are both linked to many chronic diseases, and induced by each other (150). Reactive oxygen species (ROS) could stimulate production of oxidized LDL (Ox LDL), which induced the activation of Peroxisome proliferator activated receptor γ (PPARγ), and influenced the expression of downstream genes leading to foam cell formation, inflammation and atherosclerosis. The induced macrophages secrete interleukin-1 beta (IL1b), which may stimulate the smooth muscle cells (SMCs) to over-express platelet-derived growth factor (PDGF), which may cause migration and proliferation of the SMCs. Also, Ox-LDL is toxic to macrophages, which causes the apoptosis and necrosis of foam cells and the subsequent release of their cellular contents, resulted in compose the necrotic core found in advanced lesions (151). Some radicals also stimulate nuclear factor kappa (NF-κB) and P38 Mitogen Activated Protein Kinase (p38-MAPK) leading to inflammatory responses (141). Also, cells of
the immune system such as macrophages and leucocytes are recruited to the site of damage during inflammation resulting in an over production of ROS, and consequently oxidative stress (152). Natural antioxidants are important as therapeutic agents against oxidative stress and inflammation-related diseases (143, 153). Bioactive components of antioxidant had the ability to scavenge ROS, have an indirect impact on inflammation, and are also capable of interacting directly with inflammatory pathways (152, 154).

**Cytokines, proinflammatory markers and gene expression**

The essential component of the immune response to pathogens and damaged cells is the production of inflammatory markers. Cytokines are secreted by endothelial cells, T lymphocytes, macrophages, monocytes, and platelets, Cytokines include: tumor growth factors (TGFs), TNF-α, interleukins, interferons (IFNs), chemokines and colony stimulating factors (CSFs); which participate in the control of the immune response. IL-4, secreted by T lymphocytes, increased the activity of macrophage 12/15-lipoxygenase, which synthesizes 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE) from linoleic and arachidonic acids, respectively (155). Thus, this activation via IL-4 has been shown to provide linkage for PPARγ and induce expression of CD36 in a PKC-dependent manner (156). Also, other cytokines such as TGF-β, IFN-γ and IL-10 have an effect on CD36 expression. In monocytes, IFN-γ and TGF-β decreased the expression of CD36, while IL-10 increased both the expression of CD36 and ABCA1, favoring the uptake and clearance of cholesterol by macrophages (157,158).

Adiponectin stimulates the secretion of anti-inflammatory cytokines blocking NF-kB which inhibiting the release of TNF-α, IL-6, and chemokines and reducing inflammation (159).
Adiponectin protects against FAs-mediated hepatocyte death and reduces fibrogenesis of hepatic cells (160).

High doses of dietary cholesterol cause inflammation of adipose tissue resulted in reductions in the levels of plasma high moluccular weight adiponectin. Reduced adiponectin production and signaling have been implicated in the development of NAFLD/NASH in mice and humans (161). Since the liver is a major organ involved in cholesterol metabolism, high cholesterol provided in pharmacological doses could cause hepatocyte dysfunction, fibrosis, and induction of the development of early stages of nonalcoholic steatohepatitis (162).

Cholesterol challenge diets have shown to increase levels of circulating oxidized LDL (Ox-LDL) (163). This pro-atherogenic lipoprotein is internalized by scavenger receptor B1 (SR-B1) and LDL related protein 1 (LRP-1) in macrophages leading to cell dysfunction and production of pro-inflammatory cytokines and chemo-attractants and finally the formation of foam cells, the official mark of atherosclerosis (164).

Cholesterol accumulation in adipose tissue increases macrophage infiltration which is accompanied by increased levels of inflammatory cytokines TNF-α, MCP-1, and GMF-CSF. Also, there is a reduction in the anti-inflammatory cytokine IL-10, decreased gene and protein expression of sterol regulatory binding-element protein 1 (SREBP-1) and PPAR-γ2, resulting in adipocyte dysfunction in adipose tissue from guinea pigs fed with high cholesterol (165).

Dietary cholesterol challenge, associated with a diabetogenic diet induced increased macrophage infiltration in adipose and aortic tissue leading to progress of atherosclerosis and accompanied with increased levels of systemic inflammation markers serum amyloid A and C-
reactive protein (166). A dietary cholesterol challenge also leads to arrest of adipocyte
development and induces macrophage infiltration and inflammation in adipose tissue in guinea
pigs (167). Cholesterol efflux from cells is the cause for a decrease in cholesterol content.
LXRα and LXRβ control the expression of genes implicated in cholesterol efflux and involved
in cholesterol storage, catabolism and excretion (168). In addition to ABCA1, the hepatic
expression of ABCG5 and ABCG8 were increased during a high cholesterol diet by LXRα and
LXRβ and were involved in the cholesterol catabolism into bile acids (169).

Activation of nuclear receptors; PPARγ, LXRα, LXRβ and pregnane X receptor (PXR) are
accompanied by the accumulation of hepatic TG and response elements for these receptors are
found in the promoter regions of CD36, which established this gene as a common target in the
promotion of liver steatosis (170, 171, 172, 173). PPARγ coactivator-1-beta (PGC-1β) co-
activates SREBP1s and stimulates lipogenic gene expression contributing to hepatic lipid
synthesis. However, a high fat diet increased expression of PGC-1β resulting in reduction of
hepatic fat accumulation (174). This is described by an increase in circulating TG and
cholesterol (VLDL) influenced by PGC-1β co-activation of LXRα and stimulation of
lipoprotein transport. Its overall action leads to hyperlipidemia and atherosclerosis. Hepatic
overexpression of PGC-1β with decreasing the expression and activity of PPARα and
increasing the expression of CD36 was sufficient to induce hyperlipidemia (175).

SREBP-1 has been shown to modulate the expression of the PPARγ gene at the transcription
level (176). PPARγ is involved in the transcription of several lipogenic genes that are critical
in adipocyte development (177). Inflammatory changes in adipocytes were shown to be more
receptive to TNF-α signaling and subsequent apoptosis, which might lead to macrophage
infiltration and secretion of inflammatory cytokines, which could then result in a pro-inflammatory feedback loop (166).

Although increased CD36 expression is considered proatherogenic. CD36 is an 88 kDa glycoprotein expressed on platelets, monocyte-macrophages, microvascular endothelial cells, adipose tissue, skeletal muscles and heart. CD36 deficiency is accompanied by; (1) hyperlipidemia and increased remnant lipoproteins; (2) impaired glucose metabolism based upon insulin resistance, and (3) mild hypertension, and comprises one of the genetic backgrounds of the metabolic syndrome, leading to the development of CHD (178, 179, 180).

In fatty liver and in patients that develop hepatic steatosis or hepatocyte apoptosis, the expression of CD36 is elevated compared to normal liver (181, 182). In NAFLD patients, CD36 is mainly found on the plasma membrane of hepatocytes and its elevated expression was associated with insulin resistance (183).

**Non-Alcoholic Fatty Liver Disease**

**Definition**

NAFLD is the disease affecting liver which progresses from non-alcoholic fatty liver to nonalcoholic steatohepatitis (NASH) to fibrosis and cirrhosis. It can be defined as the presence of hepatic steatosis without ballooning degeneration and inflammation in the hepatocytes with absence of significant alcohol consumption. Liver steatosis might be due to abnormal triglyceride deposition in hepatocytes resulting from imbalance between the uptake of fat and its oxidation and/or export. NASH is defined as the presence of fatty infiltration in the liver resulting in ballooning and inflammation of the hepatocytes with or without fibrosis (184,185).
Epidemiology

Obesity, diabetes mellitus, and hyperlipidemia are main disease-related factors of NAFLD (186). In the present time, NAFLD is one of the most common chronic and progressive liver diseases in developed countries, with the number of people affected increasing rapidly (187). NAFLD covers a wide spectrum, ranging from simple fat deposition changes to severe steatohepatitis with fibrosis (188).

NAFLD is the most widespread liver disease in the United States, affecting 15%-46% of adults (200, 201). Obesity is the most important risk factor for NAFLD; the prevalence of NAFLD is 4.6 times greater in the obese population, and up to 74% of obese individuals have fatty livers. The majority of patients with NAFLD have “simple steatosis” defined by excessive fat deposition within hepatocytes in the absence of substantial inflammation or fibrosis. However, around 10%-30% of patients with NAFLD develop nonalcoholic steatohepatitis (NASH), characterized by varying degrees of inflammation and fibrosis, in addition to hepatic steatosis which can progress to cirrhosis, liver failure, and hepatocellular carcinoma at different stages in patients (186, 189).

Pathogenesis

The pathogenesis of NAFLD/NASH has not been fully elucidated, but the theory of how it is formed has been recently accepted. First, excess fat accumulation occurs in hepatocytes, and then secondary activity such as oxidative stress may cause NAFLD (192). It has been suggested that lipid peroxidation is an important factor in the progression of NASH in rodent models (193).
Insulin resistance, characterized by increased hormone-sensitive lipase activity and increased lipolysis of peripheral fat, is the most consistent pathogenic factor for hepatic steatosis (194). Excess of dietary cholesterol intake (pharmacological doses) in animal models has been reported to be an important risk factor for hepatic steatosis and progression to steatohepatitis (195, 196).

**Dietary interventions**

Dietary interventions have a major role in treating obesity, insulin resistance, and NAFLD. Monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were reported to have a protective role in the progression of NAFLD (197,198).

Dietary interventions for NAFLD include modifications of nutrients intake such as carbohydrate and fat restriction; in addition, to use of antioxidants such as vitamins C, E and polyphenols, betaine, anthocyanins and curcumin (199). A carbohydrate- restricted diet was found to attenuate hepatic cholesterol accumulation induced by a cholesterol challenge in guinea pigs (188). In addition, low-carbohydrate diets showed decreased serum ALT and hepatic steatosis in humans (200).

Low carbohydrate diets have been shown to reduce serum TG and increase HDL-C. In addition, they promote weight loss and extend beneficial effects on several chronic inflammatory diseases, including atherosclerosis, diabetes, hypertension, and dyslipidemia (201). Following a low carbohydrate diet, decreased levels of cholesterol accumulation were observed in cholesterol challenged animals when compared to the unchallenged group, which may be attributed to the increased HDL-C concentrations observed in the low carbohydrate group. In addition, low carbohydrate diets have been shown to decrease levels of inflammatory cytokines
MCP-1, TNF-α, and IL-2, decrease macrophage infiltration, and increase adipocyte size in guinea pigs. In addition, low carbohydrate diets were found to decrease levels of ox LDL (202).

The international guidelines reported that lifestyle changes including diet are the only therapeutic approach recommended for fatty liver. The predictor of changes in liver fat content found in intervention studies looked to increase adherence to the Mediterranean diet and level of physical activity (203). The major physiological mechanisms include antioxidant and anti-inflammatory effects of the foods included in the Mediterranean dietary pattern (204). The Mediterranean diet has benefits with regards to decreased cardiovascular diseases and improved insulin sensitivity because of the high intake of antioxidants. Vegetables are the main source of phenolic compounds on this diet. Also, polyunsaturated fatty acids of the n-3 series from fish regulate haemostatic factors that induce protection against a variety of chronic diseases. Olive oil also represents a high intake of monounsaturated fatty acids and a good source of phytochemicals (205). Better glycemic indices, with good lipid profile and lower blood pressure has been correlated with Mediterranean diet. In one study, the Mediterranean diet had a modest impact on lipid profiles (206).

High consumption of a variety of fruits and vegetables were found to protect against CVD and development of MetS. The mechanisms of protection may depend on high amount of dietary fiber, antioxidant vitamins (A, C, and E) and carotenoids (207). Fruit and vegetable antioxidants, including vitamins and flavonoids, reduce thrombosis, improve endothelial function and contribute to an anti-inflammatory effect (208). As free radical scavengers, flavonoids inhibit lipid peroxidation, promote vascular relaxation and prevent atherosclerosis and other factors associated with the MetS (209).
Consumption of whole grains (bread, pasta, and rice) is a major characteristic of the Mediterranean diet found to protect against several metabolic disorders like obesity, diabetes and CVD by their fiber content which improved insulin sensitivity, positively influencing a promoter of the process of carcinogenesis such as insulin-like growth factor 1 (IGF-1). Fiber consumption is also effective in lowering the risk of deteriorating glucose tolerance and development of type 2 diabetes (210). The preventing chronic disease by fiber present in bran and germ depends on their bioactive compounds with antioxidant and anti-carcinogenic properties (minerals, trace elements, vitamins, carotenoids, polyphenols and alkylresorcinols). Wheat is rich source of methyl donors and lipotropes (methionine, betaine, choline, inositol and folates) which is involved in cardiovascular and hepatic protection, lipid metabolism and DNA methylation (211).

