CHRONIC CAROB MOLASSES INTAKE AND ITS IMPACT ON BLOOD LIPEMIA AND GLYCEMIA IN THE RAT MODEL

by

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CHRONIC CAROB MOLASSES INTAKE AND ITS IMPACT ON BLOOD LIPEMIA AND GLYCEMIA IN THE RAT MODEL

Abstract:

Carob molasses is one of the old traditional tasty sweets that used to be consumed frequently by the Lebanese community. It was believed to have several important medicinal functions of which the laxative effect was the most common. The present study investigates the effects of a six week period of carob molasses intake, along with a diet moderate or rich in saturated fat, upon lipemia and glycemia in the fasted state. Also, the study covers the effect of carob molasses intake upon postprandial lipemia. Results have shown that carob molasses intake do not have any significant effect on fasting serum glucose and insulin concentrations. However, a significant increase in HDL cholesterol concentration and an important improvement in the LDL/HDL cholesterol ratio were observed with carob molasses intake. No significant effects on serum total cholesterol, LDL cholesterol, triacylglycerol (TAG) and apolipoprotein B (ApoB) concentrations were recorded with carob molasses intake. On the other hand, a significant increase in stool water content was observed when carob molasses was taken with the diet regardless of the fat content in the diet. Only a significant increase in TAG but not cholesterol excretion in the stool was noted with the regular fat diet. Carob molasses intake for six weeks did not have any negative side effects as appeared from liver enzyme activities and liver fat content. Postprandially, the presence of carob molasses along with a lipid load significantly reduced serum TAG and chylomicron TAG and cholesterol 3h after intragastric instillation of the lipid load. No effect upon VLDL lipid was observed. In conclusion, carob molasses intake appeared to have a cardioprotective effects when taken on a regular basis or occasionally since it improves fasting and postprandial lipidemia and it can be used as an anti-constipation remedy without showing liver toxicity.

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GLOSSARY

Abs: Absorbance

ACAT: Acyl-CoA:cholesterol acyltransferase

ad lib: ad libitum.

ALT: Alanine Transaminase

Apo: Apolipoprotein

APS: ammonium persulfate solution

AST: Aspartate Transaminase

CETP: Cholesteryl ester transfer protein

Chol: Cholesterol

CM: Chylomicron

Conc: concentration

DTT: Dithiothreitol

EDTA: Ethylene-diamine-tetraacetic acid

ELISA: Enzyme Linked ImmunoSorbent Assay

Ext: Extract

FA: Fatty acid

GK: Glycerol kinase

GPO: Glycerol-3-phosphate oxidase

HDL: High-density lipoprotein

HTGL: Hepatic triglyceride lipase

IDL: Intermediate-density lipoprotein

LCAT: Lecithin cholesterol acyltransferase

LDH: lactate dehydrogenase

LDL: Low Density Lipoprotein

LPL: Lipoprotein lipase

MAG: Monoacylglycerol

MDH: malate dehydrogenase

NaN_3: Sodium azide

O.D: Optical Density

PAFAH: Platelet activating factor acetylhydrolase

PMSF: Phenylmethylsulfonylfluoride

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error from the mean

sGOT: Serum Glutamic Oxaloacetic Transaminase

sGPT: Serum Glutamic Pyruvic Transaminase

TAG: Triacylglycerol

TBS: Tris Buffer Solution

TEMED: Tetramethylethylenediamine

VLDL: Very Low Density Lipoprotein

Chapter 1

Introduction

1.1 Lipids

The balance of food intake and energy expenditure is crucial for survival. Hence constant body mass must be maintained by the counterbalance between the intake and storage of fats and the equivalent expenditure of stored energy (Vance and Vance, 2002). Although fat plays a vital role in meeting nutritional requirements (Gurr et al., 2002) and certain types of fat provide protection from heart disease (Hayes and Khosla, 1992), fat has come to be considered as an undesirable constituent of human diet. This is because it has been linked to obesity and cardiovascular disease (Gurr et al., 2002). The simplest form of lipids is the fatty acids with their polar hydrophilic and nonpolar hydrophobic ends (Groff and Gropper, 2000). Most stored fat is in the form of triacylglycerols, which represent a highly concentrated form of energy. They account for nearly 95% of dietary fat (Castelli and Griffin, 1997). Another important lipid is cholesterol. Although it is mainly fabricated in the liver (Figure 1.2), it also exists in lipids of meat, poultry, fish, eggs (egg yolk), dairy products, butter, lard and many processed foods. Cholesterol levels tend to be elevated in foods from animal sources that possess high levels of saturated fats (Liscum and Munn, 1999).

1.1.1 Lipid Digestion

Digestion of fats (hydrophobic) poses a problem as their digestive enzymes are hydrophilic and normally function in an aqueous environment (Groff and Gropper, 2000). Hence lipids have to be emulsified prior to digestion (Figure 1.3) (Mathews et al., 1999). This emulsification step is facilitated by bile, due to its amphipathic property (Sanders and Emery, 2003). Pancreatic lipases, primarily *lipase* and *phospholipase* A_2, then cleave fatty acids and the end

products of digestion are glycerol, fatty acids and 2-monoacylglycerol (MAG) (Dowham, 1997).

1.1.2 Lipid Absorption

The micelles stabilized by bile salts are soluble enough to penetrate the cells of the small intestine known as mucosal cells or enterocytes (Groff and Gropper, 2000). The lipid content of the micelles diffuses into the enterocytes, moving down a concentration gradient. Bile salts are returned to the liver via the portal vein (Liscum and Munn, 1999). The content of the micelles are reconstituted into triacylglycerols, phosphastidylcholine, and cholesterol esters following absorption. They are then collected as large fat particles in the cell's endoplasmic reticulum after which they become coated by a layer of proteins on their surface, forming what is called chylomicrons, to stabilize them in the aqueous environment of the circulation which they will eventually enter (Groff and Gropper, 2000). Finally, they are transported to the cell membrane and exocytosed into the lymphatic circulation (Sanders and Emery, 2003).

1.1.3 Lipoproteins

Lipoprotein particles have both lipid and protein constituents (called Apoproteins) held together by noncovalent forces. The Apo-proteins vary in each type of lipoprotein. These protein constituents are needed for recognition of the lipoprotein by receptors on the surface of cells. Their structure recognized as a two layered spheroidal microemulsion. Phospholipids, unesterified cholesterol, and proteins constitute the outer layer where as neutral lipids, mostly cholesteryl ester and triacylglycerols (TAG) make up the core. Lipoproteins differ according to the lipid/protein ratio within the particle as well as having different proportions of lipid types: triacylglycerols, cholesterol and cholesteryl esters, and phospholipids (Figure 1.1) (Groff and Gropper, 2000). Generally, they are categorized according to density, size, and/or protein constituent (Segrest et al., 2001).

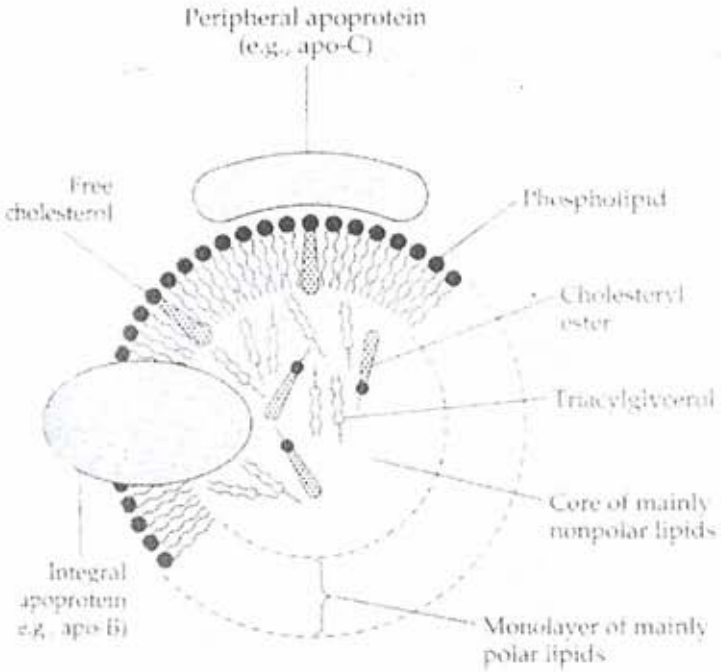

Figure 1.1 Structure of a plasma lipoprotein (Groff and Gropper, 2000).

1.1.3.1 Types of Lipoproteins

From the lowest to the highest density, the portions of lipoprotein are: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Also, there is an intermediate density particle (IDL), having a density between that of VLDL and LDL (Figure 1.2) (Groff and Gropper, 2000).

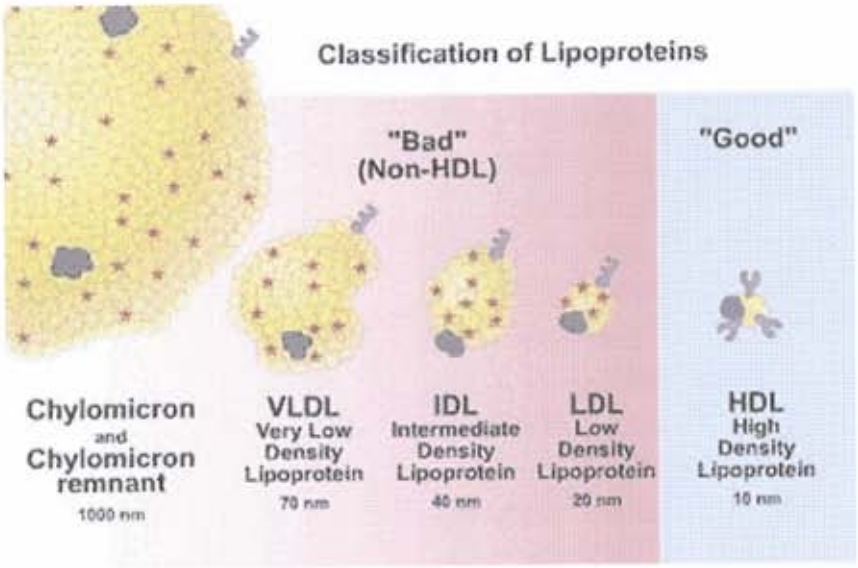

Figure 1.2 Classification of lipoproteins. In the figure, red stars reveal cholesterol, keys represent apolipoprotein B, Y symbolize apolipoprotein A, blue spots symbolize membrane proteins as well as bad and good cholesterols (Mathews et al., 1999).

1.1.3.2 Chylomicrons

These are the primary form of lipoprotein of intestinal epithelial fabrication, formed from absorbed exogenous (dietary) lipids and apoproteins (Groff and Gropper, 2000; Vance and Vance, 2002). In the intestine, they are gathered from the epithelium into the central lacteals of the villi. These large lipoproteins are then conveyed by the lymphatic system to the general circulation (Vance and Vance, 2002). Chylomicrons obtain apoC-II and apoE from plasma HDLs in the bloodstream (Mathews et al., 1999). They carry intestinal dietary fats to the other tissues (Segrest et al., 2001). The enzyme lipoprotein lipase (LPL) localized in the capillary walls of tissues, particularly adipose tissue and muscle, is activated by apoC-II on the chylomicron surface in the occurrence of phospholipid. LPL then degrades most of the triglycerides in the chylomicron to free fatty acids which are captivated by the tissues for use or for storage (Liscum and Munn, 1999). The chylomicron

remnants remaining after the action of LPL (containing some triglycerides, cholesterol and other lipids) return through the blood to the liver and kidneys (Liscum and Munn, 1999; Sanders and Emery, 2003). A considerable fraction of phospholipid, apoA and apoC is relocated to HDLs during fatty acid elimination. This deprivation from apoC-II, obstructs any further break down of the chylomicron remnants by LPL. Chylomicrons contain ApoE, ApoCII, and ApoB-48 proteins, while the remnants contain only ApoE and ApoB-48 (Mathews et al., 1999; Gurr et al., 2002).

