The Risk of Chromium Picolinate Supplementation in Normolipidemic Rats Fed Regular or High Fat Diet.

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ABSTRACT

The Risk of Chromium Picolinate Supplementation in Normolipidemic Rats Fed Regular or High Fat Diet.

By Helena Bou Farah

Chromium, an essential nutrient involved in carbohydrates and lipid metabolism, has been marketed as a dietary nutritional supplement in the form of chromium picolinate (Crp). The objective of this study is to investigate the effect of Crp supplementation on blood lipid profile in addition to other parameters in normolipidemic rats fed with either a regular or a high fat diet. Crp was administered to rats in drinking water as a low (0.285µg/100g body weight/day), in equivalence to 200µg/70kg in human, or high dose (1.43µg/100g body weight/day), in equivalence to 1000µg/70kg in human, for a period of 2 months. Control groups, however, received similar diets but with plain water. Assesment of liver enzyme activities revealed that, with respect to the control group, Crp supplementation increased SGOT activity in rats fed with either diet. It also increased ALP and LDH activities in the high fat diet group. SGPT, however, exhibited a significant decrease only with the regular fat diet. Serum insulin concentrations were not affected by Crp supplementation in all groups fed the regular fat diet. However it increased in a dose dependent manner in rats fed the high fat diet with significance reached with the high dose of Crp. Serum glucose concentrations and glucose tolerance tests were not affected by both Crp doses used regardless of the fat content in the diet. In the presence of a regular fat diet, Crp supplementation did not affect serum concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, Triacylglycerol (TAG),
very low density lipoproteins (VLDL) TAG, VLDL cholesterol, VLDL phospholipid, and VLDL apolipoprotein B (apo B). However, it increased significantly low density lipoprotein (LDL) cholesterol, LDL TAG, LDL apo B and serum total apo B with the high dose of Crp. In the presence of a high fat diet, both Crp supplementation doses increased significantly serum concentrations of total apo B, total cholesterol and TAG, but not HDL-cholesterol. No Crp-induced changes were observed in serum iron concentration, liver fat content and stool concentrations of cholesterol and TAG in all groups regardless of the fat content in the diet.

In conclusion, Crp supplementation of normolipidemic rats with the recommended dose in concomitance with a regular fat diet, may not be harmful but also not beneficial. However, high doses of Crp are not recommended because of delayed clearance and metabolism of lipoprotein particles. In the presence of a high fat diet, Crp supplementation appeared to have a negative impact on blood lipid profile, liver enzymes and hyperinsulinemia regardless of the Crp dose used. Further similar studies on human subjects may be necessary to confirm the observed effects on rats because of possible species differences.
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LDH: Lactate Dehydrogenase
LRP: Low-density lipoprotein receptor-related protein
LMWCr: Low molecular weight Chromium
MG: Monoacylglycerol
NaN₃: Sodium azide
NIDDM: Non Insulin-dependent diabetes mellitus
NIOSH: National institute of occupational safety and health
LDL: Low-density lipoprotein
LPL: Lipoprotein lipase
PMSF: Phenylmethylsulfonylfluoride
POD: Phenol oxidase
VLDL: Very low-density lipoprotein
RDA: Recommended dietary allowance
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: Standard error from the mean
Sf: Svedberg flotation rate
T3: Tri-iodothyronine
TAG: Triacylglycerol
TEMED: Tetramethylethylenediamine
SGPT: serum glutamic oxaloacetic transaminase
SGOT: serum glutamic pyruvic transaminase
Scientists for many years have recognized that very small concentrations of certain elements, such as chromium and zinc are essential for the metabolic processes involved in life. These elements were defined as "trace elements" that constitute less than 0.01 percent of an organism (Uderwood, E.J., 1971). At least fourteen different trace elements have been identified as probably essential to human metabolism or health, all but four are metals. The list includes chromium(Cr), cobalt, copper, fluoride, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc (Olwin et al., 1992).

Chromium has been marketed as Chromium Picolinate(CrP), a dietary nutritional supplement in capsules and tablets allowing the mineral to be used as an ingredient in food, beverages and nutrition bars. Chromium picolinate has good bioavailability, absorbed into the cell quite well, and more stable than other forms of chromium supplements (chromium chloride, chromium nicotinic acid). The three picolinate molecules to which chromium is bound, act as chelators, which help the chromium to be absorbed into the cells (La Bell, 2002).

CrP, an organic and low-toxic form of Cr, is used as dietary supplement to stimulate insulin activity (Evans, 1989; Walker, 1993), decrease blood glucose, cholesterol, lipid concentrations, and body fat accumulation in mammals(Evans, 1989). CrP is also believed to stimulate muscular development and increase athletic performance, and reduce depression (Austin et al., 2001).
Chromium is known as Glucose Tolerance Factor (GTF) since the trivalent form of chromium increases glucose tolerance in rats and works as a cofactor with insulin at the cellular level forming a complex at pancreatic membrane sites. (Schawrts and Mertz, 1983). These same investigators have also demonstrated a significant improvement of glucose tolerance in humans with Cr deficiency during oral supplementation with trivalent inorganic Cr. Glucose intolerant subjects are at high risk for type II diabetes and subsequent cardiovascular diseases. Subjects who do not show presence of insulin such as those with juvenile or type I diabetes do not benefit from Exogenous Cr (Mertz, 1975).

Experiments in rats show that Cr deficiency is associated with higher prevalence of aortic plaques, whereas a life time administration of this metal in trace amounts prevents the formation of atheromatous lesions, decreases serum cholesterol and triacylglycerol (TAG) levels, increases high-density lipoprotein (HDL) cholesterol (the protective portion of total cholesterol) and prolongs the life span of these animals by preventing coronary artery diseases (Olwin et al., 1994).

One of the problems in studying Cr in human body is scarcity. Parts per million is too little to assess and scientists did not have, until recently, sensitive equipments to accurately determine Cr level (Quilllin, 2003). Researchers have also found large errors when trying to repeat their experiments, due to Chromium's volatility and its potential to assume disguises in other refractory forms (Quilllin, 2003).

Several studies dealing with the effect of chromium supplementation on improving insulin sensitivity and lipid profiles in humans have had contradictory results, many have shown a positive effect on lipid parameters (Riales, et al., 1981, Lee and Reasner, 1994), but others have shown no effect on serum lipids (Anderson et al., 1983, Rabinowitz et al., 1983). Most of these studies evaluated the effect of Cr supplementation on a limited number of subjects, or on heterogeneous populations as regards to age, cigarette smoking, hypertension,
diabetes and other confounding factors such as antihypertensive medications which induce insulin resistance (Wilson and Gondy, 1995). Further complication to this issue is the difficulty of estimating Cr stores and the lack of standards for assessment of Cr tissue levels prior to supplementation, for this reason the response to chromium cannot be predicted under ordinary circumstances (Lee & Reasner, 1994).

Cr is accepted as an essential nutrient, not as a drug, its apparent effect like that of any essential nutrient, depends on the nutritional status of the test subjects. It improves an impaired function or restores it to normal, if that impairment developed because of Cr deficiency (Liu, & Morris, 1978).

1.2 PURPOSE OF THE PROJECT

The present investigation was undertaken in the aim of:

1- Studying the impact of chronic supplementation of CrP upon alterations of blood lipid and lipoprotein profile in fasted rats fed either a high or regular fat diet by determining:
   a- Plasma total cholesterol, HDL- Cholesterol, TAG and glucose concentration.
   b- Concentrations of TAG, cholesterol, phospholipids and apo B content in lipoprotein fractions: Very low density lipoprotein VLDL (Sf20-400) and Low density lipoprotein LDL(Sf0-20).

2- Insulin concentration.

3- Serum level of Liver enzymes: Alkaline phosphatase, GOT, GPT and LDH.
1.3 Chromium Picolinate

1.3.1 Introduction

Chromium Picolinate (CrP) is chromium III trispicolinate, the chromium salt of three picolinic acid molecules. The chemical formula of CrP is $C_{18}H_{12}CrN_3O_6$ and the formula weight is 418. It is present in human tissues only in trace amounts (Vincent, 2000)a.

![Chemical structure of Chromium Picolinate](image)

The chemical formula of chromium picolinate is $C_{18}H_{12}CrN_3O_6$

Formula weight is 418

Figure 1.1: Chromium Picolinate chemical formula, (Seal, 1999).

Chromium (Cr) is a metallic element, atomic number 24, atomic weight is $\sim$ 52. Chromium has several oxidative states $3^+$, $4^+$, and $6^+$. Chromium III ($Cr^{3+}$) is the oxidation state in CrP. The oxidation state determines toxicity, metabolism and excretion (Katz & Salem, 1993).
The normal range of plasma chromium values is 0.1 - 2.1 μg/ml (Cerulli et al., 1998). The content in human liver has been reported at 5.4-470 ng/g wet weight liver (0.1 - 9 μM) (Versieck, 1985).