Foods which contain oil (fish, nuts, avocado and olives) high in MUFAs have a beneficial effect on the risk of CVD (212). Fish such as sardine and anchovy have been shown to protect against CVD due to their content of MUFAs and PUFAs, especially the long-chain n-3 PUFAs. Olive oil, the main fat source of the Mediterranean diet, exerts its benefits on lipoprotein metabolism due to the high content of MUFAs (such as oleic acid) and several microcomponents that have antioxidant activity. In addition, olive oil has a role in cholesterol regulation; LDL oxidation promoting anti-inflammatory, antithrombotic and antihypertensive vasodilator effects. Replacing SFA by MUFA affects plasma lipid profile and improves insulin sensitivity (213).

**Moringa oleifera leaves**

Moringa, a plant that is a native to parts of Africa and Asia, is the sole genus in the family Moringaceae. It contains 13 species from tropical and subtropical climates that range in size
from tiny herbs to massive trees. The most widely cultivated species is *Moringa oleifera*, a multipurpose tree native to India (214). *Moringa oleifera* is the most widely known and cultivated species native to the foothills of the Himalayas in northwestern India and cultivated throughout the tropics. *Moringa oleifera* is grown for its nutritious pods, edible leaves and flowers and has many useful properties including its use as a source of food, medicine, cosmetic oil and forage for livestock. It is commonly known by its regional name as drumstick tree. It is a rapidly growing tree that is widely cultivated and has now become established in Afghanistan, Florida and East and West Africa. Its height ranges from 5 to 10 meters (214).

*Moringa oleifera* is an important plant for its contribution in bioactive compounds. *Moringa oleifera* leaves, the most used part of the plant, are rich in vitamins, carotenoids, polyphenols, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins and saponins (215). The high contribution in bioactive compounds might explain the pharmacological properties of *Moringa oleifera* leaves. Many studies *in vitro* and *in vivo* in animals confirmed numerous pharmacological properties (215).

**Traditional uses**

All parts of *Moringa oleifera* are traditionally used for different purposes, but leaves are generally the most used in human and animal nutrition and in traditional medicine. Leaves are rich in protein, minerals, beta-carotene and antioxidant compounds, which are often lacking among the populations of underdeveloped or developing countries. In traditional medicine, leaves are used to treat several sicknesses such as malaria, typhoid fever, parasitic diseases, arthritis, swellings, skin diseases, genito-urinary diseases, hypertension and diabetes (216, 217).
The different parts of *Moringa oleifera* including roots, leaves, flowers, fruits and seeds are also known to be good sources of phytochemical compounds. (214). Therefore, every part of this plant, including root, bark, gum, leaf, fruit (pods), flowers, seed, and seed oil have been used for various biological activities, including gastric ulcers (218), antidiabetic effect (219), hypotensive agent (220) cardiovascular action, liver disease (221) and hepatic and renal functions (222), regulation of thyroid hormone status (223), protecting against oxidative stress (224), treatment of inflammation (225), liver protection against hepatotoxic drugs and carbon tetrachloride (226), reduced cholesterol in the liver and kidney (227, 228, 229), antibacterial activity (230); anti-cancer (206); and hepatoprotective effects (231).

**Vitamins**

Fresh leaves of *Moringa oleifera* contain 11,300–23,000 IU of vitamin A/100 g of dry matter (232). Vitamin A participates in many physiological processes such as vision, reproduction, embryonic growth and development, immune competence, cell differentiation, cell proliferation and apoptosis, maintenance of epithelial tissue, and brain function (233). Also, fresh leaves are a good source of carotenoids with pro-vitamin A action. They contain 6.6–6.8 mg/100 g of β-carotene, greater that carrots, pumpkin and apricots (6.9, 3.6 and 2.2 mg/100 g, respectively) (234).

For vitamin C, fresh leaves contain 200 mg/100 g which is a concentration greater than oranges (235, 234). These amounts are of interest, as the vitamin C favors the conversion of cholesterol into bile acids and hence lowers blood cholesterol levels in humans and animals and increases the absorption of iron in the gut by reducing ferric to ferrous state. Also, Moringa leaves protect the body from various deleterious effects of free radicals, pollutants and toxins and act as antioxidants (236).
*Moringa oleifera* fresh leaves are a good source of vitamin E (α-tocopherol) and contain 9.0 mg/100 g similar to nuts (234). Vitamin E acts as antioxidants, but it is also involved in the modulation of gene expression and inhibition of cell proliferation (237).

**Polyphenols**

The dried leaves of *Moringa oleifera* are a great source of polyphenol compounds such as flavonoids and phenolic acids.

**Flavonoids**

Flavonoids are a sub-group of polyphenolic compounds having a benzo-γ-pyrone structure and they are synthesized in response to microbial infections (238). Epidemiological studies showed that, high intake of flavonoids has protective effects against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases. *Moringa oleifera* leaves are a good source of flavonoids (239).

Myricetin, quercetin and kaempferol are the main flavonoids found in *Moringa oleifera* leaves. In dried leaves, myricetin concentration is 5.804 mg/g of dry weight, while quercetin and kaempferol concentrations range from 0.207 to 7.57 mg/g of dry weight and not detectable amounts (ND) to 4.59 mg/g of dry weight, respectively (240, 241, 242).

**Quercetin**

Quercetin is found in dried *Moringa oleifera* leaves at concentrations 100 mg/100g as quercetin-3-O-β-d-glucoside (iso- quercetin or isotrifolin) (243, 244). Quercetin is a strong antioxidant with multiple therapeutic properties (245). It has antidyslipidemic, hypotensive,
and anti-diabetic properties in the obese Zucker rat of metabolic syndrome (246). It can reduce hyperlipidemia and atherosclerosis in high cholesterol or high-fat fed rabbits (247, 248). It can protect insulin-producing pancreatic β cells from Streptozotocin (STZ) induced oxidative stress and apoptosis in rats (249).

**Phenolic Acids**

Phenolic acids are a sub-group of phenolic compounds derived from hydroxybenzoic acid and hydroxycinnamic acid, naturally present in plants and these compounds have antioxidant, anti-inflammatory, antimutagenic and anticancer properties (250, 251). In dried leaves, Gallic acid is the most abundant, with a concentration of 1.034 mg/g of dry weight. The concentration of chlorogenic and caffeic acids ranges from 0.018 to 0.489 mg/g of dry weight and ND to 0.409 mg/g of dry weight, respectively (252,253).

**Chlorogenic acid**

Chlorogenic acid is an ester of dihydrocinnamic acid (caffeic acid) and quinic acid, is a major phenolic acid in *Moringa oleifera* leaves (254). Chlorogenic acid has a role in glucose metabolism. It inhibits glucose-6-phosphate translocase in rat liver, reducing hepatic gluconeogenesis and glycogenolysis (255). It was found to lower post-prandial blood glucose (PPBG) in obese Zucker rats (256). In oral glucose tolerance test (OGTT), chlorogenic acid reduced the glycemic response in both rats and humans; in rodents, it reduced the glucose area under the curve (257). Its anti-dyslipidemic properties are more evident as it reduces plasma total cholesterol and TG in obese Zucker rats or HFD mice (258) and reverses streptozotocin-induced dyslipidemia in diabetic rats (255).
Alkaloids

Alkaloids are a group of chemical compounds that contain mostly basic nitrogen atoms. Several of these compounds, such as $N,\alpha$-L-rhamnopyranosyl vincosamide, 4-(α-L-rhamnopyranosyloxy) phenylacetonitrile (niazirin), pyrroleumarumine 4″-O-α-L-rhamnopyranoside, 4′-hydroxy phenylethanamide-α-L-rhamnopyranoside (marumoside A) and its 3-O-β-D-glucopyranosyl-derivative (marumoside B) and methyl 4-(α-L-rhamnopyranosyloxy)-benzylcarbamate, have been isolated from Moringa oleifera leaves (259, 260).

Glucosinolates and Isothiocyanates

Glucosinolates are a group of secondary metabolites in plants. Structurally they are β-S-glucosides of thio-oxime-O-sulfates and synthesized from amino acids. 4-O-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate has been identified as the dominant leaf glucosinolate of Moringa oleifera and is accompanied by lower levels of three isomeric 4-O-(α-L-acetylrlhamnopyranosyloxy)-benzyl glucosinolates, which reflect the three position of the acetyl group at the rhamnose moiety of the molecule In young and older leaves which have around 116 and 63 mg/g of dry weight, respectively (261). Both glucosinolates and isothiocyanates are important in health promoting and prevention of disease (262).

Tannins

Tannins are water-soluble phenolic compounds that precipitate alkaloids, gelatin and other proteins. Their concentrations in dried leaves range between 13.2 and 20.6g tannin/kg (263) and in freeze-dried leaves between 5.0 and 12.0 g tannin/kg (264). They have different biological properties: anti-cancer, antiatherosclerotic, anti-inflammatory, anti-hepatoxic, antibacterial and anti-HIV replication activity (265).
Saponins

Saponins are a group of natural compounds that consist of an isoprenoidal-derived aglycone, designated genin or sapogenin, covalently linked to one or more sugar moieties (266). *Moringa oleifera* leaves are a good source of saponins. Their concentration in dried leaves is approximately 50 g/kg of dry weight, while in freeze-dried leaves it ranges between 64 and 81 g/kg of dry weight (267). Saponins have anti-cancer properties (268).

Pharmacology

Hypolipidemic effects

Many bioactive compounds found in *Moringa oleifera* leaves may influence lipid homeostasis. Phenolic compounds as well as, flavonoids, have important roles on lipid regulation (269). They are involved in the inhibition of pancreatic cholesterol esterase activity thereby reducing and delaying the cholesterol absorption, and binding bile acids by forming insoluble complexes and increasing their fecal excretion thereby decreasing plasma cholesterol concentrations (270). The extracts of Moringa showed antihyperlipidemic activity due to inhibition of lipase and cholesterol esterase thus showing its potential for prevention/treatment of hyperlipidemia (271).

*Moringa oleifera* has strong effect on lipid profile through cholesterol reducing effect. Cholesterol homeostasis is maintained by the two processes, cholesterol biosynthesis in which HMG-Co-A reductase catalyzes rate limiting process and cholesterol absorption of both dietary cholesterol and cholesterol cleared from the liver through biliary secretion. The HMG-Co-A/mevalonate ratio has an inverse relationship to the activity HMG-Co-A reductase (272). The activity of this enzyme was depressed by the ethanolic extract of *Moringa oleifera* supporting its hypolipidemic action.
Moringa leaves also contain bioactive phytoconstituent, (β-sitosterol) with cholesterol lowering effect. Cholesterol lowering action in the serum of high fat diet fed rats which might be attributed to the presence of bioactive Phyto-constituents, i.e. β- sitosterol (227).

Saponins found in Moringa leaves lowered cholesterol by preventing its absorption through binding with cholesterol in the intestinal lumen, and/or by binding with bile acids, causing a reduction in the enterohepatic circulation of bile acids and increasing their fecal excretion. The increased bile acid excretion is offset by enhanced bile acid synthesis from cholesterol in the liver and consequent lowering of the plasma cholesterol (219).

**Antioxidant effects**

Moringa leaves are a good source of natural antioxidant due to the presence of antioxidant compounds such as ascorbic acid, alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, and tannins and carotenoids (224, 273, 274), which are used in patients with inflammatory conditions, cancer, hypertension, and cardiovascular diseases (227, 275).

The beta carotene found in Moringa leaves acts as antioxidants. The antioxidants have the maximum effect on the damage causing free radicals, only when it is ingested in combination with nutrients and a group of antioxidants. A combination of antioxidants found in Moringa leaves was more effective than a single antioxidant due to antioxidant cascade mechanism (276, 232, 277).

The extract of Moringa leaves contain tannin, saponin, flavonoids, terpenoids and glycosides, which have medicinal properties. These compounds have been shown to be effective antioxidants, antimicrobial and anti-carcinogenic agents (278). Phenolic compounds act as primary
antioxidants (279). They inactivate lipid free radicals or prevent decomposition of hydroperoxides into free radicals due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet or triplet oxygen, or decomposing peroxides (280, 281).