1.1.3.3 Very Low Density Lipoproteins (VLDL)

Very Low Density Lipoproteins (VLDL) are synthesized by the liver and include triglycerides, cholesterol and other lipids to be transported from the liver to the tissues. They symbolize triacylglycerol transport mechanisms from the liver to tissues. Their production and discharge resembles the particulate secretion of intestinal chylomicrons (Liscum and Munn, 1999). The triglycerides in VLDL particles are also degraded by LPL and free fatty acids are released to tissues (Vance and Vance, 2002). VLDL particles contain ApoB-100, ApoC-I, ApoC-II, ApoC-III and ApoE. Recently freed VLDLs obtain apoCs and apoE from circulating HDLs just like growing chylomicrons. After the action of LPL, VLDL particles are converted to Intermediate Density Lipoproteins (IDL) (Mathews et al., 1999).

1.1.3.4 Intermediate Density Lipoproteins (IDL)

Intermediate Density Lipoproteins (IDL) are remnants of VLDL after the action of LPL. They can be taken up by the liver or converted to Low Density Lipoproteins (LDL) by the action of hepatic triglyceride lipase (HTGL). IDL contain ApoE and ApoB-100.

1.1.3.5 Low Density Lipoproteins (LDL)

Low Density Lipoproteins (LDL) are the remnants of IDL and contain only ApoB-100. It is usually harmless and transports about 75% of blood cholesterol to body cells. Yet, if subjected to oxidation, it can infiltrate and interact perilously with the arterial walls generating a detrimental inflammatory response (Krauss, 1995). LDL particles are recognized by LDL receptors found on the liver and extra-hepatic tissues and are internalized by receptor-mediated endocytosis (Figure 1.3). This supplies the extra-hepatic tissues with much of their cholesterol. Once inside the cell, the LDL particle is degraded and the cholesterol is esterified by the action of the acyl-CoA:cholesterol acyltransferase (ACAT) (Vance and Vance, 2002). 50% of the LDL is metabolized in the liver (Krauss, 1995). Ethanol intake, carbohydrate rich diets, high insulin and low glucagon concentrations elevate tricacylglycerol synthesis and VLDL secretion by the liver (Castelli and Griffin, 1997).

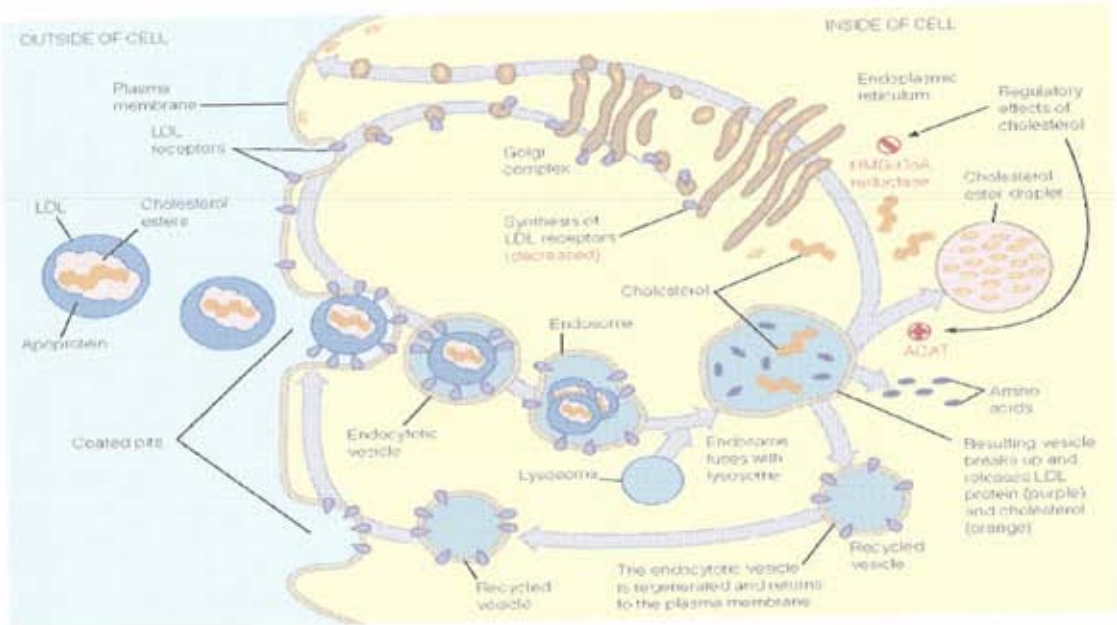

Figure 1.3 LDL receptors involved in cholesterol uptake and metabolism (Mathews et al., 1999).

1.1.3.6 High Density Lipoproteins (HDL)

High Density Lipoproteins (HDL) are the smallest (7.5-10 nm), most soluble and protein rich lipoproteins, synthesized and secreted by the liver and intestine (Vance and Vance, 2002). These recently synthesized HDLs almost lack any cholesterol and cholesteryl esters. They travel through the bloodstream gathering excess cholesterol from the tissues and blood plasma. Cholesterol picked up by HDL particles is esterified to a fatty acid obtained from phosphatidyl choline (or lecithin) via the action of plasma lecithin:cholesterol acyl transferase (LCAT) and excess cholesterol can be excreted with bile (Spring et al., 1992). LCAT activity necessitates apoA-I (located on HDLs surface) interaction (Mathews et al., 1999). HDL rich in cholesterol returns to and becomes endocytosed. They are internalized through ApoA-I-receptor interactions into macrophages. HDLs can then acquire ApoE (which then enhance their uptake and catabolism by the liver) and cholesterol from the macrophages before their secretion. They can also obtain cholesterol from cell membranes. This might lower the level of intracellular cholesterol, since the cholesterol preserved within cells as cholesteryl esters will be moved to restore the cholesterol detached from the plasma membrane (Spring et al., 1992; Liscum and Munn, 1999). HDL particles contain ApoA-I, ApoC-I, ApoC-II and ApoE proteins (Mathews et al., 1999).

1.1.3.7 Antiatherogenic Role of HDL

The role of HDL in reverse cholesterol transport and in hindering LDL oxidation, a crucial phase in atherogenesis, protects against the development

of atherosclerosis (Castelli and Griffin, 1997). A number of HDL-related enzymes are engaged in the process. Paroxonase (PON1), LCAT, and platelet activating factor acetylhydrolase (PAFAH) all contribute by hydrolyzing phospholipid hydroperoxides obtained during LDL oxidation and perform in association to avert the gathering of oxidized lipid in LDL. These enzymes account for the anti-oxidative and anti-inflammatory characteristic of HDL (Mathews et al., 1999; Groff and Gropper, 2000).

1.1.3.8 Apolipoproteins

The protein components of lipoproteins (apolipoproteins) tend to stabilize the lipoproteins as they circulate in the aqueous environment of the blood (Groff and Gropper, 2000). They are amphipathic in nature and can therefore interact with both the lipids of the lipoprotein and the aqueous surroundings (Segrest et al., 1994). Their amphipathic nature also makes them operate as detergents, and is essential in influencing and maintaining the lipoprotein size and structure (Segrest et al., 2001). A series of letters (A to E), with subclasses of each, are now designated to identify the various apolipoproteins (Groff and Gropper, 2000). Plasma apolipoproteins can be assembled into two classes, the nonexchangeable apolipoproteins [apolipoprotein (apo) B-100 and apoB-48], and the exchangeable apolipoproteins [apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE] (Segrest et al., 1992).

1.1.3.9 Apolipoprotein B-100 (apoB-100)

This is one of the largest proteins known (4,536 amino acids) (Chen et al., 1986; Knott et al., 1986). It is synthesized and used to assemble VLDL in the liver (Glickman et al., 1986). After release into the circulation, the VLDL are converted into intermediate density lipoproteins and, finally, into LDL where the apoB100 is wrapped around the spherical particle (Chatterton et al., 1995). In humans, the liver secretes exclusively apoB-100 whereas the intestine secretes apoB-48 (Vance and Vance, 2002). ApoB-100 concentrations usually vary from (60 – 120) mg/dl. The key role of both apoB isoforms is attributed to their capability to attach lipids: in the absence of either isoform, Chylomicron or VLDL particles are not produced and lipid

absorption and transfer is rigorously hindered. The capability of apoB to attach lipids is inherent in the multiple hydrophobic domains located throughout the protein (Chatterton et al., 1995).

1.1.4 Cardiovascular Disease and Lipoproteins

Atherosclerosis is a degenerative disease of vascular endothelium. Cells of the immune system and lipid material, primarily cholesterol and cholesteryl esters are the main players in the atherogenic process (Groff and Gropper, 2000). Exposure to a high level of LDL, its subsequent deposition and oxidative modification further enhance the inflammatory process. The process is marked by the uptake of LDL by phagocytic cells that become engorged with lipid, and are termed "foam cells" (Groff and Gropper, 2000; Ross, 1993). Alteration of the apoB component of LDL by oxidation accelerates phagocytic uptake. Lipid material in the form of foam cells may then infiltrate the endothelium, and as lipid accumulates, the lumen of the blood vessel involved is progressively obstructed or blocked. The deposited lipid, known to derive from blood-borne lipids, is called fatty plaque (Ross, 1993). Ever since it was discovered that plaque composition is chiefly lipid, an enormous research effort was initiated to investigate the possible link between dietary lipid and the development of atherosclerosis (Groff and Gropper, 2000).

1.2 Diabetes

Diabetes is a global problem with a social, economic and human impact. The prevalence of diabetes is increasing and an estimated 239 million people are expected to have diabetes by the year 2025. Seven million people develop Diabetes each year (Patel, 1999)

Diabetes is a common endocrine diseases characterized by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. If nor treated, diabetes can lead to serious problems on human health, but people with diabetes can take steps to control the disease and lower the risk of complications.

The beta cells in the islets of Langerhans in the pancreas are responsible for the production of insulin. Upon entrance of glucose to the blood, the pancreas should automatically produce the right amount of insulin in order to move glucose into the cells. People having type 1 diabetes fail to produce insulin, while people with type 2 diabetes do not always produce enough insulin (Johnstone et al, 2005).

1.3 Liver Hepatotoxicity

Liver hepatocellular damage can be assessed by a simple blood test to determine the presence of certain liver enzymes in the blood. Under normal circumstances, these enzymes reside within the cells of the liver. But when the liver is injured, these enzymes are spilled into the blood stream (Ahmed et al, 2007).