**Picolinic acid** a minor metabolite in the tryptophan pathway, is present in human tissues in trace amount (Rebello et al., 1982; Vincent, 2000a). Another name for picolnic acid is pyridine-2-carboxylic acid. It is recognized as low-molecular weight metal ion-binding ligand (Seal, 1998). Picolinic acid has been used as as nutritional supplement in humans and animals in conjunction with Cr. Chromium deficiency has been associated with parity, gestational diabetes, diabetes mellitus, and coronary artery disease (Wilson & Gondy, 1995). Obvious problems with low Cr also include fatigue, hypoglycemia, opacity of the cornea and congestion of blood vessels in the iris (problems very possible in diabetes), elevated levels of cholesterol, and atherosclerosis (Quillin, 2003). Because of these findings, Cr supplementation has been promoted as a health aid to the general population.

The main source of chromium is brewer’s yeast, other excellent sources include black pepper, wheat germ, potato, liver, beef, chicken, cheese, mushrooms, bananas, peas (Quillin, 2003). Cereals, refined carbohydrates and products made of white flour and white sugar are poor sources of chromium, their chromium decreases with refining and processing (Masironi et al., 1973). The widespread tendency toward increased consumption of highly processed food such as refined sugar which is not only low in Cr but also stimulates urinary Cr losses may result in a marginal intake of Cr and depletion of stores (Anderson & Kozlovsky, 1985).

There is no recommended dietary allowance (RDA) for chromium. Normal Daily recommended intakes for chromium are generally defined as shown in table 1.1 (Nisbett, 2002)
Table 1.1 normal daily recommended Cr intake (Nisbett, 2002).

<table>
<thead>
<tr>
<th>Age</th>
<th>ChromiumDose(µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3 years</td>
<td>10-80 µg</td>
</tr>
<tr>
<td>4-6 years</td>
<td>30-120 µg</td>
</tr>
<tr>
<td>7-10 years</td>
<td>50-200 µg</td>
</tr>
<tr>
<td>Adolescents and adults</td>
<td>50-200 µg</td>
</tr>
<tr>
<td>Optimum daily allowance for adults</td>
<td>150-400 µg</td>
</tr>
<tr>
<td>Tolerable upper intake levels</td>
<td>not defined</td>
</tr>
</tbody>
</table>

All clinical studies did not reveal any acute or sub-acute adverse effects or abnormalities with short term ingestion of CrP typically less than 3 month with a dose of less than 300 µg per day (Wasser et al., 1997). However, some clinical case reported acute nausea and vomiting, skin disorders and allergies, hypertension, renal failure (red urine and stool), liver failure, anemia, neuropsychiatric effects/ sleep disorders, coma at doses of 1200-2400 µg/day for more than 5 months (Cerulli et al., 1998).

1.3.2 Mode of action of Chromium

Shwarz and Mertz (1959), demonstrated the existence of a new dietary factor, which was absent in the diet of rats fed on Torula yeast as their sole protein source. Rats consuming the diet have developed an inability to remove glucose efficiently from the bloodstream, which was reversed by adding food rich in Cr, or by adding synthetic inorganic trivalent Cr complexes to the diet. A unique chromium-binding oligopeptide named low molecular weight chromium-binding substance (LMWCr) or chromodulin has been isolated and characterized
Chromium has been shown to positively correlate with potassium, fat, saturated fat, sodium, oleic acid, phosphorous, Vitamin B6, copper, protein and total carbohydrates; however, intake of other trace elements like iron and zinc did not correlate significantly with Cr intake (Anderson and Kozlovsy, 1985).

The absorption of Cr has been shown to be inversely proportional to Cr intake, although at any intake level the body absorbs dietary Cr poorly ~ 0.5-2% (Anderson & Kozlovsy, 1985). However the mechanism of Cr absorption and transport are still uncertain. In vivo administration of chromic ions to mammals orally or by injection results in the appearance of chromic ions in the iron-transport protein-transferrin (80,000-Da), a serum protein which tightly binds two equivalents of ferric iron at neutral and slightly basic PH levels (Brock, 1985).

Plasma membrane recycling of transferrin receptors is sensitive to insulin; increases in insulin results in a stimulation of the movement of transferrin receptor from vesicles to the plasma membrane (Kandror, 1999). The receptors at the cell surface can bind metal-saturated transferrin, which subsequently undergoes endocytosis with accompanying metal release at the acidic PH of the newly formed vesicles. Thus increases in insulin levels should result in increased transport of transferrin, including the portion containing bound Cr, culminating in Cr transport from the blood to insulin-sensitive cells and ultimately chromodulin (Morris et al., 1999).

In adult-onset diabetes, in which blood Cr levels are reduced and urinary Cr losses are increased (Morris et al., 1999), this transport system may be exceeding normal operation. This maybe related to patients with unexplained hepatic iron overload (characterized by a nearly constant association with diabetes) whose transferrin-bound iron levels are greatly increased (Mendler et al., 1999).

However one caution note should be addressed, the most popular form of Cr in dietary supplements, chromium picolinate, appears to be absorbed in a different
fashion from dietary Cr. Chromium tripicolinate, remarkably stable, remains intact for several hours in synthetic gastric juice (Gammelgaard et al., 1999), and days to weeks under physiologically relevant conditions (Speetjens et al., 1999). The complex also appears to pass unhindered through the jejunum and probably migrates to and is incorporated into cells in its original form (Gammelgaard et al., 1999).

Unfortunately the picolinate ligands shift the redox potential of the chromic center of the complex such that it can be reduced by biological reducing agents such as ascorbate and thiols (Speetjens et al., 1999). The resulting chromous complex can interact with oxygen catalytically, generating the hydroxyl radical (Sun et al., 2000).

1.3.4 Chromium action on insulin sensitivity, glucose tolerance and body fat

Although impaired glucose tolerance is a consequence of Cr deficiency, it has many other causes. Therefore Glucose tolerance can be improved by Cr supplementation or can be maintained inspite of a reduced insulin output only in malnourished children (Wilson & Gondy, 1995) in patients with Cr deficiency associated with total parenteral nutrition (Brown, et al., 1986, Anderson et al., 1983), and in middle age individuals with mild glucose intolerance (Anderson et al., 1983 and 1991). But those with normal glucose tolerance are not improved further (Mertz, 1993). Cr can stimulate the biological activity of insulin by increasing the insulin-sensitive cell receptors or by binding activity (Merts et al., 1974; Anderson et al., 1991).

An 8 months period of Cr supplementation (1000µg) to subjects at high risk for the development of type 2 diabetes showed no effect on body weight, abdominal fat distribution, and body mass index. However it improved insulin sensitivity in these obese subjects, suggesting that Cr may alter insulin sensitivity independent of a change in weight or body fat percentage, thereby implying a direct effect on muscle insulin action (Cefalu et al. 1999). Similar studies have shown that CrP
supplementation exhibit a positive change in body composition with (Hasten et al., 1992) and without statistical significance (Hallmark et al., 1993), while other studies failed to show any positive change (Clancey S. et al., 1994). The discrepancies may be attributed to differences in designs of the different studies.

1.3.5 Effect of Cr on atherosclerosis and blood lipid profile

Chromium deficiency has been associated with atherosclerosis. Decreased Cr concentration has been demonstrated in aortic tissue and sera of patients dying with coronary artery disease as compared with normal patients. Reversal of atherosclerosis in the aorta of cholesterol-fed rabbits by daily Cr injection has also been reported (Wilson & Gondy, 1995). These observations suggest that Cr supplementation may retard the progression of atherosclerosis in certain populations, but do not assert that it will improve atherosclerosis risk in healthy, young non-obese individuals by an improvement in serum lipids or glucose tolerance (Wilson & Gondy, 1995).

In a study done by Wilson & Gondy (1995) on 26 young adults, there were no statistically significant differences in the percentage change of fasting glucose, immunoreactive insulin IRI \( p<0.03 \), or lipids: LDL, HDL, TG, Chol: HDL \( P<0.05 \), between the Cr \( n=15 \) and the placebo \( n=11 \) groups after 90 days of supplementation with 220\( \mu \)g of Cr. However another study done by Lien and his colleagues (1999) on boilers, has shown that dietary supplements of 1600 and 3200\( \mu \)g/kg of Cr, increased significantly food consumption, body weight and lipid liver content \( p<0.05 \) and decreased abdominal fat content in these 2 groups. This supplementation has also decreased serum glucose, increased serum phospholipids content \( p<0.05 \), enhanced serum TG clearance rate, and serum HDL while reducing serum VLDL and LDL contents. Decreased insulin concentration was shown only in the group receiving 3200\( \mu \)g of Cr. The difference between boilers and mammals in terms of effectiveness of Cr
supplement might be due to the pancreatic β cells in poultry not being as active as those in mammals (Lien et al., 1999).