The radical scavenging and antioxidant activities of the aqueous and aqueous ethanol extracts of freeze-dried leaves of *Moringa oleifera* from different agro-climatic regions were investigated by Siddhuraju and Becker (282). They found that, different leaves extracts inhibited 89.7%–92.0% peroxidation of linoleic acid and had a scavenging activity on superoxide radicals in a dose-dependent manner in the beta-carotene-linoleic acid system. Iqbal and Bhanger (283) showed that, the environmental temperature and soil properties have significant effects on antioxidant activity of *Moringa oleifera* leaves.

**Anti-Inflammatory and Immunomodulatory effect**

The extract of *Moringa oleifera* leaves inhibited human macrophage cytokine production (TNF-α, IL-6 and IL-8) induced by extract of cigarette smoke and by LPS, similar to aspirin (284). Also, Waterman et al. (285) found that both *Moringa oleifera* concentrate and isothiocyanates isolated from the leaves significantly decreased gene expression and production of inflammatory markers in RAW macrophages.

The extracts of *Moringa oleifera* leaves stimulated both cellular and humoral immune response in cyclophosphamide-induced immunodeficient mice through increases in white blood cells, percent of neutrophils and serum immunoglobulins (286,287). In an animal model (mice), the anti-inflammatory and immunomodulatory effects of *Moringa oleifera* leaves were investigated *in vitro* and *in vivo*. Many bioactive compounds which are involved in the anti-
inflammatory process such as quercetin showed to inhibit activation of NF-kB and also the subsequent NF-kB-dependent downstream events and inflammation (288). In addition, flavonoids and phenolic acids were involved in the anti-inflammatory process (287).

Hepato-protective effects

Albino rats were treated with aqueous and alcoholic extract of Moringa (200mg/kg/day) for 7 days. Liver function was assessed based on liver to body weight ratio, SGPT, SGOT, ALP and bilirubin. They found that methanol extract of M. oleifera leaf have hepatoprotective effects in rats. Also, Moringa roots and flowers are reported to have hepatoprotective activity. In addition, aqueous and alcohol extracts of Moringa roots and flowers were found to have a significant hepatoprotective effect, which might be due to the presence of quercetin (224, 277). Moringa leaves have a substantial effect on the levels of SGOT, AST, SGPT, ALT, ALP and bilirubin in the serum; lipids, and lipid peroxidation levels in liver of rats (227).

In animals treated with the extract of Moringa oleifera leaves, a reduction of serum ALT, AST, ALP and BUN and creatinine was reported (289, 290). These findings were confirmed by histological examinations, which revealed an amelioration of hepatic and kidney damages induced by drugs. Similar results were found in rats co-treated with Moringa oleifera leaves and NiSO4 in order to induce nephrotoxicity (291). Also, Das et al. (288) suggested a potential role of the leaves in the prevention of NAFLD due to a reduction of ALT, AST and ALP and a lower liver damage in rats fed with high fat diet and co-treated with Moringa oleifera leaves. Also, the administration of the extract of Moringa oleifera leaves in mice was followed by decrease in serum ALT, AST, ALP, BU) and creatinine (292,293).
**Anti-hyperglycemic (Antidiabetic) effect**

Many compounds found in *Moringa oleifera* leaves might be involved in glucose homeostasis. For example, isothiocyanates have been reported to reduce insulin resistance and hepatic gluconeogenesis (294). Also, polyphenol compounds such as phenolic acids and flavonoids have effects on glucose homeostasis. These compounds have anti-diabetic effects through influencing β-cell mass and function, as well as energy metabolism and insulin sensitivity in peripheral tissues. Their effects might be due to antioxidant, enzyme inhibition, receptor agonist or antagonist activity (295, 296). Phenolic compounds, flavonoids and tannins are reported to inhibit intestinal sucrase and, slightly, pancreatic α-amylase actions (270).

The beneficial activities of *Moringa oleifera* leaves on carbohydrate metabolism have been shown by different mechanisms including preventing and restoring the integrity and function of β-cells, increasing insulin activity, improving glucose uptake and utilization (271). Hypoglycemic and antihyperglycemic activity of the leaves of *M. oleifera* might be due to the presence of terpenoids, which are involved in the stimulation of β-cells and the subsequent secretion of insulin. Also, flavonoids have been shown to play an important role in the hypoglycemic action (297).

**Hypotensive effect**

Moringa leaves contain several bioactive compounds which have been used for stabilizing blood pressure. Moringa leaves contain nitrile, mustard oil glycosides and thiocarbamate glycosides which have been shown to lower blood pressure. The isolated four pure compounds, niazinin A, niazinin B, niazimicin and niazinin A + B from ethanol extract of Moringa leaves showed a blood pressure lowering effect in rats mediated possibly through a calcium antagonist effect (224, 298).
Effects on ocular diseases

The major cause of blindness, which ranges from impaired dark adaptation to night blindness, is vitamin A deficiency. Moringa leaves, pods and leaf powder contain high concentration of vitamin A, which can help to prevent night blindness and eye problems. Also, consumption of leaves with oils improved vitamin A nutrition and delayed development of cataract (227).

Anticancer effects

The capacity of *Moringa oleifera* leaves to protect organisms and cells from oxidative DNA damage associated with cancer and degenerative diseases was reported in different studies (299). Khalafalla *et al.* (301) found that the extract of *Moringa oleifera* leaves inhibited the viability of acute myeloid leukemia, acute lymphoblastic leukemia and hepatocellular carcinoma cells. The presence of several bioactive compounds, such as 4-(α-L-rhamnosyloxy) benzyl isothiocyanate, niazimicin and β-sitosterol-3-O-β-D-glucopyranoside suggested the potential anti-cancer properties of *Moringa oleifera* leaves (302).
References


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60. DiMarco DM, Norris GH, Millar CL, Blesso CN, and Fernandez ML. Intake of up to 3 eggs per day is associated with changes in HDL function and increased plasma antioxidants in healthy, young adults. *J Nutr* 2017;247:323-329.


144. Wellen KE and Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. 


Chapter 3

Experimental Design
3.1 Background

Hypercholesterolemia is defined as high plasma cholesterol levels, and is a risk factor for many cardiovascular events. Total cholesterol levels above 200 mg/dl is considered an independent risk factor for development of peripheral vascular and coronary artery disease (CAD), and considerable attention has been directed toward cholesterol lowering therapies and alternative interventions for improving vascular function (1).

Several studies suggested that high doses of dietary cholesterol are a critical factor in the development of experimental steatohepatitis in animal models (2-5). Also, human studies support the hypothesis that dietary cholesterol plays a role in the development of steatohepatitis (6). In an epidemiological study, it was found that dietary cholesterol consumption was independently associated with the development of cirrhosis (6).

Moringa leaves extract and fraction has been shown to decrease hypertriglyceridemia and hypercholesterolemia. The anti-hypelipidemic effect might be due to decreased cholesterogenesis and fatty acid synthesis via inhibition of pancreatic cholesterol esterase (limiting the absorption of dietary cholesterol) and pancreatic lipase (involved in the inhibition of dietary triglyceride absorption) (7,8).

Phenolic compounds which are found in MO leaves have an important role in stabilizing lipid oxidation and are associated with antioxidant activity due to their scavenging ability because of their hydroxyl groups (9). Although the phenolic compounds are considered to be the major phytochemicals responsible for antioxidant activity of MO leaves, Moringa is also a good source of ascorbic acid which has antioxidant activity (10, 11). The antioxidant bioactive compounds found in MO leaves indicate scavenging activity due to hydrogen proton donation.
The beneficial effects of flavonoids present in *MO* leaves are due to their antioxidant properties. The mechanism of antioxidant action has been shown to decrease the formation of reactive oxygen species via suppression of enzymes or chelating trace elements involved in free-radical production, scavenging reactive species, protecting antioxidant defenses, decreasing alphatocopherol radicals, and activating antioxidant enzymes (13, 14).

According to Lako et al., (15), quercetin of dried *MO* leaves is present in concentrations as high as 100 mg/100 g. Quercetin is a good antioxidant with many therapeutic properties (16, 17). Quercitin has showed anti-dyslipidemic, hypotensive, and anti-diabetic effects in the obese rat model of metabolic syndrome (18). Rajanandhet al. (19) investigated the anti-inflammatory effects of *MO* leaves extract in rats fed with an atherogenic diet in order to induce hyperlipidemia. They showed that, TNF-α and IL-1 in the serum was significantly lower in rats treated with extract of *MO* leaves compared to control. The effect of supplementation of *MO* leaves concentrate in obese-induced mice with very high-fat diet was tested. A reduction of gene expression of pro-inflammatory markers, TNFα, IL-6 and IL-1β in the liver and ileum was found in tissues in mice treated with *Moringa oleifera* concentrate compared to the control group (20).

In these studies, we aim to investigate the protective effects of the bioactive components of *MO* leaves in protecting against hepatic steatosis.

3.2 Experimental diets

Isocaloric diets were formulated to meet the nutritional requirements of guinea pigs. Dietary cholesterol at 0.25% in this model corresponds to an fed amount equal to 2,500 mg/d in a human diet (21).
This project required the formulation of three different experimental diets:

(A) Control: a pelleted non-purified diet that met all the nutritional requirements for guinea pigs plus 0.25% of dietary cholesterol (w/w of diet), which is sufficient to ensure liver damage in the proposed time frame of the experiment (6 weeks) (22).

(B) Low Moringa (LM): a pelleted non-purified diet that was matched to the control taking into consideration the addition of 10% powdered MO leaves to adjust macronutrient intake (equivalent to 22g Moringa leaves/kg body weight of human).

(C) High Moringa (HM): a non-purified diet matched to control taking into consideration the addition of 15% powdered MO leaves to adjust macronutrient intake (equivalent to 33g Moringa leaves/kg body weight of human).

All ingredients were homogeneously mixed with water (5% by weight), and pelleted via cold extrusion process for all diets. After that, diets were dried at 88-92°F for 2 days to remove excess water. The composition of the MO leaves and experimental diets are presented in Table 3.1 and Table 3.2, respectively.

3.3 Animals

For this project, we used the guinea pigs model challenged with cholesterol, which has been shown to mimic humans in hepatic cholesterol and lipoprotein metabolism (23).

Twenty-four male Hartley guinea pigs weighing between 200 to 300 grams (6 weeks old) were housed in pairs in a light cycle room for a total of 12 cages. During the first week of acclimation, the animals received regular chow ad libitum and had free access to water. After one week, the cages were randomly assigned to either one of three experimental diets named "Control", "Low Moringa" (LM) (10%) and high Moringa (HM) (15 %) for a total of four cages per group.
Food consumption was monitored three times a week, weighing the food left on the feeder and subtracting that from the amount given two days prior. Once a week, guinea pigs were weighed and their body weight was monitored to ensure proper growth and health.

After 6 weeks, guinea pigs were fasted for 12 hours and anesthetized with isoflurane. Animals were sacrificed using the heart puncture technique. Blood from the heart was collected and centrifuged immediately (2000xg) to separate the plasma. Liver, heart and adipose tissue (epididymal tissue) were collected and preserved at -80° C for further analysis.

All animal experiments were performed in accordance with U.S. Public Health Service/ U.S. Department of Agriculture guidelines. Experimental protocols were approved by the University of Connecticut IACUC (Protocol number A16-003).

**Food intake and bodyweight**

During the intervention, the guinea pigs consumed an average of 20.7 and 23.6 grams of food per day, in the 10 and 15% Moringa groups, respectively, (which is equivalent to 2.07 and 3.54 gm of MO leaves/day) compared to 24.0 grams for control group. There were no differences in food intake, caloric intake, weight gain or final body weight among groups during the intervention in the control, 10 and 15% Moringa groups, respectively (Table 3.3).