Among the most sensitive and widely used of these liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST or sGOT), alanine aminotransferase (ALT or SGPT), and Lactate dehydrogenase (LDH). The enzyme aspartate aminotransferase (AST) is also known as serum glutamic oxaloacetic transaminase (sGOT); and alanine aminotransferase (ALT) is also known as serum glutamic pyruvic transaminase (sGPT).These enzymes are normally contained within liver cells. If the liver is injured, the liver cells spill the enzymes into blood, raising the enzyme levels in the blood and signaling the liver damage (Vusse, 2004)

1.4 Plant Research

Over the last decade, people tend to turn to herbs to help cure or prevent disease as they believe that any "natural" treatment is inherently safer or better than a manufactured drug (Fetrow and Avila, 2000). While there are some 300,000 chemically distinct species of plants, less than 10% have been cautiously monitored for their medicinal and toxic components (Duke, 1997). Humanity depends on a diversity of planted species, at least 6000 of which have diverse functions. It is believed that only the minority of staple crops make up the bulk food supply even though the significant involvement of

many minor species should not be underrated. Researchers have customarily concentrated on these staples while negligible, underutilized or overlooked crops have been ignored. This might hinder their development and sustainable conservation (Batlle and Tous 1997).

1.5.1 *Ceratonia siliqua* : General Information

Carob, *Ceratonia siliqua*, is a species that has a long history of use by humans. Evidence of the use of its products dates back to ancient Greece and Egypt where the plant was used as a source of food. The species itself is ancient as it survived the last ice age and flourished throughout the Mediterranean region. It is considered to be an important component of vegetation for economic and environmental reasons. Yearly production is about 310, 000 tones world wide, obtained from about 200, 000 hectares with changeable yields relying on the region and farming practices (MAPA, 1994).

The carob tree is a medium sized evergreen tree originating in the eastern Mediterranean region (Figure 1.4). It is slow growing and can reach up to 15m. Fruit production begins around the age of 15 and continues for the life of the plant. The leaves are wide, dark green and offer considerable shade. The pods (Figure 1.5) are long and leathery often growing up to 300mm long. The flowers are hermaphrodite (have both male and female organs) (Batlle and Tous 1997).

Figure 1.4 *Ceratonia siliqua* or carob tree. Left image- retrieved from www.pbase.com. Right image- retrieved from www.tomas-pavlicek-biologie.net.

Figure 1.5 Carob tree with pods (Retrieved from www.survivaliq.com).

1.5.2 Taxonomy of the Species

Kingdom: Plantae
Phylum: Anthophyta
Class: Dicotyledon
Order: *Rosales*
Family: *Leguminosae* (syn. *Fabaceae*)
Subfamily: *Caesalpinioideae*
Tribe: *Cassieae*
Genus: *Ceratonia*
Species: *siliqua*

Globally, legumes are significant portions of tropical, subtropical and temperate vegetation. Leguminosae, one of the biggest families of flowering plants, comprise 650 genera and more than 18, 000 species (Polhill et al., 1981) and is largely variable in morphology and ecology. Although the carob tree is classified as belonging to the tribe *Cassieae* of the subfamily *Caesalpinioideae*, some authors doubt this classification (Irwin and Barneby 1981; Tucker 1992a, 1992b). According to Goldbalt (1981), unlike many members of the *Cassieae* complex (where the chromosome number is 2n=48), the diploid chromosome number of *Ceratonia* is 2n=24. He therefore suggested that it might be aneuploid. Compared to all other genera of its family, Ceratonia is totally cut off or remote by considering its taxonomical classification (Zohary 1973). In addition, this genus is considered as one of the most ancient of the legume genera (Tucker 1992a). The carob is regarded as a remote remnant of a largely extinct family, Leguminosae (Hillcoat et al., 1980; Tucker 1992a).

1.5.3 Names of the Species

1.5.3.1 Scientific name: *Ceratonia siliqua* L. Derived from Greek *keras*, horn, and Latin *siliqua*, pertaining to the pod's firmness and form (Hammer et al., 1992).

1.5.3.2 Common names: kharuv (Hebrew), from which originated kharrub (Arabic) and later algarrobo or garrofero (Spanish), carrubo (Italian), caroubier (French), Karubenbaum (German), alfarrobeira (Portuguese), charaoupi (Greek), charnup (Turkish), and garrofer or garrover (Catalan) (Hammer et al., 1992).

1.5.3.3 Various names used in different regions of Italy: Ascenedda, soscella (Basilicata); carrua, carrubbi (Sicilia); carruba, sciuscella (Campania); carrubbio, carrubo, cornola, corue, pselocherato, pselocherea (Puglia); suscella (Campania and Puglia); and garrubaro, garrubbo (Calabria) (Hammer et al., 1992).

1.5.3.4 Names used in Asia: Chiao-tou-shu (China), gelenggang (Malaysia) and chum het tai (Thailand) (Kruse 1986).

Due to the alleged utilization (by St. John the Baptist) of its 'locusts' as food, the carob is as well known as St. John's bread or locust bean and from that was derived Johannisbrotbaum in German. Jewelers also utilized its even seeds as an element of weight (200 mg), the carat (Batlle and Tous 1997).

1.5.4 Parts used:

The parts used from the carob tree include the pulp and seeds. The pulp is considered as the main constituent making about 90% of the total pod mass. While the remaining 10% is mainly composed of seeds.

1.5.4.1 Edible uses:

The fruits of the carob tree can be eaten either green or after processing. The sugary pulp is a chief component of food for farm animals and also taken as snacks by people and children (Batlle and Tous 1997). The pulp can be ground into fine powder, in which sugar and protein are present (46% and 7% respectively) in addition to minute amounts of abundant minerals and vitamins, for its use in human nutrition as it is quite nutritious (Whiteside 1981). The powder, after oven drying, can be supplemented to cakes, bread,

sweets, and ice-creams or added to flavor drinks (NAS 1979). Carob powder "cocoa" has a flavor that is similar to that of milk chocolate although not as strong as dark chocolate. It is better than chocolate in that it has less calories and does not include caffeine or theobromine (Craig and Nguyen 1984; Whiteside 1981). Carob is also used to make flour and molasses. According to Merwin (1981), in some Mediterranean countries, carob pulp was amongst the first horticultural crops utilized in the production of industrial alcohol due to its rich sugar content and low price. In Egypt and some other countries, extorting carob kibbles with water generates carob syrup which makes a famous drink (Imrie 1973; Sekeri-Pataryas et al., 1973).

1.5.4.2 Other uses:

The trees are useful for decoration, landscaping, windbreaks and forestation. The leaves are useful for cattle grazing (Batlle and Tous 1997), the pods feed ruminants (Louca and Papas 1973) and non ruminants (Sahle et al., 1992), and the wood is suitable for fuel (Batlle and Tous 1997) and for making utensils (Hillcoat et al., 1980) and the seed is used for gum extraction (Batlle and Tous 1997). There are up to 15 seeds surrounded by a saccharine pulp inside the seed pod. The seeds are separated from the pulp and used for industrial carob bean gum (CBG) and locust bean gum (LBG) production, which are polysaccharides (galactomannans) contained in the endosperm of the seeds (Batlle and Tous 1997). This product is used in the manufacture of diverse food stuffs, especially confectionary. It is used as a stabilizer, emulsifier, and thickener or to prevent sugar crystallization. In nature, carob cover, leaves and beans are appealing to browsing animals (Hillcoat et al., 1980). Imrie 1973 and Sekeri-Pataryas et al. (1973) mentioned that solutions of sugar extorted from carob pods are outstanding for culturing fungi like *Aspergillus niger* and *Fusarium moniliforme* and the desiccated mycelium is platable and nourishing as it comprises about 38% crude protein. Derivatives of the carob molasses industry (milled and chopped carob pomace) were tried in Lebanon as a potting material for plants and have proven to be promising in replacing peat-based blends in nurseries (Rishani and Rice 1988).

1.5.4.3 Other Products and Uses

While food production is a very important characteristic of carob, it is by no means the only benefit that can be obtained from growing this species. Tannin is obtained from the bark of Carob. The flour made from seed pods are used to produce cosmetic face-packs. The hard wood is highly required by wood turners. Being a nitrogen fixing species, it helps improve soil fertility, and grows well on low rainfall marginal land and is used for land amelioration (Batlle and Tous 1997).

1.5.5 Carob products

Carob is a useful, multipurpose tree to humans as there are a wide range of products derived from its fruits and timber. Primarily, foods for both human and animal consumption are obtained from its seeds, pulp and seed pods (Batlle and Tous 1997).

Carob molasses, a common traditional food in the Lebanese community, is prepared in the following manner: Collected pods are left to dry, pressed by a stone, piled to ferment, then soaked in water in order to extract the sugar and other water soluble materials. The juice is finally boiled until it becomes thick and red.

1.5.5.1 Properties

The pulp's chemical composition relies on harvesting duration, cultivar and origin (Albanell et al. 1991; Calixto and Cañellas 1982; Davies et al. 1971; Orphanos and Papaconstantinou 1969; Vardar et al. 1972). It is rich in overall sugar composition (chiefly sucrose, glucose, fructose and maltose) (Table 1.1) (48-56%) and includes cellulose and hemicellulose (~18%). According to Puhan and Wielinga (1996), every 100 g of pulp contains 1100 mg potassium, 307 mg Calcium, 42 mg Magnesium, 13 mg Sodium, 0.23 mg Copper, 104 mg Iron, 0.4 mg Manganese, and 0.29 mg Zinc. Vohra and Kratzer (1964) reported that feeding experiments revealed that carob pulp is moderately low in metabolizable energy and comprise digestible proteins (only 1-2%). The lipids comprise saturated and unsaturated acids in almost equal amounts

(Rendina et al., 1969). The proteins are difficult to digest as they are attached to fiber and tannins (Loo 1969).

Table 1.1 Average constituent of carob pulp

Composition	Percentage
Overall sugars	48-56
Sucrose	32-38
Glucose	5-6
Fructose	5-7
Pinitol	5-7
Condensed tannins	18-20
Non-starch Polysaccharides	18
Ash	2-3
Fat	0.2-0.6

Source: Puhan and Wielinga 1996

In addition to tyrosine and phenylalanine (Charalambous and Papaconstantinou 1966), pod extracts contain the amino acids alanine, glycine, leucine, proline and valine (Vardar et al., 1972). Ripe carob pods are rich in concentrated tannins which make up 16-20% of the dry weight (Würsch et al., 1994). NAS (1979) indicated that in food assessment, carob pods resemble most cereal grains (NAS 1979).

1.5.5.2 Plant Chemical Composition

Carob comprise: alanine, alpha-aminopimelic-acid, amino acids, arginine, ash, aspartic acid, benzoic acid, butyric acid, capronic acid, carubin, catechin tannin, cellulose, ceratoniase, ceratose, chiro-inositol, concanavalin-A, fat, formic acid, fructose, D-galactose, gallic acid, beta-D1,6-DI-O-galloylglucose, beta-D-glucogallin, glucose, glutamic acid, glycine, gum, hemicellulose, histidine, hydroxyproline, invert sugars, isobutyric acid, isoleucine, leucine, leucodelphinidin, lignin, lysine, D-mannose, methionine, mucilage, myoinositol, pectin, pentosane, phenylalanine, pinitol, primverose, proline,

protein, saccarose, saponin, serine, starch, sucrose, sugars, tannin, threonine, tocopherol, tyrosine, valine, water, xylose. The pods contain 19.2% antioxidant polyphenols and have been recommended for utilization as food or food ingredient (MAPA, 1994).