Lee & Reasner (1994) studied the effect of Cr supplementation on non-insulin dependent diabetic Hispanic population (NIDDM). All the patients were almost certainly Cr deficient having plasma chromium levels undetectable <0.2μg/l. The 28 patients were supplied with 200μg/day of CrPic for 2 months. It was the first study to show a significant reduction in TAG levels by 17.4% when compared to placebo. No differences were noted in LDL and HDL cholesterol levels (Lee & Reasner, 1994). This percentage reduction in TAG level in NIDDM patients should be associated with a reduction in their risk of atherosclerosis.

In the study performed by Press and his colleagues (1990) on 28 volunteer subjects who received 200μg/day of Cr tripicolinate for 42 days, the levels of total cholesterol, LDL, and ApoB the principal protein of the LDL fraction, decreased significantly, a reduction of 16%. The concentration of ApoA the principal protein of the HDL fraction was also significantly increased p=0.03 while the subjects were ingesting Cr. However HDL levels were increased but not significantly during Cr ingestion (Press et al., 1990). These observations show that Crp is efficacious in lowering blood lipids in humans.

An Experiment done by Besong and his colleagues on Steers fed with a high fat diet (5.6% of dry matter) and 0.8mg/kg of CrPic for 9 weeks showed no significant effect of Cr on all TAG, HDL and LDL levels and glucose concentrations, however total cholesterol increased during the experiment for the control group due to supplementation with fat and not altered in the CrP group (Besong et al., 2001).
1.3.6 The Toxicology of Chromium

Resolution of the paradox that Cr is both a chemical carcinogen and an essential nutrient lies in the chemical speciation of the Cr. The essentiality of Cr resides in its trivalent form (Cr\(^{3+}\)) which is associated with the GTF. The carcinogenicity of Cr, on the other hand, appears to reside with some of the hexavalent Cr (Cr\(^{6+}\)) compounds of limited solubility (Katz & Salem, 1993). The soluble hexavalent Cr compounds are rapidly taken via the sulfate transport systems of the cells and subsequently reduced to Cr\(^{3+}\). Hexavalent Cr compounds are generally 10-100 times more toxic than the trivalent Cr. The National Institute of Occupational Safety and Health (NIOSH) Registry data on the acute oral toxicity of trivalent Cr compounds range from 1900-3300mg/kg body weight, in comparison a hexavalent Cr toxicity is reported to be 50-150mg/kg body weight. (registry of Toxic Effect of Substances, 1986). In addition humans cannot oxidize the nontoxic trivalent dietary Cr to its hexavalent form, therefore the toxicity of dietary Cr is virtually nonexistent (Finney et al. 1997).

The initial signs of ingesting hexavalent Cr compounds by humans are abdominal pain, vomiting, diarrhea and intestinal bleeding, followed by renal failure resulting from tubular necrosis (Michie et al., 1991), hepatic failure secondary to primary hepatocellular damage, encephalopathy, met-haemoglobinemia and hemolysis are frequent complications.

Some evidence have shown that hexavalent Cr can inhibit benzpyrene hydroxylase activity, such compounds capable of such inhibition are carcinogenic (Chretien et al., 1974).

Trivalent Chromium (Cr\(^{3+}\)) crosses the cell membrane slowly and is therefore unlikely to reach the nucleus of intact cells. Hexavalent Cr crosses the cell membrane readily by active transport; once inside the cell, it is partially reduced to the trivalent state which can form complexes with DNA, and increase the non-complementary nucleotide incorporation into DNA producing genotoxic effects (Rafetto et al., 1977). Exposure of cells from rat liver and kidney to hexavalent Cr...
lead to increased cross-linking in DNA (U.S. Environmental protection agency, 1985). Ames test has been positively reported for hexavalent but not for trivalent Chromium (Petrilli & De Flora, 1978; Gentile et al., 1981). The literature on the toxicology of Cr is incomplete in some areas and inconsistent in others. However it does appear that trivalent Cr is less toxic than hexavalent Cr compounds and speciation is a prerequisite to risk assessment (Katz & salem, 1993).

1.3.7 Liver Blood Enzymes
An initial step in detecting liver damage is a simple blood test to determine the presence of certain liver enzymes in blood. These enzymes are produced by other tissues in the body. Under normal circumstances, they reside within the cells of the liver but when the liver is injured or damaged which alters the permeability of the cell membranes, these enzymes are spilled into the blood stream raising the enzyme level in blood (Braunwald et al, 2001). Among the most sensitive and widely used of these liver enzymes are the aminotransferases which catalyze chemical reactions in the cells in which an amino group is transferred from a donor molecule to a recipient molecule. Some of the commonly used enzymes in assessing liver function are:
SGOT: (serum glutamic oxaloacetic transaminase), also known as AST (aspartate aminotransferase).
SGPT: (serum glutamic pyruvic transaminase), also known as ALT (Alanine aminotransferase).
GGTP or GGT (gamma-glutamyl transferase or transpeptidase).

SGOT is normally found in a diversity of tissues including liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged for example, its serum level rises with heart attacks. It is therefore not highly indicator of liver damage. The normal range in human is from 5-40 units/liter of serum (Braunwald et al., 2001).
SGPT is normally found in the liver, and therefore serves as a specific indicator of liver status. The normal range in human is from 7-56 units/liter of serum. GGPT is normally produced by the liver, pancreas heart and brain, and is a very sensitive enzyme for early detection of liver disease or damage. Many different impairments may cause an elevation such as: hepatitis, cirrhosis, fatty liver, carcinoma, alcohol use, some medications such as barbiturates, tranquilizers, dilantin, digoxin, and cholesterol lowering drugs e.g. Mevacor, and a variety of infiltrative diseases (Braunwald et al, 2001).

In this study, detection of liver enzymes concentration in blood was used as a method to detect if the chronic treatment with chromium picolinate caused any impairment in liver function or liver cell damage.

1.4 Intestinal Lipids

1.4.1 Intestinal Lipid Digestion and Absorption

Dietary lipids circulating in the blood include: TAG, phospholipids, cholesterol and cholesterol esters. Following the ingestion of lipids, bile salts, synthesized in the liver and stored in the gallbladder, are released into the small intestine to solubilize or emulsify the dietary lipids, mostly constituted of TAG. The emulsification of dietary lipids renders them accessible to pancreatic lipases as indicated by the following reactions:

\[
\begin{align*}
\text{Triacylglycerol} & \xrightarrow{\text{Pancreatic lipase}} 2 \text{fatty acids} + 2 \text{-monoacylglycerols} \\
\text{Phospholipids} & \xrightarrow{\text{Pancreatic phospholipase A2}} \text{FFA} + \text{Lysophospholipid}
\end{align*}
\]

The short chain fatty acids, having fewer than 10-12 carbons, are directly absorbed into the epithelial cells of villi by simple diffusion, on the other hand, 2-
monoacylglycerol, long chain fatty acids (12 or more carbons), cholesterol and
lysophospholipids require bile for their adequate absorption (Tortora and Grabowski, 1993).

1.4.2 The Function of serum Lipids

Lipids serve important physiologic functions within the human body. Cholesterol provides an essential stabilizing function for cell membrane and facilitates membrane transport. It is required for the synthesis of bile acids and serves as a precursor for the biosynthesis of adrenal and sex hormones (Bondy & Kingsbury, 2003). TAG, light in weight relative to their energy content, are stored in the adipose cells and offer an ideal means for energy storage and production. Cardiac and skeletal muscle cells extract TAG from circulating lipoproteins and convert it to fatty acids and glycerol through lipolysis. Fatty acids are a major energy source for the muscle cells. In a number of cells, such as muscle and liver cells, fatty acids can also be converted into glucose through gluconeogenesis (Bondy & Kingsbury, 2003).

1.5 Structure and Classification of lipoproteins and Apolipoproteins.

Lipids are insoluble in water, therefore they are transported in the plasma as large complex particles called Lipoproteins (Miles, 2003). These lipoproteins consist of a core of hydrophobic lipids containing cholesterol esters and TAG surrounded by a shell of phosphatidyl glycerols, free cholesterol and apoproteins. These apoproteins have an important role in lipid transport and metabolism since they have specific structural domains that are recognized by cell receptors (Dominiczak, 1997). The affinities of apoproteins for the surface components of the lipoprotein change during lipoprotein metabolism. Apoproteins often diffuse from one lipoprotein to bind to another. Only apoprotein B maintains its association with the lipoprotein during the cycle of lipoprotein metabolism (Miles, 2003).
The Lipoproteins are classified according to their densities. The lowest density lipoproteins are chylomicrons followed by the chylomicron remnants, very low density lipoproteins VLDLs, intermediate density lipoproteins IDLs, Low density lipoproteins LDLs, and high density lipoproteins HDLs. The density of these lipoproteins is related to the relative amounts of lipids to proteins in their complexes. The higher the relative protein content the higher the density of the lipoprotein (Miles, 2003).