**Liver and adipose tissue**

There was no difference in weights of liver or epididymal adipose tissue in the control, 10 and 15% Moringa groups, respectively during the intervention. Data are shown in Table 3.3.
3.4 Discussion

The use of suitable animal models is essential to understand the mechanisms by which Moringa affects plasma and hepatic lipids. The similarity between guinea pigs and humans in terms of lipoprotein and hepatic cholesterol metabolism is due to: 1) higher concentrations of free compared to esterified cholesterol in the liver (24); 2) guinea pigs have plasma cholesteryl ester transfer protein (CETP) activity (25), a critical component for human reverse cholesterol transport (26) and lecithin: cholesterol acyltransferase (LCAT) (27), and lipoprotein lipase (28) activities that contribute to remodeling of plasma lipoproteins; 3) guinea pigs exhibit moderate rates of hepatic cholesterol synthesis and catabolism (29,30); 4) there is no Apo B mRNA editing in liver (31); 5) guinea pigs require dietary vitamin C, an important anti-oxidant that may play a role in oxidation and atherosclerosis (32). 6) guinea pigs respond to dietary fat saturation, dietary cholesterol, dietary fiber by alterations in LDL cholesterol (30); 7) guinea pigs are good models for studying the mechanisms by which statins, and other drugs lower plasma LDL cholesterol (33).

No significant difference was observed between groups in food intake and body weight in the present study. Similar to our results, Ghebreselassie et al. (34) reported that, there was no significant change in body weight of mice treated with doses of 600 and 750 mg/kg of Moringa extract. In addition, no significant change in weight of liver and kidney was observed in all animals. This might suggest a non-toxic effect of the Moringa extract. In contrast, lower food intake has been found in rats treated with MO leaves extract when compared to the group feed with only high-fat diet (35). Therefore, the body weight gain in these groups was significantly lower than the rats feed with only high-fat diet. However, in our study there were no differences in body weight associated with Moringa intake.
3.5 Strengths and limitations

Strengths of this study design include the use of an animal model that has similar cholesterol metabolism to humans (36). The guinea pigs size also facilitates the sacrifice process and tissue collection. Guinea pigs have been used numerous times to evaluate the effects of cholesterol, dietary fat, polyphenols and carotenoids among others on various measures of health (37, 38, 39). Another strength is that the *Moringa oleifera* leaves used in this study were added to the food preparations similar to what humans do in their diet (40, 41).

The main limitation of the study design was the lack of analyses of the bioactive components of *MO* leaves although the concentrations most commonly found have been reported in other studies (15, 42)
### Tables

**Table 3.1 Composition of dried *Moringa oleifera* leaves**

<table>
<thead>
<tr>
<th>Component (g)</th>
<th>g/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate(^2)</td>
<td>30</td>
</tr>
<tr>
<td>Fiber</td>
<td>44</td>
</tr>
<tr>
<td>Protein</td>
<td>5</td>
</tr>
<tr>
<td>Fat</td>
<td>3</td>
</tr>
<tr>
<td>Moisture</td>
<td>10</td>
</tr>
<tr>
<td>Minerals</td>
<td>8</td>
</tr>
<tr>
<td>Quercetin (41)(^3)</td>
<td>0.0207</td>
</tr>
<tr>
<td>Chlorogenic acid (41)(^3)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

---

1. Proximate analysis of Moringa were made by CIAD (Centro de Investigacion en Alimentos Desarrollo, Hermosillo, Mexico)
2. 2 g sugar and 28 g starch
3. Chlorogenic acid and quercetin values were taken from the literature
Table 3.2 Composition of Experimental Diets\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Low Moringa (10%)</th>
<th>High Moringa (15%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g</td>
<td>% Energy</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>22</td>
<td>23</td>
<td>21.5</td>
</tr>
<tr>
<td>Moringa Protein</td>
<td>0.5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Carbohydrate\textsuperscript{3}</td>
<td>41</td>
<td>41.9</td>
<td>38</td>
</tr>
<tr>
<td>Carbohydrate from Moringa</td>
<td>3</td>
<td>3.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Fat\textsuperscript{4}</td>
<td>15.1</td>
<td>35.1</td>
<td>14.8</td>
</tr>
<tr>
<td>Moringa fat</td>
<td>0.3</td>
<td>0.6</td>
<td>0.45</td>
</tr>
<tr>
<td>Vitamins\textsuperscript{4}</td>
<td>1.1</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Minerals\textsuperscript{4}</td>
<td>8.1</td>
<td>-</td>
<td>7.3</td>
</tr>
<tr>
<td>Moringa Minerals</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>10</td>
<td>-</td>
<td>8.1</td>
</tr>
<tr>
<td>Moringa fiber</td>
<td>4.4</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Guar Gum</td>
<td>2.5</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>Energy Density (Kcal/g)</td>
<td>3.87</td>
<td>3.87</td>
<td>3.87</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Moringa leaves were provided by Scientech Health International S.A de CV (Mexico City, Mexico).
\textsuperscript{2}Percent energy from macronutrients is the same for all diets
\textsuperscript{3}Mixture of starch/sucrose
\textsuperscript{4}Fat mix was palm kernel: oil: olive oil: safflower oil (50:25:25), high in lauric and myristic acids
\textsuperscript{5}Composition of vitamin and mineral mixes were formulated according to guinea pig requirements.
Table 3.3 Food intake, initial body weight, weight gain, final body weight, liver and adipose tissue weights of guinea pigs fed a hypercholesterolemic diet with no Moringa (Control), Low Moringa (LM) or High Moringa (HM)\textsuperscript{1}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food Intake (g)</td>
<td>24.0±2.3</td>
<td>20.7±7.8</td>
<td>23.6±2.9</td>
</tr>
<tr>
<td>Energy intake (Kcal)</td>
<td>92±9</td>
<td>81±30</td>
<td>91±11</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>334±15</td>
<td>315±13</td>
<td>339±17</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>535±44</td>
<td>543±55</td>
<td>507±43</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>201±49</td>
<td>229±49</td>
<td>167±49</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>27.9±3.0</td>
<td>28.4±5.2</td>
<td>25.5±3.9</td>
</tr>
<tr>
<td>Adipose weight (g)</td>
<td>55.9±13.9</td>
<td>53.6±24.7</td>
<td>59.5±18.9</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>2.3±0.5</td>
<td>2.4±0.3</td>
<td>2.3±0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Values are expressed as mean ± SD for n = 8 guinea pigs per group.
3.6 References


35. Bias S, Singh GS and Sharma R. Antiobesity and Hypolipidemic Activity of Moringa oleifera Leaves against High Fat Diet-Induced Obesity in Rats. *Advances in Biology*. **2014**; 1-9


Chapter 4

Effects of *Moringa oleifera* leaves on plasma lipids, lipoprotein subfractions and size and on parameters of reverse cholesterol transport
4.1 Background

The liver plays a pivotal role in lipid metabolism. It extracts cholesterol from intestinal chylomicrons and excretes it back into the intestines with bile acids. It biosynthesizes TG and cholesterol and packages them as VLDL, that is secreted into the bloodstream. Through the LDL receptor (LDLR), plasma LDL as well as IDL or VLDL are removed from circulation. HDL mediates the reverse transport of cholesterol from extra-hepatic tissues to the liver (1). Lipoprotein metabolism is important because disturbances in the lipoprotein profiles are considered a risk factor for many chronic diseases such as cardiovascular disease, metabolic syndrome, NAFLD and type 2 diabetes mellitus (T2DM) (2). Guinea pigs have been shown to be a suitable animal model for evaluating lipoprotein profile altered by cholesterol challenges; their cholesterol metabolism is similar to the human situation making them ideal models to understand alterations of metabolic pathways caused by dietary interventions (3, 4, 5).

Moringa leaves have been shown to have hepatoprotective activity and decrease lipid profile in the serum of high-fat diet fed rats which may be attributed to the presence of β – sitosterol (6, 7). The aqueous extract of Moringa leaves has been found to lower the blood sugar and inhibit the growth of pathogenic microorganisms in diabetic rats (8, 9). In addition, flavonoids and saponins lower cholesterol absorption by the inhibition of cholesterol micellar solubility, thus, these bioactive compounds found in aqueous extract have hypolipidemic effects (10). Chumark et al. (11) examined the therapeutic potential of Moringa oleifera leaves on dyslipidemia induced in rabbits on a high- cholesterol (5%) diet (HCD) for 12 weeks. By the end of the regimen, relative to rabbits on a normal diet, HCD-fed rabbits experienced several-fold increases in the plasma levels of total cholesterol (TC), HDL-C, LDL-C, and TG. When these HCD rabbits were concomitantly fed a M. oleifera aqueous leaf extract, at the daily dose of 100mg/kg-BW for the duration of the protocol, these increases were reduced: for TC and
lipoprotein-cholesterol by about 50%, for TG by 75%. The anti-dyslipidemic effects of MO leaves were also examined in rats fed a high-fat diet (HFD) containing 16% fat, with or without an aqueous extract of *Moringa leaves* for 30 days. In treated rats, the plasma total cholesterol (TC) was reduced (12). In another study, Jain et al. (13) fed albino rats for 30 days, a diet contained 26% fat, with or without a methanolic extracts of *Moringa oleifera* leaves. At the highest dose (600 mg/kg-BW), *Moringa oleifera* leaves treatment reduced plasma TC, LDL-C, VLDL-C, and TG. Interestingly, serum HDL-C was unchanged by HFD diet alone; however, HDL-C was increased in rats fed leaf extract-supplemented HFD, significantly reducing the TC/HDL-C ratio. Flavonoids and saponins present in the leaves of Moringa oleifera are reported to increased HDL-C concentration and decrease LDL and VLDL cholesterol in hypercholesteremic rats (14). Polyphenols can protect LDL from oxidation through inhibition of lipid peroxidation by acting as chain breaking peroxy radical scavengers and also inhibit hepatic lipid synthesis (15, 16).

Therefore, these studies suggest that MO leaves can alter plasma lipids and lipoprotein metabolism. Based on these data, we hypothesized that Moringa leaves would decrease plasma total cholesterol, LDL cholesterol and triglycerides in guinea pigs fed a high cholesterol diet for 6 weeks.

### 4.2 Materials and Methods

#### 4.2.1 Plasma Lipids

Plasma HDL-C, TC, LDL-C, total triglycerides and glucose were measured with direct enzymatic reactions and photometric detection using the Cobas c111 analyzer (Roche Diagnostics). LDL cholesterol was calculated by the Friedwald equation as previously reported (3).
4.2.2 Plasma insulin

To measure plasma insulin, a Rat/Mouse Insulin kit (Crystal Chem. Dowers, Grove, IL), which was found to cross-react with guinea pig insulin was used for this assay. 5 µl of plasma were used for this assay. The standard curve consisted of concentrations ranging from 0.156 to 10 ng/mL. The CV for this assay was 10%.

4.2.3 Lipoprotein size and number

Lipoprotein size and number were measured by nuclear magnetic resonance (NMR) spectroscopy. This technique can quantify the size and concentration (“number”) of lipoprotein particles expressed each as an average particle size or as lipoprotein particle concentration (17, 18) and calculate the cholesterol contained in lipoproteins. (19). Each lipoprotein subclass signal emanates from the aggregate number of terminal methyl groups on the lipids contained within the particle, with the cholesteryl esters and triglycerides in the particle core each contributing three methyl groups, and the phospholipids and unesterified cholesterol in the surface shell each contributing two methyl groups. Because the methyl signals from these lipids are indistinguishable from each other, they overlap to produce a bulk lipid particle signal (20). The amplitude of each lipoprotein subclass signal serves as a measure of the particle concentration of that subclass (21). The NMR LipoProfile test is a diagnostic tool used to identify and manage risk for lipid-related heart disease (19, 20). Samples were analyzed for lipoprotein size and number at LipoScience Inc (Raleigh, NC)

4.2.4 LCAT and CETP activities

LCAT activity was determined by use of a commercially available fluorometric assay kit (Cell Biolabs Inc., San Diego, CA). The relative LCAT activity was assessed by the strength of the fluorescence signal following an incubation period. CETP activity was measured by use of a
commercially available assay kit (BioVision Inc., Milpitas, CA). The decrease in fluorescence intensity over time was used to calculate CETP activity after incubation of plasma with a self-quenching donor molecule acceptor molecule as previously reported by DiMarco et al (22).

4.2.5 Ox LDL

Plasma ox LDL were analyzed using a mouse competitive ELISA (Mercodia, Uppsala, Sweden) which is based in the mouse monoclonal antibody 4E6, which is directed against a conformational epitope in oxidized ApoB-100. We have successfully used this method to measure ox LDL in guinea pigs (23).