1.5.6 Cultivation Details

C. siliqua prefers sandy, medium and clay loam soils but can tolerate poorer soil conditions including rocky areas. If the species is to grow well, good drainage and full to semi-sun are also preferred. It requires a subtropical Mediterranean climate with cool but not cold winters, mild to warm springs and warm to dry summers. It can survive extreme temperatures from 40 to -7°C (Batlle and Tous 1997). It will tolerate a pH of 6.2-8.6. This species is extremely resistant to drought and irrigation is not required (Tous and Batlle 1990).

Its edible seeds and seed pods makes it often planted in warm temperate zones. In an appropriate environment, full-grown trees can furnish up to 400 kilos of seedpods per year. The seed is extremely even in size and weight. A symbiotic association exists between carob and certain soil bacteria. The bacteria form root nodules and fix atmospheric nitrogen some of which is utilized by the growing plant as well as by nearby ones (Batlle and Tous 1997).

1.5.7 Medicinal Uses

Apart from the health benefits obtained by substituting carob for Cocoa and synthetic sweeteners in our diet, as it has less calories, carob also has excellent nutritional value. Along with up to 8% protein, it contains minerals like magnesium, calcium, iron, phosphorus, potassium manganese, barium, copper, nickel and vitamins like A, B1, B2, B3, and D (Batlle and Tous 1997).

1.5.7.1 Astringent, Demulcent, Emollient, and Purgative Effect

The pulp component of the seedpods is very healthy and due to its elevated sugar content, it has a sweet-tasting flavor. It is also a mild laxative and at the

same time styptic (astringent) and when utilized in a decoction, will counteract diarrhea and gently aid in rinsing and alleviating irritations within the gut. As its influences seem contradictory, carob is a model of how the body reacts to herbal medicines in diverse ways, based on the herb preparation and specific medical trouble. The seedpods are also utilized to treat coughs. In addition, the demulcent and emollient flour produced from the ripe seedpods is utilized in the treatment of diarrhea (Batlle and Tous 1997).

1.5.7.2 Anti-diarrheal Effect

A study of the anti-diarrheal effects of carob bean juice conducted on 80 hospitalized children suffering from acute diarrhea and dehydration revealed that carob bean juice decreased diarrhea duration by 45%, stool production by 44% and reduced the requirement of oral rehydration solution (ORS) by 38% (compared with children receiving only ORS) (Brown 1996).

Some trials showed that 15 and at least 20g of carob powder a day could be used for treating diarrhea in children and adults respectively. It can also be combined with either applesauce or sweet potatoes (Batlle and Tous 1997).

1.5.7.3 Lipid Lowering Effect

A carob pulp preparation rich in insoluble dietary fiber and polyphenols has been confirmed to reduce lipids in humans. The daily consumption of 15g of carob powder for 4-weeks declines in mean total and in LDL cholesterols were noted by 7.1% and 10.6% respectively. However, the consumption of bread or fruit snack-bar containing carob fiber diminished LDL cholesterol by 10.5 ± 2.2 %. The ratio of LDL/ HDL cholesterol was slightly decreased by 7.9 ± 2.2 % in the carob fiber group compared to the placebo group. In addition, carob fiber consumption lowered triglycerides in females by 11.3 ± 4.5%. Lipid lowering effects were more prominent in females than in males (Zunft 2003).

In an open human intervention trial with 49 healthy volunteers having mild to moderately raised cholesterol levels; the carob fiber was taken three times a day (in breakfast cereals, fruit bars and drinking powders) in addition to the normal diet. There were highly significant reductions both in total cholesterol and LDL of up to 7.8% and 12.2% respectively after 8 weeks.

A randomized, placebo-controlled and double-blind human trial confirmed clearly the activity of carob fiber in lowering cholesterol. 58 volunteers with elevated serum cholesterol levels (200–299 mg/dl) consumed (after 2 week run-in) both bread and a fruit snack-bar with or without carob fiber for 6 weeks. Carob fiber consumption was found to reduce the serum level of LDL cholesterol by 10.5 ± 2.2%, the serum triglycerides by 18.1% ± 4.5% and the LDL/HDL ratio by 8.2%.

1.6 Aim of the Project

Carob molasses used to be one the old traditional sources of food in the Lebanese community. However, the consumption of carob molasses has declined tremendously over the last few decades, especially when it comes to city dwellers where the availability of fast food in each and every alley has become easily accessible. Also, one should not forget the infiltration of the Westernized dietary life style in the community and the ongoing replacement of the old traditions that makes part of the healthy Mediterranean dietary life style. If we take into consideration the taste, cost, availability and medicinal effect of such a nutritious food, it is worth investigating its potential importance and benefits on the human health.

The present study aims at investigating the effect of chronic intake of carob molasses upon lipemia and glycemia. In addition to other effects, the study also included the potential role of carob molasses as a remedy in either constipation or diarrhea. Parameters were assessed after a six weeks period of chronic consumption of carob molasses in the rat model, taking into consideration the use of a regular or a high-fat diet.

Parameters studied are:

a. Serum: total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triacyglycerol (TAG) and glucose.
b. Serum GOT, GPT, and LDH activity.
c. Serum Insulin.
d. Plasma Apo B100, TAG and Cholesterol.
e. Fecal Cholesterol, TAG, and water content.
f. Liver TAG.

Chapter 2

MATERIALS AND METHODS

2.1 Animal Treatment

Male Sprague-Dawley rats weighing 230-250g (Lebanese American University stock) are used in all experimental procedures. Animals were maintained and experimental protocols complied with the Guide for the Care and Use of Laboratory Animals (National Research Council of the United States 1985). Animals were maintained at an ambient temperature of 20 - 22°C, and 12 hours photoperiod, during the whole study. All animals were sacrificed using diethyl ether, at the end of the procedures described, without recovery from anaesthesia.

2.2 Group Allocation

In order to study the effect of a six weeks period of ingestion of carob molasses upon blood lipid profile, 48 rats were randomly allocated into 2 major groups each of 24 rats, where each group received either a regular or a high fat diet. These 2 groups were then subdivided into 2 further groups of 12 rats each. One group served as a control group while the other received commercial carob (Al-Rabih) molasses via drinking water (1% w/v) *ad lib* and was referred to as experimental group. All diet were similar except the high fat diet to which 5% w/w of coconut oil was added to increase the percentage fat. To increase the atherogenicity of the high fat diet, coconut oil was chosen due its high saturated fat content. Table 2.1 summarizes group allocation.

Table 2.1: Allocation of animals into the different groups.

	Regular Fat Diet		High Fat Diet	
Group	**Control**	**Experimental**	**Control**	**Experimental**
Food	6.5g/kg BW rat chow diet	6.5g/kg BW rat chow diet	6.5g/kg BW rat chow diet containing 5% w/w coconut oil	6.5g/kg BW rat chow diet containing 5% w/w coconut oil
Drink	Plain water	Water + Carob Molasses (1% w/v)	Plain water	Water + Carob Molasses (1% w/v)

2.3 Sample Collection

After 6 weeks of dietary treatment, rats were fasted for 18 hours before being sacrificed. Animals were rapidly anesthetized using diethyl ether, a midline abdominal incision was made for about two-thirds of the length of the abdomen and the inferior vena cava was exposed by exteriorizing the intestine. With the use of 10 mL syringes, about 8 mL of blood were withdrawn from the inferior vena cava and transferred into 2 tubes, (about 4 mL in each tube), one containing disodium ethylene-diamine-tetraacetic acid (Na_2EDTA 1mg/mL blood) for collection of plasma, and the other being plain for collection of serum. In order to collect serum, tubes were incubated at 4 °C for 30 minutes to allow the clotting process, then they were centrifuged (Sigma Laborzentrifugen 2K15) at a speed of 5000rpm for 20 minutes at a temperature of 10°C. Plasma was collected by immediate centrifugation at 4°C. 2 mL of plasma are used to isolate lipoproteins for total Apo B quantification as described later.

Sera samples were divided into aliquots and kept on ice or in freezer for later use in assessing lipid profile (Total cholesterol, HDL cholesterol, LDL cholesterol, and Triglycerides), glycemic profile (Glucose and Insulin) and liver enzymes activities (SGPT and SGOT).

2.4 Serum assays

2.4.1 Determination of Serum Glucose

The glucose assay kit (Spinreact, S.A, Espana) used was based upon a colorimetric method. Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The hydrogen peroxide formed reacts under catalysis of peroxidase with phenol and 4-aminophenazone to a colored compound. The intensity of the color formed is proportional to the glucose concentration in the sample. Samples, were run in duplicates.

2.4.2 Determination of Serum TAG

The TAG assay kit (Spinreact, S.A, Espana), used is based upon a colorimetric method. TAG are enzymatically hydrolyzed to glycerol and free fatty acids. The glycerol liberated reacts with Glycerol Kinase (GK) and Glycerol-3-Phosphate Oxidase (GPO) yielding H_2O_2. The H_2O_2 concentration is determined through the Trinder's reaction. Samples, along with standards, were run in duplicates.

2.4.3 Determination of Serum Cholesterol

The cholesterol assay kit (Spinreact, S.A, Espana) used was based upon a colorimetric method. The cholesterol present in the sample originates a colored complex, according to the following reaction:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenona} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-Aminophenazone} \xrightarrow{\text{PCD}} \text{Quinonimine} + 4\,H_2O$$

The intensity of the color formed is proportional to the cholesterol concentration in the sample. Samples and standards were run in duplicates.

2.4.4 Determination of Serum HDL-Cholesterol

The very low density (VLDL) and low density (LDL) lipoproteins from serum or plasma are precipitated by phosphotungstate in the presence of magnesium ions. After centrifugation, the clear supernatant containing high density lipoproteins (HDL) is removed and used for the determination of HDL cholesterol using the cholesterol assay kit as described in the previous section.

2.4.5 Determination of Serum LDL-Cholesterol

Concentration of LDL cholesterol was determined according to the Friedwald equation:

LDL-Cholesterol = Total-Cholesterol – (TAG/5) – HDL-Cholesterol

2.4.6 Determination of Serum LDH

Using the LDH assay kit (SPINREACT), Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate by NADH, according to the following reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

The rate of decrease in concentration of NADPH, measured photometrically, is proportional to the catalytic concentration of LDH present in the sample.

2.4.7 Determination of Serum GOT-AST

Using the GOT-AST assay kit (SPINREACT), Aspartate aminotransferase (AST) formerly called glutamate oxaloacetate (GOT) catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate forming glutamate and

oxaloacetate. The oxaloacetate produced is reduced to malate by malate dehydrogenase (MDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of AST present in the sample.

2.4.8 Determination of Serum GPT-ALT

Using the GPT-ALT assay kit (SPINREACT), Alanine aminotransferase (ALT) or Glutamate pyruvate transaminase (GPT) catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and pyruvate.
The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in sample.

2.4.9 Insulin determination using Enzyme Linked Immunosorbent Assay (ELISA) technique from rat sera

This technique (Rat/Mouse Insulin ELISA kit, LINCO-USA), is a sandwich ELISA based, sequentially on: 1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, 2) wash away of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine.
The enzyme activity is measured spectrophotometrically by the increased absorbancy at 450nm, corrected from the absorbancy at 590nm, after acidification of formed products. Since the increase in absorbancy is directly proportional to the amount of captured insulin in the unknown sample, the latter

can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

2.5 Determination of fecal water, cholesterol and triacylglycerol (TAG) content

Fresh stools were collected from rats of the four groups. Stools were dried in an oven (Imperial V Laboratory Oven) for 24 hours at 65°C and water content was then calculated. The weighed dried stools were ground using a mortar and pestle and a sample of 0.1g was then homogenized using (Ultra-TURRAX T25 IKA®-WERKE) in 1ml hexane and incubated in the oven at 40°C for 5 minutes. The solution obtained is then centrifuged (Sigma Laborzentrifugen 2K15) at 5000rpm for 10 minutes at room temperature. 200μl from each sample is pipetted into clean tubes to assess triacylglycerol and another 200μl for cholesterol content. Hexane (200μl) was evaporated at 80°C after which TAG and cholesterol contents were estimated using TAG and cholesterol assay kits (Spinreact, S.A, Espana) (Daher et al., 2003). Final results were presented as concentration of TAG or Cholesterol in mg/g of dry stool.