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>protein %</th>
<th>Lipids %</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Cholesterol</td>
<td>Phospholipid</td>
<td>TAG</td>
<td></td>
</tr>
<tr>
<td>Chylomicron</td>
<td>1</td>
<td>99</td>
<td>4</td>
<td>7</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>8</td>
<td>92</td>
<td>22</td>
<td>16</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>21</td>
<td>79</td>
<td>46</td>
<td>22</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Lp(a)</td>
<td>36</td>
<td>64</td>
<td>37</td>
<td>18</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>50</td>
<td>50</td>
<td>20</td>
<td>26</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>FFA-albumin</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

1.5.1 CHYLOMICRONS
Chylomicrons are produced in the intestinal lumen following the absorption of digested fat. They are transported in the blood to tissues such as skeletal muscle, fat and liver (Bondy & Kingsbury, 2003). Chylomicrons are the largest lipoproteins and have the lowest protein to lipid ratio, and hence the lowest density of all lipoproteins. The hydrophobic molecules are enclosed inside the chylomicrons while the phospholipids and proteins are on the surface so that the hydrophilic surfaces are in contact with water (Miles, 2003).
The principle apoproteins of nascent chylomicrons are apo B-48, apo A-I, apo A-II and apo A-IV. In circulation, the nascent chylomicrons acquire apo C and apo E from plasma HDL in exchange for phospholipids (Table 1.3 Dominoicz, 1997). The acquisition of apo-CII from HDL is essential to activate Lipoprotein lipase, LPL. Chylomicrons bind to membrane bound LPLs located on the adipose and muscle tissues where the TAG are hydrolyzed into FA (Olson, 1998). The affinities of apoproteins for the surface components of the lipoprotein change during lipoprotein metabolism. Apoproteins often diffuse from one lipoprotein to bind to another. Only apoprotein B maintains its association with the lipoprotein during the cycle of lipoprotein metabolism (Miles, 2003). The FAs are transported into adipose cell where they are once again resynthesized into TAG and stored. In the muscle, the FAs are oxidized to provide energy. As the tissues absorb the FAs, the chylomicrons progressively shrink by transferring a substantial portion of its phospholipids and apoproteins A and C to HDL, and are reduced down to cholesterol enriched remnants. The Apo C proteins are continuously recycled between chylomicrons and HDL. The remnants lacking apo A and C proteins do not bind to LPLs in the capillaries. The remnants are rapidly absorbed by the liver (Miles, 2003).

1.5.2 Very Low Density Lipoproteins.
VLDL is the main secretory lipoprotein of the liver, it contains cholesterol, phospholipids and triglyceride. The primary apoprotein of VLDL is B-100 (Olson, 1998). The liver secretes VLDLs via exocytosis. Like chylomicrons, VLDLs undergo constant changes in the plasma. First, the nascent VLDL acquires apo C and E from HDL. VLDLs bind to the same membrane bound LPLs located on the adipose and muscle tissues where the TAG is hydrolyzed into fatty acids. The fatty acids are transported into the adipose cells where they are once again resynthesized into triacylglycerols and stored. In the muscle the fatty acids are oxidized to provide energy (Schaefer, 2002). As the tissues absorb the fatty acids and monoacylglycerols, the VLDLs progressively shrink forming intermediate density lipoproteins (IDLs) by transferring a substantial portion of its
phospholipids and apoprotein C to HDL. LDLs can bind to receptors of liver cells where they are absorbed in an analogous manner to chylomicrons, or they can be further catabolized by LPLs, eventually losing apo- E to form LDLs (Miles, 2003).

1.5.3 Low Density Lipoproteins

LDL is a cholesterol rich lipoprotein, which contains almost exclusively apo B-100. LDL is the principal plasma cholesterol carrier to most tissues of the body. LDL (bad cholesterol) is positively related to coronary heart disease. High levels of LDL are associated with the formation of atherosclerotic plaques that occlude blood vessels causing heart attack and strokes (Bondy & Kingsbury, 2003). The LDLs bind to specific cell receptors located on the plasma membrane of target cells. The LDL binding domain has negatively charged residues which interact with positively charged arginine and lysine residues of apo-B 100 (Miles, 2003). Once the vesicle is inside the cell, the LDL receptors are recycled to the cell surface, the vesicles fuses with lysozymes which then degrade the lipoprotein to its primary components, fatty acids, glycerol, cholesterol and amino acids. The cholesterol is used for membrane or steroid synthesis. Approximately 75% of the LDLs are absorbed by the liver (Olson, 1998).
Low Density Lipoprotein

Figure 1.2: Low density Lipoprotein structure. (Miles, 2003).

1.5.4 High Density Lipoproteins

High density lipoproteins are secreted by the liver and intestinal cells. The nascent HDLs are disk-shaped, but they become spherical as they acquire free cholesterol from cell membranes and TAGs from other lipoproteins (Miles, 2003). The function of HDLs (good cholesterol) is to remove excess cholesterol and carry it to the liver to be metabolized into bile salts, which implies the inverse relationship between HDL and the incidence of heart diseases (Schaefer, 2002). HDL contains enzymes that either esterify cholesterol or transfer cholesteryl esters to the liver. Lechithin-cholesterol acyltransferase (LCAT) is a peripheral enzyme activated by apo A-I. It facilitates the storage and transport of excess cholesterol by catalyzing the transfer of long chain fatty acids from phospholipids to cholesterol to form cholesteryl esters (Olson, 1998).

Cholesteryl esters occupy the core of the HDL, and are exchanged between lipoproteins via a process that is mediated by cholesteryl ester transfer protein.
(CEPT) which is another peripheral enzyme circulating with HDL. CEPT promotes the net transfer of cholesteryl esters from HDL to LDL, IDL and VLDL in exchange for TAGs. This process partially help in transforming VLDLs and IDLs into LDLs. As the HDLs grow in size they acquire apo-E which increases their binding affinity towards receptors in the liver where they are absorbed and catabolized (Miles, 2003).

Table 1.3 Lipoprotein Classification and characteristics (Dominiczak, 1997).

<table>
<thead>
<tr>
<th>Lipoproteins</th>
<th>Major Lipid component</th>
<th>Major Apoproteins</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>TAG</td>
<td>ApoB-100, ApoC-I, C-II, C-III, ApoE</td>
<td>Liver</td>
</tr>
<tr>
<td>IDL</td>
<td>CE</td>
<td>ApoB-100, ApoE, ApoC</td>
<td>Catabolism Of VLDL</td>
</tr>
<tr>
<td>LDL</td>
<td>CE</td>
<td>ApoB-100</td>
<td>Catabolism Of IDL</td>
</tr>
<tr>
<td>HDL</td>
<td>CE,PL</td>
<td>ApoA-I, A-II, A-IV, ApoC-I, C-II, C-III, ApoE</td>
<td>Intestine, liver, other</td>
</tr>
</tbody>
</table>

^CE: Cholesterol ester
2.1 Animal treatment

Male Sprague-Dawley rats, *Rattus norvegicus*, 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). All animals were sacrificed using diethyl ether, at the end of the procedures described, without recovery from anaesthesia.

2.1.1 Chromium picolinate treatment

Male rats (n=30) weighing 200-250 g (Lebanese American University stock) were maintained at an ambient temperature of 20-22°C and were fed the same standard rat chow diet until 18 hours prior to experimentation where they had free access to water (control group) or water + CrP (Chromium Picolinate groups). Fasted rats were divided into three groups of 10 rats each: control, low dose CrP (0.286μg/70kg body weight), high dose Cr P (1.43μg/70kg body weight) for 8 weeks. In a second experiment, the rats were treated similarly except that the diet was enriched with olive oil (5% w/w).

2.2 Blood study

Fasted rats (18 hours) were anaesthetized using diethyl ether and placed supine on a homeothermic table. Anesthesia was maintained by the use of a nose cone. 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.
Using 10ml syringes about 9 ml of blood were withdrawn from the inferior vena cava and transferred into plain and disodium ethylene-diamine-tetraacetic acid (Na₂EDTA, 1 mg/ml) containing tubes. After 30 min centrifugation was carried at a speed of 2000g for 20 minutes at a temperature of 4°C, then plasma and serum were collected and divided into aliquots and used for lipoprotein fractions isolation (VLDL, Sf 20-400; LDL, Sf 0-20) as described in the following section, and blood lipid analysis (serum TAG, total cholesterol, HDL cholesterol and glucose). Similarly, TAG, cholesterol and phospholipid concentrations were measured for the lipoprotein fractions after density gradient ultracentrifugation of the plasma samples.

2.3 Fecal study

Procedure:
Fresh stools, collected from the rats of each of the four groups, were dehydrated in the oven (Imperial V, # 3476-1) at 60°C for 24 hours. The dried stools were then ground using a mortar and a pestle and samples weighing 0.2 g were used for TAG and cholesterol extraction. Extraction was performed as described by Daher et al. (2003). Briefly, samples were extracted for 4 hours using 3 ml of n-hexane at 50°C with constant agitation. All samples were then centrifuged at 5000 g for 20 minutes. Supernatants containing the TAG and cholesterol were isolated and dried at 80°C until complete evaporation of n-hexane. Following that, TAG and cholesterol contents were calculated using TAG and cholesterol assay kits (Spinreact, S.A, Espana).