4.2.6 Statistical Analysis

Differences between groups were analyzed by one-way ANOVA and LSD post hoc analysis. P < 0.05 was considered to be significant. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.). All data are presented as mean ± SD.

4.3 Results

4.3.1 Plasma lipids and glucose

There were no differences in plasma total cholesterol, VLDL, LDL, HDL, triglycerides, glucose or insulin among the control, low moringa or high moringa groups (Table 4.1).

4.3.2 Lipoprotein size and subfractions

Total VLDL or total LDL and all subfractions (large, medium and small) were not different between groups. Regarding HDL, total HDL, large and small HDL were not affected, while medium HDL was lower in both groups of moringa compared to control group (P<0.05). There were no differences in VLDL, LDL or HDL size among groups (Table 4.2).
4.3.3 LCAT and CETP activity

LCAT activity was higher in both groups of moringa compared to the control group (P<0.05) (Table 5). In addition, no differences were found in CETP activity or in the concentration of oxidized LDL between groups (Table 4.3).

4.4 Discussion

4.4.1 Plasma lipids and lipoproteins

Unexpectedly, no changes in plasma lipids, glucose or insulin were observed among groups. It is well known that Moringa oleifera leaves contain 24.3% of fiber (24), thus we were expecting to observe decreases in plasma cholesterol due to fiber intake. However, we observed no changes in plasma total or LDL cholesterol suggesting that the fiber present in Moringa did not disrupt micelle formation or cause interruption of the enterohepatic circulation of bile acids as we have observed in other guinea pig studies (25).

Moringa leaves, however, resulted in lower concentrations of the medium HDL particles, which have been shown to decrease reverse cholesterol transport (RCT) (1). In other study conducted with low carbohydrate diets observed significant increases in HDL cholesterol, while medium HDL decreases (26).

4.4.2 LCAT and CETP activities

To further confirm that RCT might have been enhanced by Moringa leaves, we observed higher LCAT activity. LCAT activity was increased by 55% (p< 0.05) by ML intake while CETP activity was not different among groups. These results suggest a potential role of ML in modulating RCT. The key function of lecithin-cholesterol acyltransferase (LCAT) is to form cholesteryl ester and, at a constant apoA-I concentration, larger discoidal HDL particles are the
preferred substrate (27). The lipid transfer proteins, cholesteryl ester transfer protein (CETP) are involved in moving cholesteryl ester, triacylglycerol and phospholipid molecules among HDL and other lipoprotein particles (28, 29). In one study, over-expression of LCAT increased HDL-C, but this elevation in HDL-C did not increase RCT from macrophages to feces (30). An explanation for this observation is that increased esterification by LCAT resulted in an increase in the production of mature HDL particles and a reduction in lipid-poor preβ-HDL. Thus, the level of LCAT activity determines the array of HDL particles present in the plasma, and this in turn influences the efficiency of the various efflux pathways in the removal of cell cholesterol. In addition, liver expression of CETP promotes macrophage RCT and this effect is dependent on the LDL receptor (30). Thus although we did not observe changes in atherogenic lipoproteins, the observed changes in these mediators (LCAT, and HDL size) of RCT suggest that Moringa may also protect against atherosclerosis. Further studies are necessary to confirm this theory.

However, there was no difference in oxidized LDL in plasma of guinea pigs consuming moringa leaves or control diets suggesting that that Moringa leaves do not exert an antioxidant effect in plasma in these animals that also did not change the concentrations of plasma lipids due to Moringa treatment

4.5 Conclusion

Although Moringa leaves had no effect on the dyslipidemias induced by the high cholesterol diets in guinea pigs, Moringa appeared to affect HDL metabolism by decreasing the medium HDL, which has been considered a less protective HDL and by increasing LCAT activity. These results suggested a potential role of Moringa leaves in modulating the parameters involved in RCT.
Table 4.1 Plasma lipid profile, glucose and insulin of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%)\(^1\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>294.6 ± 167.0</td>
<td>192.9 ± 84.7</td>
<td>251.6 ± 70.3</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>11.0 ± 2.9</td>
<td>10.6 ± 5.0</td>
<td>11.9 ± 4.0</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>244.5 ± 166.4</td>
<td>155.0 ± 73.9</td>
<td>187.7 ± 73.4</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>39.1 ± 28.3</td>
<td>27.4 ± 24.2</td>
<td>52.0 ± 30.3</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>55.9 ± 13.9</td>
<td>53.6 ± 24.7</td>
<td>59.5 ± 18.9</td>
</tr>
<tr>
<td>Plasma Glucose (mg/dL)</td>
<td>158.4 ± 16.5</td>
<td>154.4 ± 23.4</td>
<td>164.6 ± 18.9</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>32.4 ± 13.9</td>
<td>34.0 ± 10.7</td>
<td>30.3 ± 11.0</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at P<0.05
Table 4.2 Lipoprotein subfractions in guinea pigs fed a hypercholesterolemic diet (0.25 g/100 g) with no Moringa (control), Low (10%), or High Moringa (15%)1

<table>
<thead>
<tr>
<th>(nmol/l)</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total VLDL (nmol/L)</td>
<td>55.2 ±128.1</td>
<td>45.4 ±21.4</td>
<td>61.5 ± 1.7</td>
</tr>
<tr>
<td>Large VLDL (nmol/L)</td>
<td>1.7 ± 1.3</td>
<td>1.1 ± 0.9</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>Medium VLDL (nmol/L)</td>
<td>14.3 ± 7.0</td>
<td>13.7 ± 7.6</td>
<td>16.7 ± 7.2</td>
</tr>
<tr>
<td>Small VLDL (nmol/L)</td>
<td>39.1 ± 21.2</td>
<td>30.6 ± 14.0</td>
<td>16.7 ± 7.2</td>
</tr>
<tr>
<td>Total LDL (nmol/L)</td>
<td>531 ± 219</td>
<td>563 ± 173</td>
<td>709 ± 284</td>
</tr>
<tr>
<td>IDL (nmol/L)</td>
<td>63 ± 31</td>
<td>80 ± 33</td>
<td>107 ± 52</td>
</tr>
<tr>
<td>Large LDL (23-30 nm)</td>
<td>148 ± 61</td>
<td>102 ± 46</td>
<td>104 ± 30</td>
</tr>
<tr>
<td>Small LDL (18-23 nm)</td>
<td>320 ± 160</td>
<td>382 ± 123</td>
<td>498 ± 252</td>
</tr>
<tr>
<td>Total HDL (μmol/L)</td>
<td>1.5±0.6</td>
<td>0.9 ± 0.7</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Large HDL (10-13 nm)</td>
<td>0.19±0.13</td>
<td>0.14±0.09</td>
<td>0.21±0.08</td>
</tr>
<tr>
<td>Medium HDL (8.2-10)</td>
<td>0.56±0.55a</td>
<td>0.11±0.21b</td>
<td>0.21±0.13b</td>
</tr>
<tr>
<td>Small HDL (7.3-8.2)</td>
<td>0.8±0.6</td>
<td>0.6±0.7</td>
<td>1.0±0.5</td>
</tr>
<tr>
<td>VLDL size (nm)</td>
<td>51.0 ± 5.7</td>
<td>46.4 ± 5.5</td>
<td>47.4 ± 5.5</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>20.8 ± 0.6</td>
<td>20.3 ± 0.5</td>
<td>20.3 ± 0.7</td>
</tr>
<tr>
<td>HDL size (nm)</td>
<td>9.5 ± 1.0</td>
<td>10.1 ± 0.7</td>
<td>9.7 ± 0.5</td>
</tr>
</tbody>
</table>

1Values are means ± SD, n=8; Means in the same row without a common letter differ at P<0.05
Table 4.3 Lecithin Cholesterol Acyltransferase (LCAT), Cholesteryl ester transfer protein (CETP) activity and Oxidized LDL

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT activity (Fluorescence, RFU)</td>
<td>195.4 ± 46.8^a</td>
<td>243.6 ± 34.2^b</td>
<td>237.5 ± 20.3^b</td>
</tr>
<tr>
<td>CETP Activity (pmol/L/h)</td>
<td>19.4 ± 13.7</td>
<td>10.8 ± 3.8</td>
<td>14.1 ± 12.2</td>
</tr>
<tr>
<td>Oxidized LDL (mg/L)</td>
<td>3.9 ± 4.8</td>
<td>2.4 ± 3.5</td>
<td>7.1 ± 3.5</td>
</tr>
</tbody>
</table>

^1 Values are means ± SD, n=8; Values in the same row with different superscripts are significantly different at P<0.05
4.6 References


Chapter 5

The effects of Moringa leaves on hepatic steatosis
5.1 Background

Hepatic steatosis is defined by triglyceride accumulation in hepatocytes as a result of an imbalance between the uptake of fat and its oxidation and export. The most consistent underlying pathogenic factor is insulin resistance, which predisposes to lipolysis of peripheral fat with mobilization to and uptake of fatty acids by the liver (1,2). The pathogenesis of hepatic triglyceride accumulation is that obesity and insulin resistance increase the release of free fatty acids (FFA) from adipocytes through increased hormone-sensitive lipase activity (2). Triglyceride accumulation in hepatocytes increases the vulnerability of the liver to secondary insults through effects of cytokines or oxidative stress (3) since the circulating FFA is not only substrate for triglyceride synthesis but may also be directly cytotoxic. The apoptosis of hepatocytes correlates with progressive inflammation (steatohepatitis), fibrosis, cirrhosis and hepatocellular carcinoma (4, 5). As stated previously, cholesterol feeding could induce dyslipidemia and insulin resistance. In animal models an excess of dietary cholesterol also appears to be an important risk factor for hepatic steatosis and progression to steatohepatitis (3). ALT and AST are enzymes involved in amino acid metabolism and can be used as indicators of liver damage (6). Other studies have also shown reduction of serum ALT and AST in animals treated with the extract of Moringa leaves (7). Pari and Kumar (8) found a reduction of ASP and AST with Moringa intake and histological evaluation of liver further documented a recovery from liver damage with Moringa leaves. These results suggest that Moringa leaves exert protective effects in liver without inducing toxicity. Also, Das et al. (9) reported a reduction of ALT, AST and ALP and a lower liver damage in rats fed with high fat diet and co-treated with MO leaves, suggesting a potential role of the leaves in the prevention of NAFLD. The hepatoprotective effect may be related to antioxidant properties and anti-inflammatory effects (10).
Previous studies showed that a cholesterol challenge leads to hepatic steatosis in animal models (11) and reported that Moringa decreased lipid profile of liver, heart and aorta in hypercholesteremic rabbits and increased the excretion of fecal cholesterol (12,13). This hepatoprotective activity of Moringa has been found in different studies. Ethanolic extracts of Moringa leaves have shown a protective effect against drug-induced liver damage in rats (8). Further, histological examinations confirmed an amelioration of the hepatic damages induced by drugs (14). Moringa leaves also showed the ability to reduce carbon tetrachloride-induced liver fibrosis and control the rise of serum amino transferase activities and globulin level (10). In addition, Moringa showed to protect liver exposed to ionizing radiation by increased antioxidant enzymes and inhibited translocation of nuclear factor kB (NF-kB) from cytoplasm to the nucleus (15).

Phenolic acids, including chlorogenic acid (CGA), from Moringa leaves have been shown to have antioxidant, anti-inflammatory and anti-hyperglycemic properties (16). CGA from other dietary sources has been recognized as an important modulator of metabolic pathways related to lipid synthesis, lipoprotein uptake and inflammation. It has been reported that CGA markedly decreased the concentration of TC and TG in plasma and liver, which is most probably due to the increase of the fatty acid utilization in the liver and the alteration of the activities of enzymes involved in lipid metabolism (17, 18, 19, 20, 21). Gebhardt (22) demonstrated that CGA can indirectly yet efficiently inhibit \( \beta \)-hydroxy-\( \beta \)-methyl glutaric acyl coenzyme A reductase (HMG CoA reductase) in primary cultured rat hepatocytes and inhibit the synthesis of cholesterol. In addition, Wan et al. (20) suggested that the hypocholesterolemic functions of CGA are most likely due to the increase in fatty acid utilization in the liver via the upregulation of peroxisome proliferation-activated receptor \( \alpha \) mRNA. Waterman et al. (23)
also evaluated the effect of supplementation of 5% moringa concentrate (MC, delivering 66 mg/kg/d of moringa isothiocyanates) in obese-induced mice with very high-fat diet for 12 weeks. They found a reduction of gene expression of pro-inflammatory markers, TNFα, IL-6 and IL-1β in the liver and ileum tissues in mice treated with Moringa concentrate compared to the control group.