2.6 Liver Lipid Content Determination

A liver tissue sample was collected from each fasted rat during blood collection for analysis and estimation of total fat content. Liver tissue was first washed in 0.9% NaCl solution, blot dry using a filter paper, and then exactly 1 g of the tissue was weighed. Liver fat extraction procedure was conducted according to the method of Folch et al. (1957).

The assay procedure is as follows:

- The tissue was homogenized with Chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture). After dispersion, the whole mixture was agitated during the 15-20 minutes in an orbital shaker at room temperature.
- The homogenate was centrifuged to recover the liquid phase.

- The solvent was washed with 4 ml of 0.9 % NaCl solution. After vortexing, the mixture was centrifuged for 10 min at 2000 g to separate the two phases.
- After centrifugation and siphoning of the upper phase, the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator.

Finally, the mass of remaining fat of each sample was determined.

2.7 Isolation of Lipoproteins for ApoB Quantification

Each 2 mL of the collected plasma is placed in the bottom of a 10 mL polycarbonate ultracentrifuge tube (Sorvall, Kendro Laboratory products) on ice. Crystalline NaCl (0.14g/ml) was added to the plasma samples and vortex mixed. To minimize the proteolytic degradation, a preservative solution containing aprotonin was added (2mg/mL), and phenylmethylsulfonylfluoride (PMSF), 10mM dissolved in 2-propanol, at concentrations of 5 μL/mL plasma and 10 μL/mL plasma respectively. A 4 ml NaCl solution (density= 1.065g/ml) 0.01% (w/v) Na_2EDTA and 0.02% sodium azide (NaN_3) with the pH adjusted to 7.4 was layered on top of the plasma in the ultracentrifuge tube using the peristaltic pump machine (Watson-Marlow Limited, England). The tubes were then centrifuged for 20 hours at 15°C in (Sorvall Discovery™ 90SE) at 50000rpm. After which, the upper 500μl layer, containing chylomicrons, VLDL and LDL lipoproteins, are carefully aspirated and placed in Eppendorf tubes to be used as, described later, for gel electrophoresis.

2.7.1 Preparation of samples for apo B100 analysis

100 μl from the samples, containing lipoproteins, were mixed with 200 μl of sample buffer (ratio 2:1) as described below:

Sample buffer is a solution containing 0.15M sodium phosphate, 12.5% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) mercaptoethanol, 0.001% (w/v) bromophenol blue, adjusted to pH=6.8.

The Eppendorf tubes containing the lipoprotein samples and the sample buffer were denatured at 90 °C for 4 min using Eppendorf tube heater, and then centrifuged (Heraeus Sepatech BIOFUGE 13R) for 5 min at 5000rpm. Samples were then stored at -20°C for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis.

2.7.2 Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.7.2.1 Stock solutions (Hames, 1990)

0.5 M Tris buffer (pH 6.8): 6.02 g Tris was dissolved in approximately 80 ml distilled water, pH adjusted to 6.8 with 12 M HCl. Then distilled water was added to a final volume of 100 ml. This solution is stable for several weeks at 4°C.

3M Tris buffer (pH 8.8): 36.6 g Tris was dissolved in about 80 ml distilled water and pH adjusted to 8.8 with 12 M HCl. Distilled water was added to a final volume of 100 ml. This solution is stable for several weeks at 4°C.

1.5 % ammonium persulfate solution (APS): 0.15 g of ammonium persulfate was dissolved in 10 ml of distilled water.

Protogel: 30 g acrylamide and 0.8 g N, N'-bis-methylene acrylamide were dissolved in approximately 70 ml of distilled water; the final volume was adjusted to 100 ml.

Urea/SDS/dithiothreitol solution: 19.2 g urea, 12.3 mg dithiothreitol and 0.08 g lauryl sulfate SDS were dissolved in 30 ml of distilled water and the final volume was made up to 40 ml.

Electrode buffer (pH 8.3): 99.4 g glycine and 21.4 g Tris were dissolved in 2 L of distilled water, then 7 g of SDS were added to the Tris/Glycine solution and the final volume made up to 7 L with the addition distilled water.
Water-saturated n-butanol: 90 ml n-butanol + 10 ml water.

2.7.2.2 SDS-PAGE preparation

Following the method of Hames (1990) according to the original method of Laemmli (1970), linear gradient (5-20%) polyacrylamide gels were prepared to allow an increased protein sieving effects and a better separation of a larger range of protein molecular weights. A 5 % acrylamide resolving gel, a 20 % acrylamide resolving gel and a stacking gel, to be layered on top of the resolving gels, were prepared as shown in Table 2.2.

Table 2.2: Gel mixtures for 5-20% gradient gels (4 gels)

	Resolving Gel		Stacking Gel
	5%	20%	
0.5M Tris	-	-	7.5ml
3M Tris	7.4ml	7.4ml	-
Protogel	10ml	40ml	3.7ml
Urea/SDS/DTT	6ml	600µl	0.6ml
1.5%APS	1.4ml	1.4ml	2.3ml
Sucrose	-	9g	-
Water	40.4ml	5.4ml	15ml
TEMED	46 µl	46 µl	22.5 µl

The electrophoresis apparatus (Hoefer SE 600, Amersham Pharmacia Biotech) was assembled using the side spacers and clamps according to the manufacturer's instructions. Clamped glass plates were tested to ensure they are water tight and thus prevented leakage. Immediately before pouring the resolving

gels, 46 μl tetramethylethylenediamine (TEMED) was added to each of the gels to initiate polymerization. The 20% gel was placed in the mixing chamber and the 5% gel in the reservoir chamber of a gradient forming apparatus (Coleparmer, USA). Using a peristaltic pump, the gels were poured into the glass chambers while stirring of the 20% chamber. After the resolving gel has been poured, it was overlayered with water-saturated butanol and left to polymerize. After about 45 min, the water-saturated butanol was discarded and the gel top was rinsed several times with distilled water. The remaining water droplets were removed with a filter paper. A fifteen-well comb was inserted between the glass plates leaving about 0.5 cm between the bottom of the combs and the resolving gel. Before overlayering the polymerized resolving gel with the stacking gel, 22.5 μl of TEMED were added to the stacking gel. The poured stacking gel was left for about 30 min to polymerize then the comb was carefully withdrawn. Electrode buffer was added at this stage to the wells of the gel in order to avoid any later disturbance in the wells. Protein samples were loaded in the wells along with the molecular weight marker and the protein standard, after which the upper and lower electrode chambers were filled with electrode buffer.

2.7.2.3 Loading of samples

Samples stored at -20°C are thawed in preparation for loading. Molecular weight markers, (Sigma Chemical Co.Ltd) were dissolved and denatured according to the supplier's instructions. Using disposable gel loading tips, 10 μl of molecular weight marker solution was loaded into the first gel lane, 5 μl, 15 μl and 30 μl of standard solution were added respectively into lanes 2, 3 and 4 of the gel. The remaining lanes were loaded with 100 μl of the lipoprotein samples under examination.

2.7.2.4 Electrophoresis

After loading all samples, electrodes of the upper chamber lid were properly attached, and the water-cooling system and power supply (Consort E455, Belgium) were then switched on. Electrophoresis was carried out at 220V,

120mA, 20W for both gel chambers. Four hours later, the power is switched off, the water-cooling system disconnected and the gel carefully removed for the staining-destaining steps.

2.7.2.5 Staining and quantification of proteins

The staining solution was prepared by dissolving Coomassie Blue R-250 (1g/L) in water (450 ml/L), methanol (450 ml/L), and glacial acetic acid (100 ml/L).

The protein destaining solution contained (800ml/L) water, (100ml/L) methanol and (100ml/L) glacial acetic acid.

Staining and destaining were carried out in an automated gel stainer-destainer (Hoefer processor plus, Amersham Pharmacia Biotech) for about 17 hours. Then, the gel slabs were carefully removed, placed between two transparencies, labelled and scanned. The image saved was used for quantitation of apo B100 bands using 1-D Advanced software (Advanced American Biotechnology, 1166E Valencia Dr, #6C, Fullerton CA. 92831). A standard curve was then constructed for every gel slab and the concentrations of apo B100 were estimated accordingly.

2.8 Postprandial Study:

2.8.1 Group allocation and fat loading

Two groups of 8 animals each were allocated into control and experimental group. Fasted animals (300-325g) of both groups received 5 ml of a lipid emulsion solution by intragastric gavage. In the control group, the lipid emulsion contained 20% olive oil in 0.9% NaCl. In the experimental group, the lipid emulsion contained 20% olive oil and 5% carob molasses in 0.9% NaCl. All emulsions were prepared by sonication for 2 min using an ultrasonic homogenizer.

2.8.2 Sample Collection & Blood Study

After 3h of lipid loading, animals were anesthetized using diethyl ether and serum and plasma samples were collected as described previously. Serum samples were used for routine lipid analysis while plasma samples were used for the isolation of chylomicron and VLDL lipoprotein fractions.

2.8.3 Isolation of Lipoproteins: Chylomicrons and VLDL

Each 2 mL of the collected plasma is placed in the bottom of a 10 mL polycarbonate ultracentrifuge tube (Sorvall, Kendro Laboratory products) on ice. Crystalline NaCl (0.14g/ml) was added to the plasma samples and vortex mixed. To minimize the proteolytic degradation, a preservative solution containing aprotonin was added (2mg/mL), and phenylmethylsulfonylfluoride (PMSF), 10mM dissolved in 2-propanol, at concentrations of 5 μL/mL plasma and 10 μL/mL plasma respectively. A 2.5 ml NaCl solution (density= 1.065g/ml) containing 0.01% (w/v) Na_2EDTA and 0.02% sodium azide (NaN_3) with the pH adjusted to 7.4 was layered on top of the plasma in the ultracentrifuge tube. Another 2.5 ml NaCl solution (density= 1.006g/ml, 0.01% (w/v) Na_2EDTA and 0.02% sodium azide (NaN_3) with the pH adjusted to 7.4) was layered on top of the previous layer in the ultracentrifuge tube using the peristaltic pump machine (Watson-Marlow Limited, England)

The tubes were first centrifuged for 30 min at 15°C (Sorvall Discovery™ 90SE) at 28000 rpm. The upper 500μl layer, containing the chylomicron fractions, were carefully aspirated and placed in Eppendorf tubes to be used for lipid analysis. Tubes were then over layered with 0.5 ml of NaCl solution (density= 1.006g/ml) and centrifuged for 15 h at 50000 rpm. Another upper 0.5 ml, containing the VLDL fractions, were carefully aspirated, placed in Eppendorf tubes and used for lipid analysis.

Serum, chylomicron and VLDL samples collected were analyzed for their TAG and cholesterol content.