2.4 Isolation of lipoprotein fractions

Each 2 ml of collected plasma was placed in the bottom of a 10 ml polycarbonate ultracentrifuge tube (Sorvall, Kendro, Laboratory Products) put on ice. Proteolytic
degradation was minimized by adding a preservative solution containing aprotinin, 2 mg/liter, and phenylmethylsulfonylfluoride (PMSF), 10 mM dissolved in 2-propanol, at concentrations of 5 µl/ml plasma and 10 µl/ml plasma respectively. Solid KBr (0.34 g/ml) was added to the plasma samples and vortex mixed in order to increase the density to 1.21 g/ml. A 2 ml NaCl solution (1.006 g/ml NaCl was prepared, containing 0.01% (w/v) Na₂ EDTA and 0.02% sodium azide (NaN₃) 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 gradients were then centrifuged for 24 hours at 15°C in the F-28/13 Supraspeed fixed-angle rotor (Sorvall RC 28S centrifuge) at 28000 rpm. The top 0.5 ml TAG-rich lipoprotein (VLDL) fraction and the lower layer (LDL) (V=1.5ml) were aspirated. The lipoprotein fractions collected were divided into two parts: one sample (V = 300 µl) was used for apo B48 and apo B100 analysis and the remaining volume was placed in 1.5 ml Eppendorf tubes stored at -20°C for subsequent analysis.

### 2.5 Preparation of samples for apo B48 and apo B100 analysis

Samples containing the different lipoprotein fractions were delipidated according to Karpe et al. (1996) in a methanol-diethyl ether solvent system. A volume of 300 µl of the lipoprotein fraction sample was injected into 4 ml ice cold methanol in a 10-ml glass tube with a syringe (1ml) to ensure efficient delipidation.

A volume of 4 ml of ice-cold diethyl ether was then added to the methanol and the delipidation mixture was centrifuged for 30 min at 4000g at -4°C. After removal of the solvent, another 4 ml of ice-cold diethyl ether was immediately added, and the sample was vortexed and centrifuged for 20 min under the same conditions. After centrifugation, the diethyl ether was removed and the protein material was dissolved in 50-150 µl of sample buffer at room temperature for 30
min: 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) bromomophenol blue, adjusted to pH=6.8. The dissolved protein mixture was then transferred to an Eppendorf tube, denatured at 90°C for 3 min after which it was centrifuged for 3 min at 13,000g. Samples were then frozen at -20°C for later analysis by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

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

2.6.1 Principle

Similarly to many other important biological molecules, proteins as amphoteric compounds have their net charge determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electric field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The net charge carried by a protein is in addition dependent on its size, the charge carried per unit mass of molecule differing from a protein to another. Consequently, at any given pH other than their isoelectric point, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of the molecules. Separation of proteins by SDS-PAGE electrophoresis is based on coating all proteins with SDS so that all proteins have a uniform charge. Separation is therefore dependent on the size of the protein-sodium dodecyl sulfate complex. Hence, in SDS-PAGE separations, migration is determined not by intrinsic electric charge of polypeptides but by molecular weight (Laemmli, 1970; Horton et al., 2002). In SDS PAGE, the polyacrylamide gel may be prepared so as to provide a wide variety of
electrophoretic conditions. The pore size of the gel may be varied to produce
different molecular sieving effects for separating proteins of different sizes. In this
way, the percentage of polyacrylamide can be controlled in a given gel. In
polyacrylamide gels, the effective pore size is inversely related to acrylamide
concentration in the polymerization mixture (Hames, 1990). SDS, an anionic
detergent with a long hydrophobic tail, binds to the hydrophobic side chains of
amino acids at a constant ratio of 1.4 g of SDS to 1 g of polypeptide. This confers
a net negative charge to the polypeptide in proportion to its length and to the
molecular weight of the protein (Hames, 1998). This ratio ensures that all SDS-
protein complexes have a similar mass:charge ratio; hence, eliminating the
intrinsic charge of the protein as a factor affecting migration (Rybicki and Purves,
2001). Moreover, SDS blocks hydrophobic interactions, and substantially unfolds
the protein molecules, minimizing differences in molecular form by eliminating the
tertiary and secondary structures (Lodish et al., 2000). When proteins are treated
with 2-mercaptoethanol or dithiothreitol (DTT) (in addition to SDS), all disulphide
bonds between cysteine residues are broken, producing subunits of proteins
(Hames, 1998). Urea may also be employed to disrupt hydrogen bonds.
Following SDS-PAGE separation, the protein bands are visualized directly on the
polyacrylamide gel by staining with Coomassie Blue. A concentration of 0.2-0.5
µg of any protein can be detected in a sharp band using this dye. Staining is
quantitative up to 15-20 µg for at least some proteins (Hames, 1990). Not all
proteins have the same chromogenicity with this dye, therefore, standards of the
same proteins are usually included in the same gel for appropriate quantification.

2.6.2 Stock solutions

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

0.5 M Tris buffer (pH 6.8): 6.02 g Tris was dissolved in approximately 90 ml distilled water and the pH adjusted to 6.8 with 12 M HCl. Then the solution was diluted with distilled water to a final volume of 100 ml. This solution is also stable for several weeks at 4°C.

Water-saturated n-butanol: 90 ml n-butanol + 10 ml water.

Electrode buffer (pH 8.3): 42.6 g glycine and 9.18 g Tris were dissolved in 2 L of distilled water, then 3 g of SDS were added to the Tris/Glycine solution and the final volume made up to 3 L with the addition of 1 L distilled water.

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. The solution was then diluted with distilled water to a final volume of 100 ml. This solution is stable for several weeks at 4°C.

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

2.6.3 SDS-PAGE preparation

2.6.3.1 Preparation and pouring of resolving and stacking gels

The electrophoresis apparatus (Hoefer SE 600, Amersham Pharmacia Biotech) was assembled according to the manufacturer's instructions. Clamped glass
plates were tested to ensure they are water tight. Gels were prepared according to the method of Hames (1990). A linear gradient (5-20%) polyacrylamide gels (resolving gels) were prepared as described in Table 2.1. Immediately before pouring the resolving gels, 11.5 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 (Colepermer, 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 overlayed 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 overlaying the stacking gel (Table 2.1), 11.25 µl of TEMED were added to the stacking gel. The poured stacking gel was left for about 30 min to polymerize after which the combs were carefully withdrawn. Electrode buffer was added, protein samples along with molecular weight marker and apo B standard were loaded in the wells. Molecular weight markers, (Sigma Chemical Co.Ltd) were prepared according to the supplier's instructions. 10 µl of molecular weight marker solution was loaded in first lane, then 0.2, 0.8, and 3.2 µg of standardized apo B samples were loaded in lanes 2-4. The remaining lanes were loaded with the lipoprotein fractions under examination. All samples were run in duplicate. 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 gels. Four hours later, the power supply was switched off, the water-cooling system disconnected and the gel carefully removed for staining and destaining.
Table 2.1 Gel Mixtures for 5-20% gradient gels.

<table>
<thead>
<tr>
<th></th>
<th>Resolving Gel</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% Gel</td>
<td>20% Gel</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>4.5 g</td>
<td>-</td>
</tr>
<tr>
<td>3M Tris</td>
<td>3.7 ml</td>
<td>3.7 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris Buffer</td>
<td>-</td>
<td>-</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Protogel</td>
<td>5 ml</td>
<td>20 ml</td>
<td>3.7 ml</td>
</tr>
<tr>
<td>Urea/SDS/DTT</td>
<td>3 ml</td>
<td>300 μl</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>1.5% APS</td>
<td>0.7 ml</td>
<td>0.7 ml</td>
<td>2.3 ml</td>
</tr>
<tr>
<td>Water</td>
<td>20.2 ml</td>
<td>2.7 ml</td>
<td>15 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>23 μl</td>
<td>23 μl</td>
<td>22.5 μl</td>
</tr>
</tbody>
</table>

|                      |               |               |
|                      | Stacking Gel  |               |
|                      |               |               |
|                      |               |               |
2.6.3.2 Preparation of standard apolipoprotein B100

Since rat apo B48 standards are difficult to prepare, human apo B100 was used as a standard for apo B48 quantification. Kotie et al. (1995) and Van Beek et al. (1998) have shown that apo B100 and rat apo B48 have similar chromogenicity when stained with Coomassie Blue R-250, hence validating the use of human apo B100 as a standard. Apo B100 standards prepared from human LDL (1.030<d<1.040 g/ml) isolated from fasting human plasma samples by the density gradient ultracentrifugation procedure described by Karpe et al. (1996). Briefly, apo B100 was derived from LDL isolated from fasting human plasma samples by the density gradient ultracentrifugation procedure. The LDL subfraction was recovered from a 1 ml portion located 5.5 to 6.5 ml below the top of the gradient after 16 hour of ultracentrifugation. Fast and careful desalting was achieved by passing the LDL through a PD-10 column (Pharmacia). The total protein content was then determined using the Lowry assay (1951) with addition of SDS (final concentration 1 %) to the reagent to reduce turbidity. The mean of several determinations was taken as the final protein value. After delipidation of LDL as previously described, the dissolved protein mixture was denatured at 95°C for 5 minutes. Batches of the delipidated and denatured apo B100 protein standard can be stored at -20°C and thawed immediately before use.