Quercetin, another bioactive component in Moringa has been shown to improve hepatic lipid accumulation in various pathways, such as the synthesis, transport and oxidation of long-chain fatty acids and TG by suppressing the expression of two lipogenic genes SREBP-1c and FAS. As a result, quercetin has the therapeutic potential for preventing or treating NAFLD and IR-related metabolic disorders (24).

5.2 Materials and methods

5.2.1 Hepatic Lipids

Ten mg of liver for each sample was homogenized with 200 µL of chloroform/ isopropanol/MP40 (7:11:01). The slurries were centrifuged and the supernatant was dried at 50°C overnight. The quantification for total and free cholesterol was done with enzymatic assays kits (Wako Diagnostics) according to Carr et al (25). The esterified cholesterol fraction was calculated manually by subtracting free cholesterol from total cholesterol (26). Liver triglycerides were analyzed by enzymatic methods (Wako Diagnostics, CA) (27).

5.2.2 Oxidized LDL

Tissue total protein was extracted using RIPA buffer and total protein concentrations of the lysates were determined using the BCA Protein Assay Kit (Cell Signaling Technologies Inc,
Beverly, MA). Hepatic oxLDL was measured with a specific guinea pig OxLDL ELISA kit (MyBioSource), which measures a specific fraction of the apo B molecule (28).

5.2.3 Hepatic inflammatory cytokines

The potential of the Moringa leaves as an anti-inflammatory agent was evaluated by measuring the cytokines in liver homogenates. To ensure that there was no interference with other liver components in the assay, the protein was extracted and quantified as detailed before for the oxLDL measurement. IL-1 beta, IL-6, IL-10, IFN-gamma, MCP-1, and TNF-alpha were measured using Luminex technology with the MILLIPLEX MAP Rat Cytokine Immunoassay kit (Millipore corporation, Charles, MO, USA) (27).

5.2.4 Liver histology

Small pieces taken from the same liver section were immersed in 10% buffered formalin. Formalin-fixed livers were paraffin embedded and sections 3–5 μm were prepared and stained with hematoxylin and eosin and scored by a pathologist blinded to the treatment.

5.2.5 Liver enzymes

Both AST and ALT were measured with direct enzymatic reactions and photometric detection using the Cobas c111 analyzer (Roche Diagnostics).

5.2.6 Gene expression

Total RNA was extracted from the liver using the GenElute Mammalian Total RNA Purification Kit, according to the vendor’s specifications (Sigma-Aldrich). Samples were lysed and homogenized in guanidine thiocyanate and 2-mercaptoethanol to release RNA and inactivate RNases. Resulting lysates were spun through a filtration column to remove cellular
debris and shear DNA. The filtrate was then applied to a high capacity silica column to bind total RNA, followed by washing and elution. To check the integrity of the extracted RNA, samples were electrophoresed through a 1% agarose gel at 125V for 45 minutes. cDNA was synthesized for each gene using cDNA synthesis kit following the manufacturer’s instructions. cDNA was then amplified using Real Time PCR. The primers used for β-actin, a house-keeping gene, were taken from guinea pig sequences as previously reported (29). The genes selected to be measured in this study were the following: DGAT2, CD68, PPARɤ, SREBP1 and LDL-R.

5.2.7 Statistical Analysis
Differences among groups in hepatic lipids, liver enzymes, inflammatory cytokines and gene expression of regulators of lipid synthesis and uptake were analyzed by one-way ANOVA and LSD post hoc analysis. Pearson correlations were calculated between plasma liver enzymes and oxidized LDL P < 0.05 was considered to be significant. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.). All data are presented as mean ± SD.

5.3 Results
5.3.1 Hepatic Lipids
Hepatic total cholesterol, cholesteryl esters (CE) and triglycerides (TG) were significantly lower (P<0.05) in the group fed the HM diet, while the concentrations of hepatic lipids in the LM group were not different compared with HM or control groups. Free Cholesterol concentration was significantly lower in both Moringa groups compared to control group (Table 5.1).
5.3.2 Oxidized LDL

As shown in Table (5.1), there were no significant differences in the concentration of OxLDL between both Moringa groups and control.

5.3.3 Liver histology

There was a lower degree of steatosis and lipid accumulation in livers from the guinea pigs fed the HM diet compared to the control and the LM group (Figure 5.2). Liver samples from guinea pigs fed the control diet showed most lipid accumulation while the LM group exhibited less accumulation than the control in agreement with hepatic cholesterol and triglyceride concentrations.

5.3.4 Liver enzymes

Both moringa groups had lower concentration of ALT than the control group, while no difference in the AST between groups were observed (P<0.05) (Table 5.2). A significant correlation was found between liver enzymes and oxidized LDL (Figure 5.1)

5.3.5 Hepatic inflammatory cytokines

Hepatic cytokines; interleukin 1 beta (IL-1β), interleukin 10 (IL-10) and Interferon gamma (IFNγ) were lowest significantly (P<0.05) in group fed the HM diet, intermediate in the low moringa group and highest in control group. There were no differences in hepatic cytokines; interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor alpha (TNFα) among different groups (Table 5.3).
5.3.6 Gene expression

Guinea pigs group fed on high Moringa (HM) diet had lower levels of LDL receptor (LDL-R) gene expression compared to control and LM groups. Guinea pig fed the moringa leaves had lower gene expression of cluster of CD68 and SREBP1 compared to control (p<0.05). DGAT2 and PPARγ were lowest in the HM group; intermediate in LM and highest in the control group (P < 0.05) (Figure 5.3).

5.4 Discussion

In the current study, we have demonstrated that Moringa leaves (ML) effectively reduce hepatic steatosis in guinea pigs most likely by the action of polyphenols present in ML, mainly CGA and quercetin, which have been shown to alter gene expression of major regulators of hepatic cholesterol and TG synthesis and uptake. Also, we observed that, there was a dose response with the HM diet having better effects overall on liver health.

5.4.1 Hepatic lipids

The cholesterol challenge used in the present study was found to cause hepatic steatosis in guinea pigs as previously reported (11). Accumulation of lipids both cholesterol and TG were observed in the control animals, while the intake of ML resulted in lower concentrations of cholesterol and TG in a dose-dependent manner. CGA is one of the bioactive components present in ML (30). Other investigators have reported that CGA markedly decreases the concentration of total cholesterol and TG in liver (18) and in agreement with our results, these hypolipidemic effects have also been observed in cells (31).

It is well known that hepatic steatosis is defined as accumulation of TG and fatty acids in the hepatocytes and the presence of increased hepatic free cholesterol (FC) concentration could
accelerate the development of NAFLD and NASH and also contribute to lipotoxicity and liver injury (32,33). Thus the observed decreases in FC by Moringa intake confirm that Moringa can be successfully used to prevent hepatic steatosis.

Moringa leaves have also been reported to decrease lipid accumulation in liver, heart and aorta in hypercholesteremic rabbits and increase the excretion of fecal cholesterol (13). CGA has also been demonstrated to inhibit β-hydroxy-β-methyl glutaric acyl coenzyme A reductase (HMG CoA reductase), the rate limiting enzyme of cholesterol synthesis and improved lipid metabolism by the transcriptional activation of AMPK and inhibition of downstream targets, such as SREBP2 and HMGCR, to suppress the TC synthesis and TG levels in liver cells. CGA also increased ABCA1 and CYP7A1 to enhance TC efflux and bile acid transport, synthesis, and excretion (22, 34).

5.4.2 Oxidized LDL
OxLDL is a mixture of lipoproteins with heterogeneous modifications such as oxidation of phospholipids, modification of apolipoprotein B (apoB) with malondialdehyde and aggregation of apoB. These particles are defined as biomarker for atherosclerosis and in addition they can exhibit pro-inflammatory, immunogenic, apoptotic, and cytotoxic activities for other diseases, including NAFLD (35,36). OxLDL particles were showing to be trapped in the lysosome compartment leading to presence of cholesterol crystals which can activate the inflammasome and induce the activation of NF-κB signaling, therefore, OxLDL derived lysosomal lipid accumulation can been linked to hepatic inflammation, liver fibrosis and the progression of NASH (37,38). In our study, no significant effect of Moringa leaves in altering the concentration of OxLDL in the liver was observed when compared to control group. Thus the
protective effects of Moringa in decreasing hepatic steatosis are not related to OxLDL concentrations in the liver

5.4.3 Liver histology and liver enzymes
Histology evaluation indicated low hepatic lipid accumulation in the high moringa group, intermediate in the low moringa group and high in the control group in total agreement with hepatic lipids and inflammatory markers. When there is an injury to the liver, there is an increase in serum concentrations of amino-transferases: AST and ALT. These enzymes are highly concentrated in the liver although AST is also diffusely represented in the heart, skeletal muscle, brain and kidneys suggesting that an elevation in plasma ALT is more specific for liver injury (6).

Quercetin has been shown to inhibit the development of NASH by reducing macrovesicular steatosis, microvesicular steatosis, hepatocellular ballooning and inflammatory infiltrate. This effect was accompanied by a significant reduction in AST and ALT, lipid peroxidation, inflammation and DNA damage (39). Moringa leaves in the present study were also found to have a hepatoprotective effect, which could be partly due to the presence of quercetin, a well-known flavonoid with hepatoprotective activity (8, 9, 40).

It is well known that, high levels of AST and ALT in serum are indicators for liver dysfunction. High concentration of *Moringa oleifera* leaves were shown to improve liver functions (41). Confirmed by Buraimoh (42) and Halaby et al (43), Moringa leaves have shown significant hepato-protective activity and can protect the liver from chemically induced damage. Other studies showed a reduction of serum ALT and AST in animals treated with the extract of *Moringa oleifera* leaves (7, 44, 45).
5.4.4 Hepatic inflammatory cytokines

The many bioactive compounds naturally present in *Moringa oleifera* leaves, such as flavonoids and phenolic acids, might be involved in the anti-inflammatory process. Among them, quercetin inhibited activation of NF-kB and also the subsequent NF-kB-dependent downstream events and inflammation (46).

In the present study, the HM moringa group, the inflammatory cytokines, IL-1β and IFNγ were 27 and 21% lower, respectively compared to the control (p< 0.05), while the concentrations for these cytokines were intermediate for the low Moringa group. IL-1β is an important marker for hepatic damage because in liver disease, IL-1β promotes the recruitment of inflammatory cells to the liver, which contributes to fibrosis, an important characteristic of the progression from hepatic steatosis to steatohepatitis (47).

The anti-inflammatory effects of extract of *MO* leaves in rats fed with an atherogenic diet in order to induce hyperlipidemia were investigated by Rajanandh *et al.* (48). They showed serum TNF-α and IL-1 was significantly lower in rats treated with extract of *MO* leaves compared to control. Also, a reduction of gene expression of pro-inflammatory markers, TNFα, IL-6 and IL-1β was found in the liver and ileum tissues in mice with very high-fat diet which treated with *Moringa oleifera* concentrate (23). Moreover, both *MO* leaves extract and quercitin down-regulated the expressions of iNOS, interferon gamma (IFN-γ) and C reactive protein (CRP) compared to the group only fed with high-fat diet. Finally, release of serum inflammatory cytokines TNF-α and IL-6 potently decreased in the group treated with the extract of *MO* leaves compared to a high fat fed group (46). Mahajan *et al.* (49) reported that *MO* leaves were effective in blocking production of several cytokines including TNF-α, IL-4, and IL-6 and
might be due to additional mechanisms such as mast cells stabilization and antispasmodic activities, which could be another protective effect of Moringa (50).

5.4.5 Gene expression

It has been reported that hyperinsulinemia alters hepatic cholesterol homeostasis by increasing nuclear SREBP-2 and LDL-R (30). SREBP-2 is a transcription factor that regulates cholesterol biosynthesis (3) and the LDL-R in the liver is crucial for cholesterol regulation (51). DGAT1 is the enzyme that catalyzes the final step in triglyceride synthesis, and it has been reported to be decreased after carotenoids treatment and high fat diets (3, 52).