2.9 Data handling and statistical methods

Values of the different tested parameters within each group are presented as mean ± SEM. Comparison between each two groups was made by independent t-test. A p value of less then 0.05 ($p<0.05$) was considered significant.

Chapter 3

RESULTS

3.1 Effects of Carob Molasses on Blood lipid profile in Rats fed either a regular or high-fat diet

Animals were maintained for a period of six weeks on water and carob molasses and had either a regular or a high-fat diet. At the end of the treatment, all rats were fasted for 18 hours, and rapidly blood was collected. Concentrations of the following are measured in serum: glucose, TAG, cholesterol, HDL cholesterol, LDL cholesterol, SGOT-AST, SGPT-ALT and LDH. ApoB 100 concentration in lipoprotein fractions is determined by SDS-"PAGE after being obtained by ultracentrifugation of plasma samples. Fecal Cholesterol, TAG, and water content as well as serum Insulin concentrations,

3.1.1 Serum Lipid Profile

Table 3.1 summarizes the mean serum concentration values in mg/dl of glucose, TAG, cholesterol, HDL-cholesterol, LDL-cholesterol and the ratio LDL/HDL in control and experimental groups.

3.1.1.1 Serum Glucose

There is no significant difference in the concentration of serum glucose between the control and the carob treated group in both regular and high-fat diet ($p>0.05$).

3.1.1.2 Serum TAG

There is no significant difference in the concentration of circulating TAG between the control and the carob treated group in both regular and high-fat diet ($p>0.05$).

3.1.1.3 Serum total Cholesterol

The concentrations of circulating cholesterol showed no significant difference ($p>0.05$) between the control and experimental groups of the regular and high-fat diet.

3.1.1.4 Serum HDL-Cholesterol

There is a significant ($p<0.05$) difference in the concentrations of HDL-cholesterol between the control and experimental groups of both the regular and high-fat diet.

3.1.1.5 Serum LDL-Cholesterol

There is no significant difference ($p>0.05$) in the concentrations of circulating LDL-cholesterol between the control and the carob groups of both the regular and the high-fat diet.

3.1.1.6 LDL/HDL ratio

Calculation of the LDL/HDL-cholesterol ratio shows 14.3 and 8.5% improvement in the ratios with carob intake with the regular and high-fat diet respectively.

3.1.1.7 Plasma ApoB Concentration

Figure 3.1 shows the plasma ApoB concentration of animals in the different groups. Assessment of plasma ApoB concentration revealed that the intake of carob molasses along with the diet has no significant effect on the total plasma ApoB content. However, it was evident that increasing fat saturation in the diet increase the relative ApoB concentration.

3.1.3 Serum Enzymes

Table 3.2 summarizes the serum enzyme activities of sGOT, sGPT and LDH in the control and experimental groups of both regular and high-fat diet.

When compared with the control group the experimental groups did not show any significant difference in the serum enzyme activities of serum GOT, GPT and LDH when either a regular or a high-fat diet was administered to the animal along with or without carob intake.

Table 3.1: Glucose (mg/dL), TAG (mg/dL), Cholesterol (mg/dL), HDL-Cholesterol (mg/dL) levels in serum after six weeks chronic consumption of carob molasses in rats fed a regular or high-fat diet; values denote mean ± SEM (n=12)

	Regular-Fat Diet		High-Fat Diet	
Parameter	**Control**	**Carob**	**Control**	**Carob**
Glucose	99.5 ± 4.2	109.7 ± 5.7	121.8 ± 10.7	123.4 ± 5.7
TAG	57.3 ± 3.8	54.8 ± 3.5	78.3 ± 5.7	68.8 ± 7.1
Cholesterol	49.6 ± 2.2	48.0 ± 1.6	51.2 ± 3.0	46.2 ± 2.4
HDL-Cholesterol	22.1 ± 1.4	26.6* ± 1.64	22.8 ± 1.0	28.4* ± 2.0
LDL-Cholesterol	13.4 ± 2.2	12.3 ± 1.5	9.0 ± 0.9	8.8 ± 1.4
LDL/HDL	0.606	0.46	0.395	0.310

* Significant difference ($p<0.05$) between the experimental group and the control group.

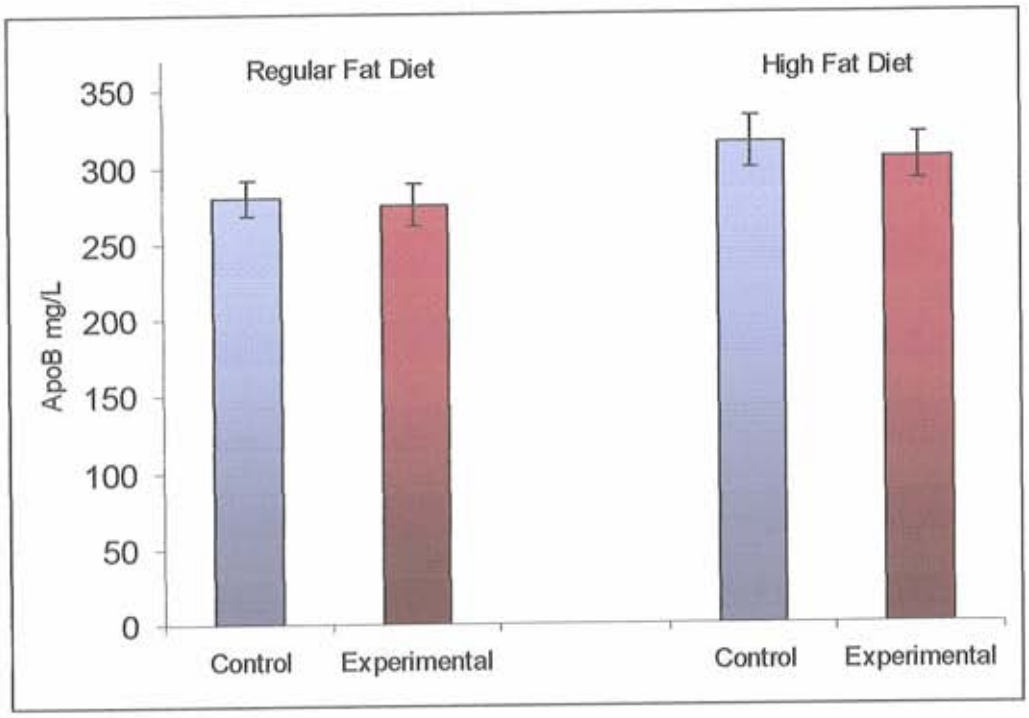

Figure 3.1 Rat plasma ApoB concentration (mg/L) as determined in fasted rats subjected to a six weeks period of *Carob* treatment. Bars denote mean ± SEM (n=12).

Table 3.2: sGOT, sGPT, and LDH activities in serum after six weeks consumption of carob molasses in rats fed regular or high-fat diet. Values denote mean ± SEM (n=12)

	Regular-Fat Diet		High-Fat Diet	
Parameter	**Control**	**Carob**	**Control**	**Carob**
sGOT	74.5 ± 4.5	73.1 ± 3.8	79.0 ± 4.5	78.1 ± 4.1
sGPT	29.4 ± 2.8	28.1 ± 3.5	25.0 ± 1.8	24.8 ± 9.5
LDH	902.8 ± 123.3	861.3 ± 91.3	757.3 ± 74.0	748.8 ± 95.2

3.1.4 Serum Insulin

Figure 3.2 shows the serum insulin concentration of animals in the different groups. No significant changes in the serum insulin concentration were observed among the different groups in both the regular and high-fat diet groups.

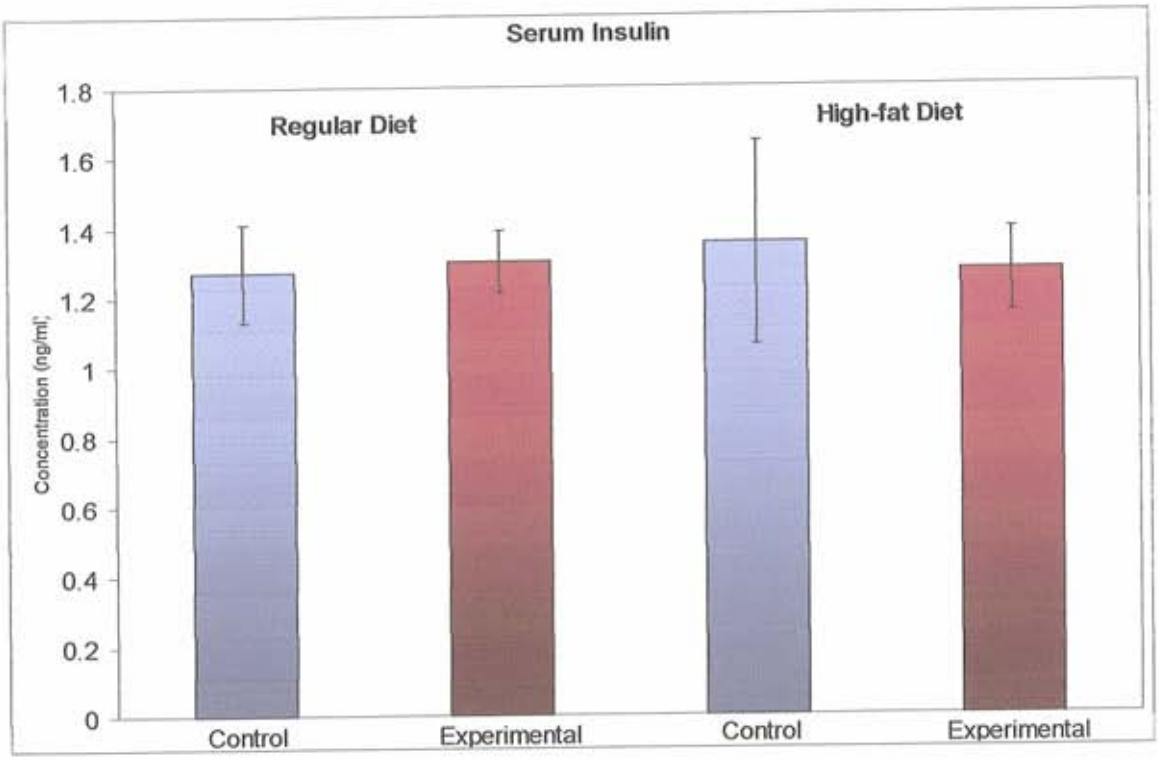

Figure 3.2 Rat Serum Insulin concentrations (ng/ml) as determined in fasted rats subjected to a six weeks period of *Carob* treatment. Bars denote mean ± SEM (n=12).

3.1.5 Stool Study

The following results summarize the stool TAG and cholesterol content in mg/g of dry feces as well as the stool-water content in the control and experimental groups.

3.1.5.1 Stool TAG content

The carob group showed a significant increase ($p<0.05$) in the stool TAG content with respect to the control group in the presence of the regular-fat diet while no significant difference was noted in the high-fat diet group. Results are shown in Figure 3.3.