2.6.4 Staining and quantitation of proteins

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). To remove any insoluble material, the solution was filtered using Whatman No.1 filter paper. The protein destaining solution consisted of (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 16 hours. Then, the gel slabs were carefully removed, placed between two transparencies, labelled and scanned. The image saved was used for quantitation of apo B48 and 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 B48 and apo B100 were calculated accordingly.

2.7 Serum Assays

2.7.1 Determination of plasma TAG

Principle
The TAG assay kit (Spinreact, S.A, Spain) used was based upon a colorimetric method. The 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 H₂O₂. The H₂O₂ concentration is determined through the Trinder’s reaction. The enzymatic reaction sequence employed in the assay was as follows:

$$\text{Triacylglycerol} + \text{H}_2\text{O} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone-P} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + 4-\text{AP} + \text{p-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinonimine(red)} + \text{H}_2\text{O}$$
Procedure
Samples, along with standards, were run in duplicates, and were mixed with the working reagent and incubated 10 minutes at room temperature. The absorbances (Abs.) of the unknown and the standard samples were measured against Blank reagent at $\lambda = 505$ nm (Helios-W spectrophotometer, UVG 101103). Color of the mixtures was varying from light pink to dark red, and it was stable for about 30 min.

Calculation
Plasma TAG concentration was calculated using a TAG standard of 200 mg/dl concentration according to the following equation:

Sample conc. (mg/dl) = (Abs. of unknown x conc. of standard) / Abs. of standard

2.7.2 Determination of plasma cholesterol
Principle
The cholesterol assay kit (Spinreact, S.A. Spain) used was based upon a colorimetric method. Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters and $\text{H}_2\text{O}_2$ is formed in the subsequent enzymatic oxidation of cholesterol by cholesterol-oxidase according to the following equations:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Esters + $\text{H}_2\text{O}$</td>
<td>$\text{CHE}$</td>
</tr>
<tr>
<td>Cholesterol + $\text{O}_2$</td>
<td>$\text{CHOD}$</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$ + 4 AP + Phenol</td>
<td>$\text{POD}$</td>
</tr>
</tbody>
</table>
Procedure
Samples, along with standards, were run in duplicates, and were mixed with the working reagent and incubated for 10 minutes at room temperature. The absorbances of the samples (unknown) and the standard samples were measured against Blank reagent at $\lambda = 505$ nm (Helios-γ spectrophotometer, UVG 101103). Color of the mixtures was varying from light pink to dark red, and it was stable for about 30 mn.

Calculation
Plasma cholesterol was calculated using a cholesterol standard of 200 mg/dl concentration according to the following equation:

$$\text{Cholesterol conc. (mg/dl)} = \frac{\text{Abs. of unknown} \times \text{conc. of standard}}{\text{Abs. of standard}}$$

2.7.3 Determination of plasma HDL cholesterol

Principle
LDL and VLDL are specifically precipitated by phosphotungstic acid and magnesium ions and can then be removed by centrifugation. HDL remain in the supernatant. Determination of HDL cholesterol is performed using the clear supernatant.

Procedure
Samples, run in duplicates, were mixed with the precipitating reagent (Phosphotungstic Acid 14 mmol/L, Magnesium chloride 2 mmol/L), allowed to stand for 10 minutes at room temperature and centrifuged at 12000 rpm for 4 minutes. The clear supernatant was used for cholesterol determination using the cholesterol assay kit as described in the previous section.
Calculation
Plasma HDL-cholesterol concentration was calculated according to the following equation:
HDL-cholesterol (mg/dl) = Absorbance of sample x 320 at λ = 505 nm

2.7.4 Determination of plasma LDL cholesterol, direct.

Principle
In the Dialab (IVD, Germany) method, non LDL-lipoproteins are enzymatically processed, while LDL is selectively protected with reagent 1 (protective reagent). In the second step, LDL is released and selectively determined.

Procedure
The samples and calibrator are mixed with reagent 1, incubated for 5 min at 37°C and A1 absorbance is read at 650nm against the blank reagent1. Then reagent 2 is added to the blank, samples and calibrator, mixed and incubated for 5 min at 37°C. A2 is also read at 650nm.
Calculation

\[ \Delta A = [(A1-A2)_{\text{sample or calibrator}}] - [(A1-A2)_{\text{blank}}] \]

\[ \text{LDL-C [mg/dl]} = \frac{\Delta A_{\text{sample}} \times \text{Conc. cal[mg/dl]}}{\Delta A_{\text{calibrator}}} \]

2.7.5 Determination of serum glucose

Principle

The glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The formed hydrogen peroxide reacts under catalysis of peroxidase (POD) with phenol and 4-aminophenazone to a red-violet quinonimine dye as an indicator. The enzymatic reaction sequence employed in the assay was as follows:

\[
\begin{align*}
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} & \xrightarrow{\text{GOD}} \text{Gluconic Acid} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + \text{Phenol} & \xrightarrow{\text{POD}} \text{Quinonimine (red)} + 4\text{H}_2\text{O}
\end{align*}
\]

Procedure

Samples, run in duplicates, were mixed with the working reagent, and incubated 10 minutes at room temperature. The absorbances of the unknown and the standard samples were measured against the Blank reagent at \( \lambda = 500 \) nm (Helios-γ spectrophotometer, UVG 101103).

Calculation

Plasma glucose concentration was calculated using glucose standard of 100 mg/dl concentration according to the following equation:

\[ \text{Glucose conc. (mg/dl)} = \frac{(\text{Abs. of unknown} \times \text{conc. of standard})}{\text{Abs. of standard}} \]
2.8 Lipoprotein fraction assays

2.8.1 Determination of phospholipids

Principle
Phospholipids are determined after enzymatic reaction in the presence of phospholipase. The formed choline reacts under catalysis of choline oxidase with \( O_2 \) & \( H_2O \) to form hydrogen peroxide \( (H_2O_2) \) which reacts then with 4-aminophenazone and dichlorophenol under catalysis of peroxidase (POD) to a red-violet quinonimine dye as an indicator.

\[
\begin{align*}
\text{Phospholipids} + H_2O & \xrightarrow{\text{Phospholipase}} \text{Choline} + \text{Phosphatidic acid} \\
\text{Choline} + 2O_2 + H_2O & \xrightarrow{\text{Choline Oxidase}} \text{Betaine} + 2H_2O_2 \\
2H_2O_2 + 4\text{-aminophenazone} + \text{dichlorophenol} & \xrightarrow{\text{POD}} \text{Quinonimine} (\text{red}) + 4 \text{H}_2\text{O}
\end{align*}
\]

Procedure
Samples, run in duplicates, were mixed with the working reagent, and incubated for 5 minutes at 37 °C. The absorbances of the unknown and the standard samples were measured against the Blank reagent at \( \lambda = 505 \text{ nm} \) (Helios-γ spectrophotometer, UVG 101103).

Calculation
Lipoprotein phospholipid concentration was calculated using phospholipid standard of 200 mg/dl concentration according to the following equation:

\[
\text{Phospholipid conc. (mg/dl)} = \frac{\text{Abs. of unknown x conc. of standard}}{\text{Abs. of standard}}
\]
2.8.2 Determination of TAG

The TAG concentration in the different lipoprotein fractions, VLDL (Sf 20-400), and (LDL Sf 0-20) was measured using the TAG assay kit. This TAG determination is based upon the same principle as the colorimetric method described in section 2.7.1 for serum samples.

2.8.3 Determination of Cholesterol

The cholesterol concentration in the different lipoprotein fractions, VLDL (Sf 20-400), and (LDL Sf 0-20) was measured using the cholesterol assay kit. This determination is based upon the same principle as the colorimetric method described in section 2.7.2 for serum samples.

2.8.4 Determination of Serum Apolipoprotein B

PRINCIPLE
The assay of apoB using the DIALAB (IVD, Germany) test kit is based on turbidometric measurement. Turbidity is caused by the formation of antigen-antibody insoluble immuno complexes. The formation of the complexes is accelerated and enhanced by PEG (phosphate buffer saline).

Procedure
The samples, calibrators, and the control were mixed with the buffer solution, incubated for 5 minutes at room temperature, and the absorbance Δ1 read at 340nm. The second step consists of adding the antibody reagent, mix and incubate at room temperature for 5 minutes and the second absorbance Δ2 read at 340nm.
Calculation

Calculate $\Delta A = (\Delta 2 - \Delta 1)$ of the calibrators versus assigned concentration values on a linear graph paper. Calculate $\Delta A$ optical densities of samples and controls and read values in mg/dl on the reference curve.