However, decreased expression of hepatic HMG-CoA reductase is the primary response employed by the animals to achieved cholesterol homeostasis in the face of a dietary cholesterol challenge. This decrease in hepatic HMG-CoA reductase expression was accomplished by a decrease in translational efficiency of the HMG-CoA reductase mRNA (53). In the current study, no differences in HMGCR mRNA expression were observed between experimental groups. However, the regulation of this enzyme is post-translational, not only transcriptional. Thus, it is possible that Moringa might have affected the regulation of HMG-CoA reductase post-translationally.

The LDL receptor (LDL-R) is an important regulator used by the liver to control cholesterol levels in the body (54). Some studies have found that animals challenged with high-cholesterol diets increased the expression of hepatic LDL-R and they have suggested that this response might be utilized by the liver to help maintain cholesterol homeostasis (55). Increased expression of the hepatic LDL receptor can help maintain cholesterol homeostasis by removing LDL cholesterol from the blood. The increased dietary cholesterol reaches the liver by way of
chylomicron remnants. The excess hepatic cholesterol can be incorporated into VLDL and processed to intermediate density lipoprotein (IDL) and LDL by the action of lipoprotein lipase (56). In this study, however, high concentration of *Moringa oleifera* leaves decreased the level of LDL-R gene expression. This decrease in the LDL-R might be associated with the decreases in hepatic cholesterol and the lack of effect of Moringa on plasma LDL cholesterol.

Interestingly, guinea pig consumed the HM had lower expression of LDL-R compared to the other groups. The hepatic LDL-R is the major mechanism by which the body removes LDL cholesterol from plasma and it has been shown that LDL-receptors (57) and LDL uptake are up-regulated by soluble fiber (58,59). In this study, there were no differences in plasma cholesterol among groups, while there was a very significant lowering of hepatic cholesterol in the HM group suggesting that the decreased expression of LDL-R might have contributed to the lack of effect in lowering plasma LDL-C and to the very significant lowering of hepatic cholesterol.

SREBPs regulate the expression of target genes involved in cholesterol, fatty acid and triglyceride syntheses. SREBP-1c and -2 predominates in the liver. SREBP-1 is associated with fatty acid synthesis genes, while SREBP-2 has a preference for cholesterol synthesis genes; however, all 3 isoforms can drive the expression of HMG-CoA-R by binding to its SRE in the proximal promoter region of this gene (60,61). We observed decreases in SREBP-1c expression in guinea pigs fed *Moringa oleifera* leaves compared to control. Gene expression of CD68 and SREBP-1c were 60% lower in both the LM and HM groups compared to the control (p< 0.01). CGA as has been shown in other studies could have also had an effect in decreasing TG via reductions in SREBP1c (24).
DGAT is the enzyme that catalyzes the final step in TG synthesis by adding a free fatty acid to sn-1, 2- diacylglycerol (62). The enzyme has two isoforms, DGAT1 and DGAT2, however, DGAT2 is the one primarily expressed in the liver (63). Studies with animal models have suggested that this enzyme plays an important role in the development of hepatic steatosis (32). In this study, the *Moringa oleifera* leaves groups showed a significant reduction of hepatic gene expression of DGAT2 when compared to controls and this can explain the differences observed in steatosis in the liver. The decreases in hepatic TG can also be associated with the lower expression of DGAT2. The flavonoid quercetin has been shown to reduce TG synthesis in Caco-2 cells in part, via reduction of DGAT activity (64). Since *Moringa oleifera* leaves have significant concentrations of quercetin, we can speculate that this flavonoid might have been involved in the decreased expression of DGAT-2 further contributing to the observed lower concentrations of TG in the guinea pigs fed the ML (30).

PPARγ is expressed at high levels in adipose tissue, macrophages, endothelial cells and large intestine but is also found in liver (65). PPARγ plays a major role in lipid and glucose metabolism, in macrophage foam cell formation and inflammatory response and in adipogenesis (66). Activation of PPARγ in adipocytes increased FA storage and TG synthesis through increased expression of its target genes and increased secretion of adipokines resulting in decreased lipid accumulation in other tissues. PPARγ expression level in hepatocytes was linked with liver steatosis in rodents (67). Normal mice fed a HFD had an increased expression of PPARγ2 in the liver (68). In our study, we showed that PPARγ were decreased in *Moringa oleifera* leaves groups compared to control group (P < 0.05) (Figure 5.3).

**5.5 Conclusions**

In conclusion, a challenge with dietary cholesterol has a profound effect on hepatic cholesterol homeostasis, which results in elevated hepatic total cholesterol, free cholesterol and
triglyceride concentrations. However, the polyphenols present in *Moringa oleifera* leaves, possibly chlorogenic acid and quercetin, regulated these genes involved in lipid synthesis and uptake, resulting in lower concentrations of hepatic lipids. The lower lipids in liver due to *Moringa oleifera* leaves intake were associated with lower inflammation caused by cholesterol challenge and decreased hepatic steatosis.
TABLES

Table 5.1 Concentration of hepatic total cholesterol, free cholesterol, cholesteryl ester and Oxidized LDL of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%)¹

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/g)</td>
<td>10.9±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.0±1.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.2±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free Cholesterol (mg/g)</td>
<td>4.5±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2±1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesteryl Esters (mg/g)</td>
<td>6.3±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8±1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.8±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/g)</td>
<td>50.9±11.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.0±8.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.4±11.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidized LDL (ng/g)</td>
<td>2.7±1.4</td>
<td>1.9±0.9</td>
<td>2.3±0.5</td>
</tr>
</tbody>
</table>

¹ Values are means ± SD, n=8; Values in the same row with different superscripts are significantly different at P<0.05
Table 5.2  Plasma ALT and AST of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%)\(^1\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>239.3±144.6 (^a)</td>
<td>109.4±43.7 (^b)</td>
<td>141.9±73.6 (^b)</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>552.9±479.4</td>
<td>276.9±146.5</td>
<td>299.4±195.1</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at P<0.05
Table 5.3 Hepatic cytokines of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%)\(^1\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (ng/g)</td>
<td>196.4±49.2(^a)</td>
<td>180.3±40.9(^{ab})</td>
<td>143.1±31.5(^b)</td>
</tr>
<tr>
<td>IL-6 (ng/g)</td>
<td>30.1±3.7</td>
<td>27.5±4.9</td>
<td>37.3±16.4</td>
</tr>
<tr>
<td>IL-10 (ng/g)</td>
<td>233±54(^a)</td>
<td>208±42(^{ab})</td>
<td>160±38(^b)</td>
</tr>
<tr>
<td>IFNγ (ng/g)</td>
<td>194.4±20.5(^a)</td>
<td>173.4±39.2(^{ab})</td>
<td>153.4±42.3(^b)</td>
</tr>
<tr>
<td>MCP-1 (ng/g)</td>
<td>11.4±1.1</td>
<td>10.3±1.2</td>
<td>21.9±21.9</td>
</tr>
<tr>
<td>TNFa (ng/g)</td>
<td>31.1±4.9</td>
<td>27.4±4.4</td>
<td>35.6±19.3</td>
</tr>
</tbody>
</table>

\(^1\) Values are means±SD, n=8; Values in the same row with different superscripts are significantly different at P<0.05 using LSD as post-hoc test. IFNγ = Interferon gamma; MCP-1= monocyte chemoattractive protein-1.
Figure 5.1 Correlation between liver enzymes Alanine Aminotransferase (ALT) ($r=0.47$, $P < 0.05$) and Aspartate Aminotransferase (AST) ($r=0.48$, $P < 0.05$) and oxidized LDL of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%).
Figure 5.2 Histological evaluation of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%) (200x magnification)
Figure 5.3 Hepatic relative gene expressions of male guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%). Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.
5.6 References


Chapter 6

The Effects of Moringa leaves on Adipose tissue
6.1 Background

Adipose tissue has a major role in the development of several metabolic-related diseases, such as atherosclerosis, coronary heart disease (CHD) and type 2 diabetes (1). Adipose tissue is the main site for triglyceride and cholesterol storage in humans as well as in other animal species (2). Excessive accumulation of cholesterol can be toxic to several tissues, including heart, aorta, and liver (3, 4, 5).

Several studies showed that increased cholesterol accumulation in epididymal adipose tissue impaired adipocyte differentiation and maturation, induced insulin resistance and secretion of pro-inflammatory cytokines, and stimulated macrophage (Mφ) recruitment in LDL receptor knock-out mice (LDLR -/-) (4). In addition, in adipose-derived stromal cells, cholesterol accumulation leads to impairment of adipocyte development and function (6). Release of cytokines, such as tumor necrosis factor (TNF)-α, resulted in infiltration of Mφ, neutrophils, T-cells, and other immune cells (7).

Obesity leads to hypertrophied adipocytes due to high TG and cholesterol accumulation, resulting in abnormal cellular cholesterol distribution (8, 9). Overall, these features triggered by lipid accumulation are hallmarks of dysfunctional adipocytes (10).

In liver, a major organ involved in cholesterol metabolism, hypercholesterolemia caused hepatocyte dysfunction, fibrosis, and induction of the development of early stages of nonalcoholic steatohepatitis. So, it is important to evaluate the effects of hypercholesterolemia on other major organs of cholesterol metabolism and storage, such as adipose tissue (11).
It has been reported that obesity leads to hypertrophy of adipocytes due to high TG and cholesterol accumulation resulting in abnormal cellular cholesterol distribution with decreased plasma membrane (PM) cholesterol and increased fluidity in these cells (12, 13). Overall, these characteristics triggered by TG and cholesterol accumulation are hallmarks of dysfunctional adipocytes (14).

In guinea pigs, we have demonstrated that high hepatic cholesterol concentrations induce the development of the initial stages of nonalcoholic steatohepatitis and fibrosis (11). Our previous study indicated that Moringa leaves (ML) prevented hepatic steatosis and reduced the level of pro-inflammatory cytokines compared to a control diet where no moringa was provided. ML has also been shown to reduced lipid accumulation in adipocytes in other animal species (15).

Guinea pigs have several similarities to humans in lipoprotein and cholesterol metabolism and are considered a good model for study of diet-induced atherosclerosis (16, 17). For this study, we are using guinea pigs as animal models to study adipose tissue inflammation induced by a high cholesterol diet as well as the potential beneficial effects of Moringa leaves in protecting against the recruitment of macrophages and the release of inflammatory cytokines.

In a study conducted in rats, fat accumulation in the thoracic and abdominal regions due to a high fat, high cholesterol diet was reported. The observed increases in body weight were postulated to be due to excessive energy intake, which led to adipose tissue accumulation (18). In addition, positive correlations between daily lipid intake and body mass index as well as fat deposition have been reported (19). The reduction in these measures in obese rats resulted from treatment with *Moringa oleifera*, which may be associated with less utilization of dietary lipids. Dongmeza and co-workers found (15) that a high level of moringa extract or its fractions such
as saponins and tannins resulted in reduced energy leading to decreased protein and lipid biosynthesis. Therefore, moringa has the ability to reduce body lipid and consequently energy retention.

The objective of the present study was to evaluate the effects of a high cholesterol dietary challenge on cholesterol tissue accumulation and inflammation in guinea pigs and whether ML would protect against these metabolic alterations.

We hypothesized that high dietary cholesterol would induce cholesterol accumulation, and inflammation in adipose tissue and that ML would protect against these metabolic abnormalities.

6.2 Materials and Methods

6.2.1 Cholesterol in adipose tissue

Epididymal adipose tissue (0.12 g) was homogenized and 15 ml of chloroform-methanol 2:1 (v/v) were added and tubes were incubated at 60°C overnight. After adding 1.6 mL of 0.05% sulfuric acid (H₂SO₄) to each tube, samples were centrifuged at 3,000 rpm for 10 min. at room temperature and dried under nitrogen. Total cholesterol was measured using enzymatic kits (WakoChemicals USA, Inc., Richmond, VA) according to Carr et al (20).

6.2.2 Inflammatory Cytokine Concentration in the adipose tissue

Tissue total protein was extracted using RIPA buffer and total protein concentration of the lysates was determined using the BCA Protein Assay Kit (Cell Signaling Technologies Inc, Beverly, MA) (21). Using the same concentration of protein for all samples, the following cytokines were measured using Luminex technology (Luminex MAGPIX System, Austin, TX)
with the MILLIPLEX MAP Rat Cytokine Immunoassay kit (Millipore corporation, Charles, MO, USA): IL-1β, IL-6, IL-10, IFN-γ and monocyte chemotactic protein 1 (MCP-1), as previously described (19).