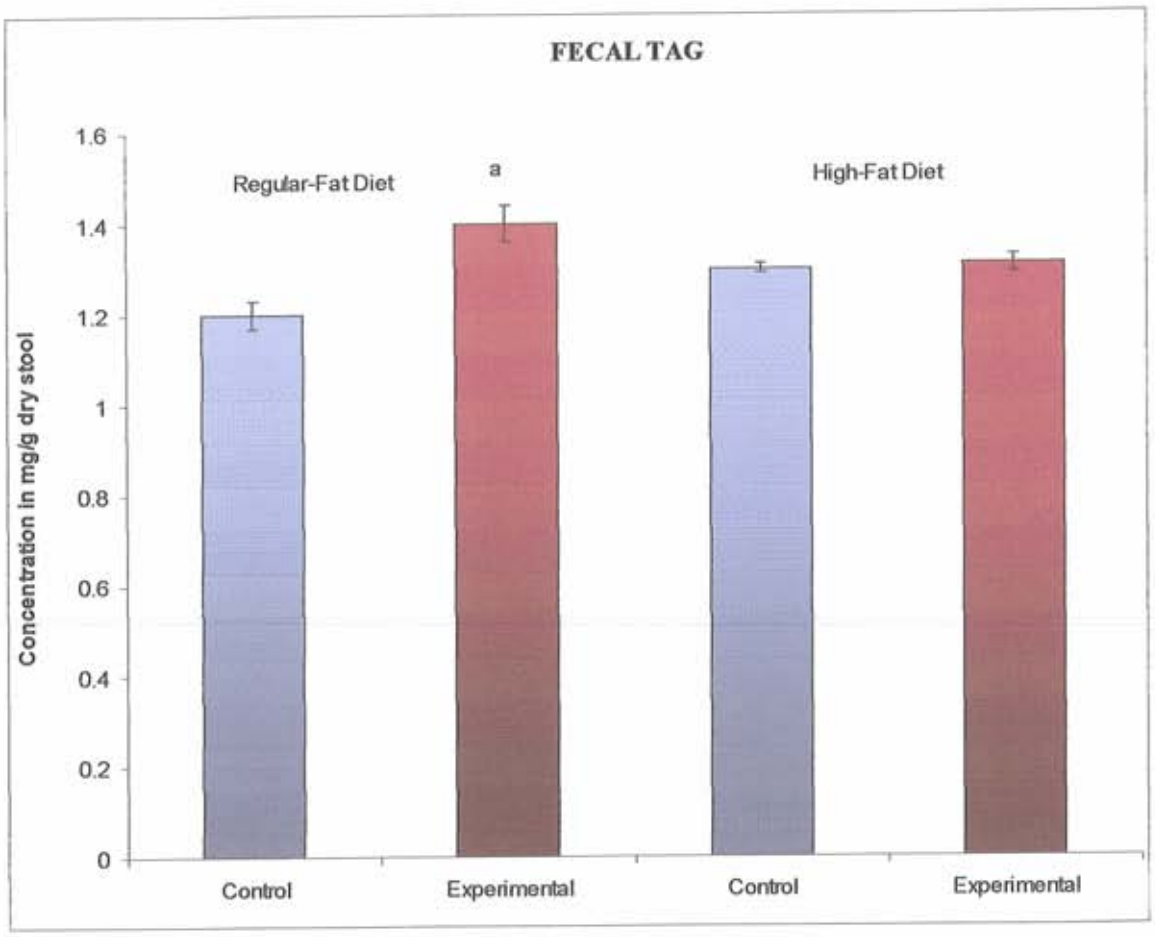

Figure 3.3 Rat Fecal Triglyceride content (mg/g) dry stool, six weeks after Carob treatment. Bars denote mean ± SEM (n=8)

[a] Significant difference ($p<0.05$) between the experimental group and the control group.

3.1.5.2 Stool Cholesterol content

No significant changes were observed in fecal-cholesterol content in the different groups when either a regular or a high-fat diet was administered to the animals. Data are shown in Figure 3.4.

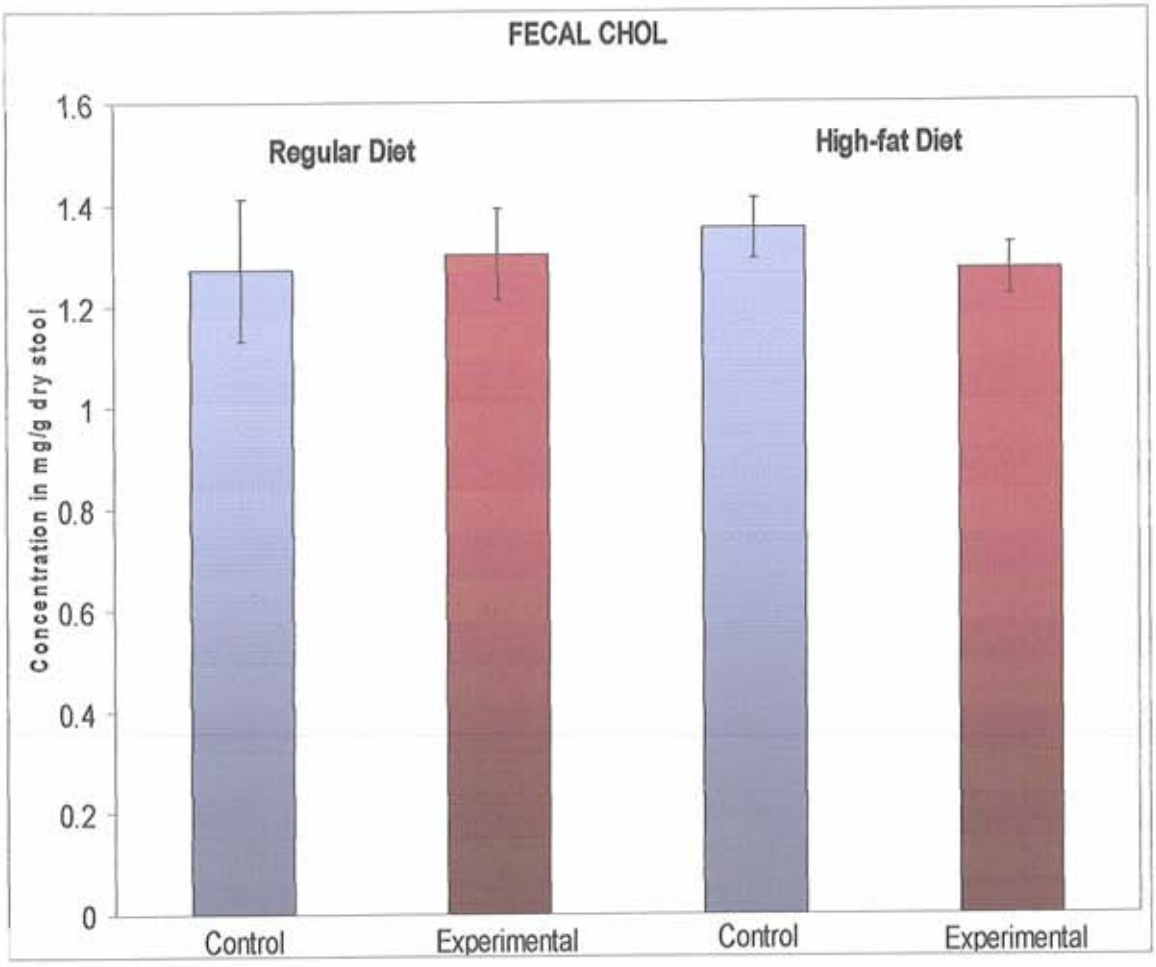

Figure 3.4 Rat Fecal Cholesterol content (mg/g) dry stool, six weeks after Carob treatment. Bars denote mean ± SEM (n=8)

3.1.5.3 Stool Water content

Determination of the stool water content revealed that the carob treated groups showed a significant increase ($p<0.05$) in the stool water content with respect to the control group in the presence of either a regular or a high-fat diet. Data are presented in Figure 3.5.

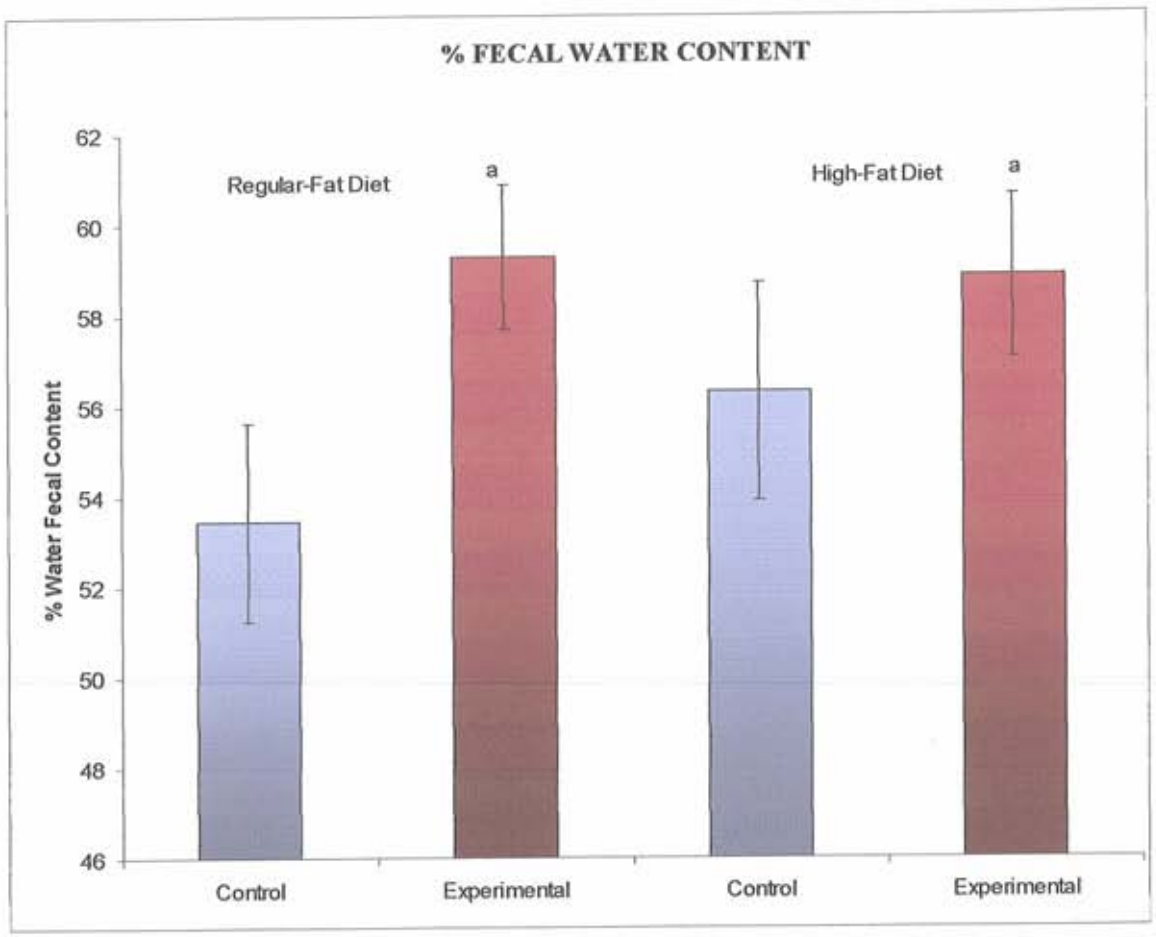

Figure 3.5 Rat Fecal Water content six weeks after Carob treatment. Bar denote mean ± SEM (n=8)

[a] Significant difference ($p<0.05$) between the experimental group and the control group.