2.9 Determination of Serum iron

Principle

The iron test kit (Spineract, S.A. Spain) is a colimetric test without depolarization. In serum iron is bound to transferin. In weakly acidic solution, the iron dissociates from this complex and the serum proteins remain in solution. In this test the iron is reduced with ascorbic acid and then converted to a complex by a specific colour reagent of ferrozine.

Procedure

Samples were mixed with the working reagent, and incubated for 5 minutes at 37 °C. The absorbances of the unknown and the standard samples were measured against the Blank reagent at $\lambda = 562$ nm (Helios-$\gamma$ spectrophotometer, UVG 101103).

Calculation

The iron concentration was determined using an iron standard sample of concentration 100μg/dl according to the following equation:

$$\mu g/dl \text{ Fe} = \frac{\text{Ab sample} - \text{Ab sample blank} \times \text{Standard conc(μg/dl)}}{\text{Ab Standard}}$$
2.10 Rat insulin Elisa

Principle
The rat insulin Elisa (DRG instruments GmbH, Germany) is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the samples reacts with peroxidase conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtiter wells. The unbound enzymes labelled antibodies are all removed in a simple washing step. The bound conjugate is directed by reaction with 3,3',5,5'-tetramethybenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint results, that is read spectrophotometrically.

Procedure
The serum samples and the standards are dispensed in the appropriate wells. The Anti-insulin –HRP conjugate is added into the wells and incubated in a horizontal shaker at room temperature for 120 minutes. The liquid is removed and the washing solution is added into each well and aspirated. This step is repeated 5 times after which the plate should be inverted firmly against an absorbant paper.

The peroxidase substrate is dispensed into the wells and incubated for 15 minutes at room temperature, avoiding any direct light. The stopping reagent is finally added and the absorbance is read at 450nm using the Spectra Max Plus ELISA reader, (Molecular Devices, USA).

Calculation
A standard curve is constructed using all standard points for which absorbances are < 1,5 OD unit. The insulin concentrations of the samples are determined using a computerized data reduction of absorbance for the standards versus the concentration using a cubic regression performed with the E-LizaMat 300 reader and the DGR Elisa regression program.
2.11 Liver Enzymes concentration

Principle

**Alkaline Phospahtase (ALP):** p-nitrophenyl phosphatase is converted to p-nitrophenol and phosphate by alkaline phosphatase. The increase of absorption at 405nm is proportional to the alkaline phosphatase in the sample.

**GOT-AST:** AST catalyses reaction between alpha ketoglutaric acid and L-aspartate, giving glutamate and oxaloacetate. Oxaloacetate, in presence of maleate dehydrognase (MDH), reacts with NADH giving malate and NAD. The O.D. variation is proportional to the AST activity.

**GPT-ALT:** ALT catalyses reaction between alpha ketoglutaric acid and L-alanine, giving L-glutamic acid and pyruvic acid. Pyruvic acid in the presence of Lactate dehydrogenase (LDH) reacts with NADH giving lactic acid and NAD. The O.D. variation is proportional to the ALT activity.

**Lactate dehydrogenase (LDH):** LDH catalyses the reaction between pyruvic acid and NADH. The creation of NAD allows to determine the LDH presence in the samples.

Procedure

The appropriate volume of the samples is mixed with the indicated reagent volume in the SGM, Italia test kits, they are mixed and incubated at 37 °C for 1 minute and the extinction decrease per minute, during 3 minutes is measured for GOT, GPT and LDH at 340nm. For the ALP the extinction increase per minute, during 3 minutes, is measured at 405nm.
Calculations

\[ \text{ALP U/l} = \Delta E/\text{min} \times 2750 \]
\[ \text{LDH U/l} = \Delta E/\text{min} \times 8095 \]
\[ \text{GPT U/l} = \Delta E/\text{min} \times 1746 \]
\[ \text{GOT U/l} = \Delta E/\text{min} \times 1746 \]

2.12 Folch Method

Liver lipid extraction was performed according to the method of Folch et al. (1959). Briefly, the tissue (Liver right lobe) weighing approximately 1 g is homogenized in 20ml of the solvent mixture (chloroform:methanol 2:1), using a high speed 4000rpm homogenizer (Glas-Col, USA). After the homogenization the whole mixture is agitated for 30 min in an orbital shaker at room temperature. Then, the homogenate is filtrated using a Whatman filter paper and washed with 0.2 volume (4ml for 20ml) of 0.9% NaCl solution. After vortexing for few seconds, the upper phase is removed by siphoning and the interface is washed 2 times with metanol/water (1/1) without mixing the whole preparation. Finally, the lower chloroform phase containing lipids is evaporated under vacuum in a rotary evaporator. The flask containing the lipids is weighed before and after evaporation, the mass of lipid content is computed and the percentage of lipid in the liver is calculated.

2.13 Glucose Tolerance Test

A dose of 1.1g of glucose/kg body weight was administered intragastrically using a stomach tube to fasted rats (18 hours). Animals were previously (8 weeks) receiving either plain water or water + CrP as described in section 2.1. Two hours following glucose administration, rats were sacrificed and blood samples were collected from the inferior vena cava. Serum glucose test was performed using the Spinreact Glucose test kit.
2.14 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

Following a two month period of supplementation with CrP in water, animals were sacrificed and blood samples were collected. The following parameters were measured in plasma: TAG, Total cholesterol, HDL-cholesterol, LDL-cholesterol, ApoB, Glucose, Glucose tolerance test (GTT), insulin, iron, SGPT, ALT, SGOT, and LDH. Similarly concentrations of TAG, cholesterol and phospholipids, ApoB 48 and ApoB 100, were measured in the LDL and VLDL lipoprotein fractions. The liver and stool fat contents were also determined.

3.1. Lipoprotein Fractions Assay

Fasting VLDL and LDL cholesterol, TAG and phospholipid concentrations in the control and CrP treated groups were determined in animals previously fed a regular fat diet. Data are shown in table 3.1 and 3.2 respectively.

3.1.2. VLDL TAG, Cholesterol and Phospholipids

The concentrations of TAG, cholesterol and phospholipid in the VLDL lipoprotein fraction were determined. Data have shown that all groups showed similar VLDL TAG, cholesterol and phospholipid concentrations, indicating that chronic CrP intake had no effect on these parameters (Table 3.1).

3.1.3. VLDL ApoB 48 and ApoB 100

Determination of apo B48 and apo B100 concentrations in the VLDL lipoprotein fraction revealed a dose dependent decrease in apo B48 and apo B100 concentrations with CrP intake. However, the decrease observed did not attain significance when comparing the different groups. Similar insignificant changes were observed when total VLDL apo B was calculated (Table 3.1).
**TABLE 3.1:** cholesterol (mg/dl), triacylglycerol (mg/dl), Phospholipids (mg/dl), ApoB48, ApoB100, and total ApoB (mg/dl) in the VLDL fraction obtained after density gradient centrifugation after two months of supplementation with Crp for rats fed on a normal diet (chow+water). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>69.4</td>
<td>67.0</td>
<td>71.0</td>
</tr>
<tr>
<td></td>
<td>±5.6</td>
<td>±6.7</td>
<td>±7.8</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>248.8</td>
<td>223.9</td>
<td>242.1</td>
</tr>
<tr>
<td></td>
<td>±15.5</td>
<td>±14.8</td>
<td>±17.2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>101.68</td>
<td>91.74</td>
<td>83.36</td>
</tr>
<tr>
<td></td>
<td>±11.0</td>
<td>±8.2</td>
<td>±7.8</td>
</tr>
<tr>
<td>ApoB 100</td>
<td>6.30</td>
<td>5.98</td>
<td>5.59</td>
</tr>
<tr>
<td></td>
<td>±0.52</td>
<td>±0.48</td>
<td>±0.42</td>
</tr>
<tr>
<td>ApoB48</td>
<td>2.35</td>
<td>2.14</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>±0.32</td>
<td>±0.22</td>
<td>±0.12</td>
</tr>
<tr>
<td>Total ApoB&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.65</td>
<td>8.12</td>
<td>7.39</td>
</tr>
<tr>
<td></td>
<td>±0.82</td>
<td>±0.71</td>
<td>±0.56</td>
</tr>
</tbody>
</table>

<sup>1</sup> it is the summation of ApoB100 and ApoB 48.
Low is the low dose of Crp (0.236 µg/100g)
High is the high dose of Crp (1.43 µg/100g)
3.1.4. LDL TAG, Cholesterol and phospholipid

In comparison with values of the control group, the mean LDL TAG concentrations of both the low and high dose groups have increased in a dose dependent manner. A significant ($p < 0.05$) increase was only reached between the high dose group and both the control and normal dose groups.

The mean LDL Cholesterol concentration of the high dose group showed a significant increase with respect to the control. No significant changes have been observed between the control and normal dose group.