6.2.3 Histological evaluation
Small pieces of adipose tissue samples were immersed in 10% buffered formalin. Formalin-fixed adipose tissues were paraffin embedded and 3–5 μm sections were stained with hematoxylin and eosin. Histological evaluation was performed in two separate occasions by a veterinary pathologist blinded to the treatments. A low magnification screen of the entire tissue was assessed for evidence of abnormality, and crown-like structures and signs of inflammation were quantified in 10 fields at 200X.

6.2.4 Statistical Analysis:
Differences between groups were analyzed by one-way ANOVA and Fisher’s LSD post hoc analysis. p<0.05 was considered to be significant. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.)

6.3 Results
6.3.1 Cholesterol accumulation and inflammation scores
There was no significant difference in the total cholesterol in the adipose tissue nor any difference in the inflammation scores among of guinea pigs fed high moringa, low moringa and control group as shown in Table 6.1.
6.3.2 Inflammatory cytokine concentrations in the adipose tissue

There were no significant differences in the inflammatory cytokine concentrations; IL-1β, IL-6, IL-10, IFN-γ, TNFα and MCP-1 among groups of guinea pigs fed high moringa, low moringa and control group as shown in Table 6.1.

6.3.3 Histological evaluation

Adipocytes from all guinea pigs contained abundant lipid irrespective of diet. The majority of the guinea pigs did not have signs of inflammation (Figure 1, Panel A); 2 of the guinea pigs fed the ML had inflammation (Figure 1, Panel B). Histological CLS scores are also presented in Table 6.1. A representative figure of tissue histology for most guinea pigs (Panel A) and for one of the guinea pigs showing crown-like structures (Panel B) is presented in Figure 6.1.

6.4 Discussion

6.4.1 Cholesterol accumulation in the adipose tissue

Guinea pigs are a good model for study of chronic inflammatory diseases. Previous studies showed that cholesterol challenge can induce atherosclerosis, and hepatic cholesterol accumulation (16, 22, 23). Studies showed that cholesterol synthesis in adipose tissue only accounts for 4% of that produced in the liver, therefore, most of the cholesterol in the adipocyte is delivered by lipoprotein-mediated mechanisms (24).

Cholesterol can be delivered into the adipocyte through HDL scavenger receptor B1 (SREBP1) –independent (25, 26) or –dependent mechanisms (27) and LDL receptor and oxidized-LDL (ox-LDL) scavenger receptor pathways (28, 29). HDL-cholesterol removal by SREBP1 takes place in the caveolae (a specific plasma membrane lipid raft) where SREBP1 binds HDL and extracts cholesterol ester (CE). In caveolae, CE can be taken up by HDL or internalized into
an intracellular membrane compartment (30). In the SREBP1–independent mechanism via CE transfer protein (CETP), CE is transferred from HDL to a specific plasma membrane compartment, and then apoE is secreted into this compartment and acquires CE. Finally, apoE loaded with CE is directed to the extracellular matrix and recaptured by the LDL receptor–related 1 protein (26). Another cholesterol source for the adipocyte is ox-LDL. This lipoprotein enters the adipocyte through the SREBP1, or ox-LDL receptor 1 and is further proteolytically degraded (28).

Unexpectedly, no effect of Moringa leaves on cholesterol accumulation in the adipose tissue was observed in this study. These results might be related to the lack of effect of Moringa in reducing plasma LDL cholesterol or oxidized LDL (chapter 4) that we observed or that the 6-week study period was too short to manifest any beneficial effect.

6.4.2 Inflammatory cytokine concentrations in the adipose tissue

In this study, there were no differences in inflammatory cytokine concentrations in the adipose tissue among groups. In the previous studies, cholesterol challenge diets were shown to increase levels of Ox-LDL (22). This pro-atherogenic lipoprotein is internalized by SR-B1 and LRP-1 in Mø and smooth muscle cells leading to cell dysfunction and release of pro-inflammatory cytokines and chemo-attractants and formation of foam cells which are the hallmark of atherosclerosis (31).

The amount of cholesterol in the adipose was greater than what has been observed in guinea pigs fed a low cholesterol diet (32). Cholesterol deposition in adipose tissue increased macrophage infiltration associated with high levels of inflammatory cytokines TNF-α, MCP-1, and GMF-CSF. Also, cholesterol accumulation led to decreased gene and protein expression of SREBP-1 and PPAR-γ2 resulting in adipocyte dysfunction and blunted cell development.
Expression of SREBP-1 showed to directly modulate the expression of the PPARγ gene at the transcription level (33), which directly involved in the transcription of several lipogenic genes that are important in adipocyte development (34).

Dysfunctional adipocytes are found to be more receptive to TNF-α signaling and subsequent apoptosis, which associated with macrophage infiltration and secretion of inflammatory cytokines (4).

None of these metabolic alterations were observed in the 6-week intervention in this study indicating that a longer time of feeding is needed to have the presence of dysfunctional adipocytes associated with the high cholesterol feeding. The increases in cholesterol accumulation and the release of inflammatory cytokines were present in all guinea pigs since the inclusion of ML in the diet did not affect the higher cholesterol uptake associated with the dietary intervention (32).

6.5 Conclusion

Moringa leaves had no effect in protecting the adipose tissue against inflammation in guinea pigs fed a high cholesterol diet. It is clear that Moringa exerted its main effects in the liver (Chapter 5) where substantial reductions in lipid accumulation and in inflammation were observed. The lack of effect of Moringa in reducing plasma cholesterol explains why cholesterol accumulation in the adipose was not reduced leading to inflammation and macrophage accumulation. Further studies need to be conducted with longer feeding times for Moringa to assess if plasma lipids could be reduced and therefore cholesterol accumulation in the adipose could also be lowered.
TABLES

Table 6.1 Concentration of cholesterol, histological scores and inflammatory cytokines in adipose tissue of guinea pigs fed a hypercholesterolemic diet with no Moringa (control), Low (10%), or High Moringa (15%) $^1$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low Moringa</th>
<th>High Moringa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/g)</td>
<td>11.60±1.50</td>
<td>12.77± 3.82</td>
<td>10.91±1.23</td>
</tr>
<tr>
<td>Histological scores</td>
<td>0.150 ± 0.18</td>
<td>0.125 ± 0.18</td>
<td>0.175 ± 0.31</td>
</tr>
<tr>
<td>IL-1β (ng/g)</td>
<td>30.0 ± 12.4</td>
<td>25.2 ± 7.3</td>
<td>36.2 ± 19.3</td>
</tr>
<tr>
<td>IL-6 (ng/g)</td>
<td>16.8 ± 2.3</td>
<td>15.2 ± 2.9</td>
<td>17.3 ± 3.1</td>
</tr>
<tr>
<td>IL-10 (ng/g)</td>
<td>40.7 ± 15.3</td>
<td>32.2 ± 12.1</td>
<td>47.5 ± 19.8</td>
</tr>
<tr>
<td>IFN-γ (ng/g)</td>
<td>116.3 ± 25.2</td>
<td>95.3 ± 31.8</td>
<td>107.0 ± 24.9</td>
</tr>
<tr>
<td>MCP-1(ng/g)</td>
<td>7.0 ± 1.00</td>
<td>7.0 ± 1.1</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>TNFα (ng/g)</td>
<td>14.2 ± 1.2</td>
<td>13.7 ± 2.4</td>
<td>15.0 ± 3.4</td>
</tr>
</tbody>
</table>

$^1$ Values are presented as mean ± SD for n= 8 guinea pigs per group
Figures

Fig 6.1 Representative histological evaluation of adipose tissue as observed in the majority of the guinea pigs (Panel A) and histological evaluation of one of two guinea pigs showing evidence of inflammation and macrophage infiltration (Panel B).

Panel A
Panel B
6.6 References


Chapter 7

Conclusions and future directions
7.1 Conclusions

This project demonstrated that MO leaves do protect against hepatic steatosis by reducing hepatic lipid accumulation and inflammation although no positive effects were observed on plasma lipids or adipose tissue inflammation.

However, MO leaves appeared to affect HDL metabolism by decreasing the medium HDL, which has been considered a less protective HDL and by increasing LCAT activity. These results suggested a potential role of MO leaves in modulating RCT.

Higher concentration of Moringa leaves were found to be more effective in protecting hepatic tissue against cholesterol-induced damage as the high Moringa groups showed lower concentrations of both total and esterified cholesterol, plasma alanine aminotransferase (ALT), hepatic cytokines; interleukin 1 beta (IL-1β), interleukin 10 (IL-10) and Interferon gamma (IFNγ), DGAT2 and PPARγ mRNA expression. An unexpected finding was that there were no differences in plasma or hepatic oxidized LDL (OxLDL) suggesting that Moringa leaves are not such potent antioxidants.

A challenge with dietary cholesterol has a profound effect on hepatic cholesterol homeostasis, which results in elevated hepatic total cholesterol, free cholesterol and triglyceride concentrations. However, the polyphenols present in Moringa oleifera leaves, possibly chlorogenic acid and quercetin, regulated the genes involved in lipid synthesis and uptake, resulting in lower concentrations of hepatic lipids and protection against liver inflammation.

Moringa leaves had no effect in protecting the adipose tissue against inflammation in guinea pigs fed a high cholesterol diet. The lack of effect of Moringa leaves in reducing plasma
cholesterol explains in part why cholesterol accumulation in the adipose was not reduced leading to inflammation and macrophage accumulation.

7.2 Future directions

The importance of conducting these studies is to cover the gaps in current knowledge regarding the use of Moringa leaves as medication in the prevention or treatment of NAFLD, T2DM, and CVD. With the completion of the three aims, there is a better understanding on how Moringa leaves containing bioactive compounds affected plasma lipids, hepatic steatosis and inflammation in liver and about the lack of effects in plasma and the adipose tissue. However, there are still questions that remain unanswered after the completion of the study.

An important limitation in this project was the lack of a positive control group. A similar study should be conducted using a control with guinea pigs receiving the same diet without cholesterol and without Moringa leaves. This will help us clarify how much Moringa leaves protect against hepatic steatosis by comparing the measured parameters with animals where hepatic steatosis is not induced.

Also, we have little information about the effects of Moringa leaves in guinea pigs in longer periods of time to determine if a more extended period of feeding would have an effect in decreasing plasma cholesterol as was observed in other animal studies and therefore reducing cholesterol accumulation in the adipose tissue and inflammation. Also, it is important to determine accurate concentrations of bioactive components in Moringa and which of these compounds present in Moringa leaves can ameliorate the deleterious effects of a cholesterol challenge.
The high contribution in bioactive compounds may explain the pharmacological properties ascribed to *Moringa oleifera* leaves. Many *in vitro* and *in vivo* studies in animals have confirmed these properties. However, few studies on human beings are available. Therefore, it is too early to recommend *Moringa oleifera* leaves as medication in the prevention or treatment of cardiovascular disease and dyslipidemia. Further studies aimed to confirm the pharmacological effects of moringa in humans and, at the same time, ensuring its safety during chronic or long-term use should be encouraged.

For future perspectives, a similar study should be conducted regarding the use of Moringa leaves as a medication in the treatment of obesity and CVD and to determine whether it is safe to move forward to perform clinical trials. *In vitro* studies should be done before clinical trials can be conducted.

It is unclear whether the hypocholesterolemic effect of Moringa leaves was associated with repressed *de novo* cholesterol biosynthesis, increased hepatic cholesterol clearance or increased intestinal excretion. It might be interesting to find the physiological basis of decreases in hepatic steatosis following Moringa treatment.

Besides a rationalized investigation of the therapeutic effect of *M. oleifera* leaves, it is possible to conduct an unbiased search for the affected physiological pathways. This could be achieved by globally comparing tissues or fluids of untreated and treated animals or humans.

Further exploration of the eventual use of *Moringa oleifera* therapy in humans will require that reference standards be set for its cultivation, the collection of its parts, as well as for their final conditioning under good agricultural and collection practices. Furthermore, when the pharmacological basis of the therapeutic effects on a particular pathology is rationally well
circumscribed, standardized assays should be required to evaluate relevant properties. For example, on the basis of current knowledge, it would be indicated that the content of fiber, phytosterols as well in antioxidant and anti-inflammatory activities be evaluated and documented in *Mopringa oleifera* formulations destined to the treatment of atherosclerosis or diabetes.