3.1.6 Liver Fat content

Determination of the percentage fat content of the liver showed no significant changes among the different groups. Data are presented in Figure 3.6.

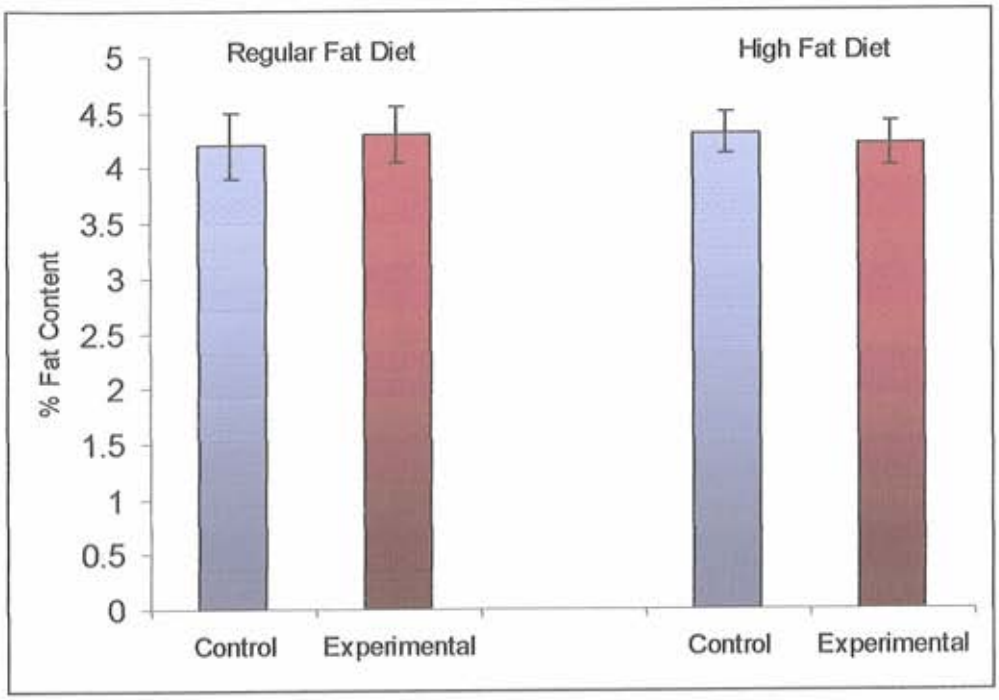

Figure 3.6 Percentage fat content in the liver six weeks after Carob treatment. Bar denote mean ± SEM (n=8)

3.2 Postprandial Effect of Carob Molasses on Blood lipid profile in Rats

Fasted rats (18 h) received an intragastric lipid load and 3 h later the concentrations of the following were measured: plasma TAG, and cholesterol; chylomicron TAG and cholesterol, and VLDL TAG and cholesterol.

3.2.1 Plasma Lipid Profile

Table 3.3 summarizes the data of the plasma lipid profile of both the control and experimental groups.

3.2.1.1 PlasmaTAG

The presence of the carob along with the lipid load resulted in a significant decrease in the concentration of plasma TAG ($p<0.05$) postprandially.

3.2.1.2 Plasma Cholesterol

The postprandial plasma concentrations of cholesterol of the different groups were not affected by the presence of carob along with the lipid load.

3.2.1.3 VLDL TAG and Cholesterol

The postprandial VLDL TAG and cholesterol concentrations of the different groups were not affected by the presence of carob along with the lipid load.

3.2.1.4 Chylomicron TAG and cholesterol

The presence of carob along with the lipid load resulted in a significant decrease in the concentration of chylomicron TAG and cholesterol ($p<0.05$) postprandially.

		Control	Experimental
Serum	TAG	153.1 ± 10.5	117.8* ± 12.4
	Cholesterol	102.1 ± 11.0	96.0 ± 15.1
VLDL	TAG	53.4 ± 5.0	50.3 ± 11.3
	Cholesterol	20.5 ± 2.8	19.6 ± 1.4
Chylomicron	TAG	94.4 ± 26.2	33.0* ± 6.2
	Cholesterol	3.7 ± 0.3	2.9* ± 0.25

Table 3.3: TAG (mg/dL), Cholesterol (mg/dL), VLDL TAG(mg/dL), VLDL Cholesterol (mg/dL), Chylomicron TAG(mg/dL), Chylomicron cholesterol (mg/dL) in rats orally fed a mixture of oil(control) vs a mixture of oil and carob molasses; values denote mean ± SEM (n=8)

* Significant difference ($p<0.05$) between the experimental group and the control group.

Chapter 4

DISCUSSION

The present study focuses on assessing the effects of a six weeks period of consumption of carob molasses on blood lipid profile, glycemia and liver enzymes. Fecal Cholesterol, TAG, and water content were determined in order to assess possible secondary effects of the carob molasses on the digestive tract. This study was conducted in the rat model and took into consideration two major types of dietary life-styles; a regular or high-fat diet.

In the present study, the long term intake of carob molasses appeared to have no significant effect on the fasting blood lipid profile except for high density lipoprotein (HDL). Serum HDL cholesterol levels were significantly increased in the carob treated rats when either a regular or a high-fat diet was administered to the animals. The increase in HDL cholesterol, also known as the good cholesterol, indicates a cardioprotective role of carob molasses when taken consistently for about a month time. Luckily, the increase in HDL cholesterol was not accompanied with an increase in LDL cholesterol. The LDL/HDL ratios were lower in the carob treated groups if compared with the control groups regardless of the amount of fat in the diet. This further reinforces the benefit of carob molasses as a dietary supplement. Although carob molasses intake appeared somehow to have a positive role in reducing fasting serum TAG only when the diet is high in fat, such an effect is limited and did not reach significance.

In the present study, carob molasses intake did not appear to affect the total plasma ApoB concentration. Hence, such a finding indicates that carob molasses has no effect on the metabolism of VLDL to LDL and the clearance of the latter molecules from the blood. Also, this reinforces our finding regarding the lack of

effect on LDL cholesterol. Previous studies have shown that carob pulp has decreased significantly TAG, total cholesterol, and LDL cholesterol levels (Zunft 2003). The differences in results, if compared with the present study may be attributed to the differences in the constituent of carob molasses and carob pulp. Up to our knowledge no studies have investigated the effect of carob molasses on serum HDL cholesterol levels. If we extrapolate the amount of carob molasses taken by the rats to human, the dose would be equivalent to 70g /70 kg body weight. Such a dose is feasible and can be accommodated as part of the daily food intake.

Results have shown no effect of carob molasses on fecal cholesterol in the presence of a regular or a high-fat diet. This indicates that carob molasses intake does not interfere with the absorption of cholesterol in the digestive system. However, carob molasses has increased significantly fecal TAG content when the animals were exposed to a regular fat diet but failed to show this effect when the fat content in the diet was elevated. Therefore, carob molasses seems to have a limited intervention with the absorption of TAG in the gastrointestinal tract, and such an intervention is only effective when the diet is not high in fat.

There are contradictory results on the effect of carob on fecal water content. Leob et al. (1989) and Brown (1996) mentioned that carob tannins have an astringent effect in the gastrointestinal tract making them useful for treating diarrhea. Similar findings were reported when carob powder was administered to children with diarrhea (Batlle and Tous 1997). However, Hostettler et al. (1995) showed that carob intake did not help adults with traveler's diarrhea. The present study did not investigate a pathological condition such as diarrhea, but it covered only normal physiological condition. Data of the present investigation revealed that carob molasses help in increasing the water content in the stools regardless of the fat content in the diet. Therefore, taking carob molasses may help in relieving the mild physiological constipation that many people in the community have. Also, it appeared that animals put on a high fat diet showed relatively more

water content in their stools with respect to animals on a regular fat diet. However, increasing water in the stool of normal animal does not allow us to extend our finding and conclude about pathological conditions such as diarrhea since the body may respond differently to herbal medicine under normal and abnormal physiological conditions.

Liver enzymes such serum GOT, GPT, and LDH are used to detect hepato-cellular damage. The activities of these enzymes were assessed in order to determine the liver function and investigate possible hepatotoxic effect that may be exerted by the long term intake of carob molasses along with the diet. Data revealed that carob molasses had no effect on the activity of the liver enzymes (GOT, GPT, and LDH) tested when either a regular or a high-fat diets is lead by the animals. Although no previous studies have dealt with such an effect to compare with, it appeared that carob molasses are safe to consume at the dose used and no hepato-cellular damage is observed. On the other hand, determination of the percentage fat content in the liver further confirms the safety of carob molasses intake.

In addition to the fasting blood lipid profile, the present study investigated the fasting glycemic profile in order to observe possible side effects that may appear following the long term intake of carob molasses, a sugar rich food. According to the results obtained the serum glucose concentration in both experimental groups of regular and high fat diets has slightly increased, but it was far from reaching significance. Determination of serum insulin concentration showed similar fasting serum insulin level in the different groups regardless of the fat content in the diet. Therefore, in spite of the high sugar content of carob molasses the glycemic profile was not badly affected in the normal rat tested. It is worth investigating in the future the glycemic profile when diabetic animals are used.

The postprandial lipid profile when carob molasses is taken along with a lipid load was also investigated in the present study. Data revealed that the presence of carob molasses reduced significantly the postprandial serum TAG concentration. The decrease in serum TAG is attributed to a decrease in chylomicron TAG rather than VLDL TAG. Chylomicrons are the initial form of lipoprotein generated from exogenous dietary lipids (Groff and Gropper, 2000). Consequently, carob molasses seems to affect either the rate at which TAG is absorbed by the intestinal enterocytes or it may be helping in speeding up the lipoprotein lipase activity and consequently the clearance of TAG from the blood. Further, studies are needed to find out which mechanism is being involved. Whatever the mechanism is, the intake of carob molasses along with a lipid load appears to help avoiding tremendous increase in serum TAG postprandially. Also, carob molasses intake appeared to reduce significantly chylomicron cholesterol. Such a decrease in chylomicron cholesterol resulted only in a mild decrease in serum cholesterol. This is attributed to the fact that chylomicrons carry only a limited amount of cholesterol in the blood unlike LDL, HDL and VLDL.

Although carob molasses has high sugar content, the glycemic profile was not badly affected in the normal rats. It is worth investigating in the future the glycemic profile when diabetic animals are used. Further studies are also needed to find out the mechanism behind reduced postprandrial chylomicron TAG and this can be done either by assessing the effect of carob molasses on rate of gastric emptying or by assessing the effect of carob molasses on lipoprotein lipase activity.

In conclusion, a six week period of consumption of carob molasses in rats subjected to a regular or high-fat diet appeared to have a cardioprotective effect since a significant increase in fasting HDL-Cholesterol was observed. HDL-cholesterol nowadays is considered a very important risk factor in assessing cardiovascular problems since it plays a profound role in the protection against

atherosclerosis and thus cardiovascular disease. Also, carob molasses helps in reducing postprandial triglyceridemia that occurs when a fat load is ingested and help in reducing the effectiveness of TAG absorption by the gastrointestinal tract. In addition, carob molasses appeared to have a laxative effect and can be used as a remedy against mild physiological constipations. No secondary harmful effects were observed in the present study. Finally, it can be interpreted that carob molasses has a positive effect on atherosclerosis, it does not cause hepatotoxicity, it has a laxative effect, and can be taken on a regular basis as a dietary supplement or with a meal to reduce high postprandial triglyceridemia.

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