The LDL phospholipid concentrations were similar in all groups and no significant changes among the different groups have been detected (Table 3.2)

3.1.5. LDL, ApoB 48 and ApoB 100.

Determination of apo B48 and apo B100 concentrations in the LDL lipoprotein fraction revealed a dose dependent increase in their concentrations with CrP intake. However, a significant increase was only reached between the high dose and control groups. Calculation of the total LDL apo B concentrations showed similar dose dependent changes, with a significant difference reached between the high dose and control groups (table 3.2).
TABLE 3.2: cholesterol (mg/dl), triacylglycerol (mg/dl), Phospholipids (mg/dl), ApoB48, ApoB100, and total ApoB (mg/dl) in the LDL fraction obtained after density gradient centrifugation after two months of supplementation with Crp for rats fed on a normal diet (chow+water). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>20.6</td>
<td>21.9</td>
<td>26.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±1.28</td>
<td>±1.37</td>
<td></td>
<td>±2.36</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>42.1</td>
<td>46.2</td>
<td>88.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>±6.2</td>
<td>±7.2</td>
<td></td>
<td>±12.2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>40.09</td>
<td>38.2</td>
<td>46.57</td>
</tr>
<tr>
<td>±3.4</td>
<td>±2.1</td>
<td></td>
<td>±4.8</td>
</tr>
<tr>
<td>ApoB 100</td>
<td>15.1</td>
<td>17.2</td>
<td>20.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±1.3</td>
<td>±1.4</td>
<td></td>
<td>±1.9</td>
</tr>
<tr>
<td>ApoB48</td>
<td>5.2</td>
<td>5.9</td>
<td>6.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±0.44</td>
<td>±0.61</td>
<td></td>
<td>±0.54</td>
</tr>
<tr>
<td>Total ApoB&lt;sup&gt;1&lt;/sup&gt;</td>
<td>20.3</td>
<td>23.1</td>
<td>26.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±1.69</td>
<td>±1.91</td>
<td></td>
<td>±2.38</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant difference (p<0.05) with respect to the control

<sup>b</sup> Significant difference (p<0.05) between the low and high dose

<sup>1</sup> It is the summation of ApoB100 and ApoB 48.

Low is the low dose of Crp (0.286 μg/100g)

High is the high dose of Crp (1.43 μg/100g)
3.2. Serum Analyses

The mean fasting serum concentrations (mg/dl) of TAG, total cholesterol, HDL-cholesterol, total apo B, glucose, and insulin were determined in all groups following Crp supplementation along with a normal or a high fat diet.

3.2.1. Serum TAG, total cholesterol and HDL cholesterol

Regular fat diet:
Results of the normal fat diet are shown in table 3.3. The mean serum TAG and total cholesterol concentrations were similar in all groups and no significant changes among the different groups have been detected. Although the HDL cholesterol concentration of the normal and high dose groups were about 17% higher than that of the control group no significant differences have been reached. Calculation of the total cholesterol / HDL cholesterol and LDL cholesterol / HDL ratios (Figure 3.1) revealed that the recommended dose of Crp (low dose group) exhibited only slight benefit on these ratios. Increasing the dose of Crp, however, showed no benefit at all.

High fat diet:
Results of the high fat diet are shown in table 3.4. The mean serum TAG concentrations of the Crp treated groups were significantly higher than that of the control group. Similar significant increases were observed with serum total cholesterol concentrations. However, no significant changes in serum HDL cholesterol concentrations were detected among the different groups. Calculation of the total cholesterol / HDL cholesterol (Figure3.2) showed that Crp intake along with a high fat diet has no positive impact on this ratio.
**TABLE 3.3:** Triacylglycerol (mg/dl), cholesterol (mg/dl), HDL-cholesterol (mg/dl), ApoB (mg/l), and glucose (mg/dl), Insulin (ng/ml), after two months of supplementation with CrP for rats fed on a normal diet (chow+water). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol</td>
<td>65.1 ± 3.17</td>
<td>68.2 ± 5.89</td>
<td>67.6 ± 4.97</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>62.5 ± 4.6</td>
<td>64.5 ± 3.42</td>
<td>69.6 ± 3.47</td>
</tr>
<tr>
<td>HDL-Chol</td>
<td>30.4 ± 2.1</td>
<td>35.4 ± 1.3</td>
<td>35.8 ± 2.5</td>
</tr>
<tr>
<td>Total ApoB</td>
<td>195.2 ± 16.4</td>
<td>217.6 ± 9.3</td>
<td>238.7 ± 18.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>91.0 ± 4.5</td>
<td>90.2 ± 4.3</td>
<td>95.6 ± 9.8</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.57 ± 0.097</td>
<td>0.44 ± 0.07</td>
<td>0.48 ± 0.07</td>
</tr>
</tbody>
</table>

*Significant difference (p<0.05) with respect to the control
Low is the low dose of CrP (0.286 μg/100g)
High is the high dose of CrP (1.43 μg/100g)
**TABLE 3.4:** Triacylglycerol (mg/dl), cholesterol (mg/dl), HDL-cholesterol (mg/dl), ApoB (mg/l), and glucose (mg/dl), Insulin (ng/ml), after two months of supplementation with CrP for rats fed a rich diet (chow+5% w/w oil). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol</td>
<td>43.5 ±3.78</td>
<td>65.2 ±11.5</td>
<td>52.1 ±2.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40.3 ±3.0</td>
<td>51.6 ±4.1</td>
<td>48.2 ±2.2</td>
</tr>
<tr>
<td>HDL-Chol</td>
<td>34.1 ±1.0</td>
<td>35.7 ±1.8</td>
<td>41.7 ±4.6</td>
</tr>
<tr>
<td>Total ApoB</td>
<td>139.5 ±7.4</td>
<td>215.4 ±20.9</td>
<td>254.2 ±9.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>89.7 ±6.1</td>
<td>93.1 ±4.2</td>
<td>92.3 ±2.4</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.49 ±0.09</td>
<td>0.74 ±0.12</td>
<td>1.04 ±0.16</td>
</tr>
</tbody>
</table>

*Significant difference (p<0.05) with respect to the control

Low is the low dose of CrP (0.286 µg/100g)

High is the high dose of CrP (1.43 µg/100g)
**Figure 3.1** Total cholesterol/HDL Cholesterol, and LDL/HDL Cholesterol ratios after 2 months of CrP ingestion in rats fed a regular fat diet.

Low dose: Low dose CrP = 0.296 μg/100g

High dose: High dose CrP = 1.43 μg/100g
Figure 3.2 Total cholesterol/ HDL cholesterol after 2 months ingestion of CrP in rats fed a high fat diet.

Low dose: Low dose CrP = 0.286 μg/100g
High dose: High dose CrP = 1.43 μg/100g
3.2.2. Serum total ApoB
The effect of CrP intake on total ApoB concentrations was also studied in the serum samples. Data have shown a dose dependent increase in total apo B concentration with CrP intake. In the regular fat diet study, a significant increase was only reached between the high dose and control groups (Table 3.3). However, in the high fat diet study, both Crp groups exhibited a significant increase in their total apoB concentration with respect to the control group (Table 3.4).

3.2.3. Serum Glucose
The mean fasting serum concentrations of glucose as determined following CrP supplementation along with either a regular or high fat diet showed similar values in the different groups, and no significant changes have been detected. Results are shown in tables 3.3 and 3.4 respectively.

3.2.4. Serum Insulin
The mean fasting serum concentrations of insulin as determined following CrP supplementation along with either a regular fat diet showed similar values in the different groups with no significant changes observed (Table 3.3). However, when the diet was rich in fat, insulin concentration increased in a dose dependent manner with CrP intake and significance was reached only between the high dose and control groups (table 3.4).

3.2.5. Serum iron test.
The mean fasting serum concentrations of iron as determined following Crp supplementation along with a normal or a high fat diet showed similar values in the different groups, and no significant changes have been detected (Fig. 3.3)
Figure 3.3 Serum Iron concentrations in rats fed either a regular or high fat diet, after 2 months of CrP supplementation. Values denote ±SEM (n=10).

Control: Control Group
Low: Low dose CrP (0.286 µg/100g)
High: High dose CrP (1.43 µg/100g)
3.3. Enzymes

Regular fat diet

Table 3.5 summarizes the activities of ALP, SGPT, SGOT and LDH in the control and experimental groups when the animals were receiving a regular fat diet. Data have shown that the serum concentrations of ALP and LDH were similar in all groups; however, both the low dose and high dose groups exhibited a significant increase in the concentration of SGOT when compared with the control group. On the contrary, the serum concentration of SGPT was lower in both experimental groups with respect to the control group with significance reached only with the low dose group.

High fat diet:

Table 3.6 summarizes the activities of ALP, SGPT, SGOT and LDH in the control and experimental groups when the animals were receiving a high fat diet. Data have shown that the serum concentrations of ALP and LDH were significantly higher in both CrP groups with respect to the control group. The serum concentration of SGOT in the high dose group was significantly higher than both the low dose and control groups. No significant changes in serum SGPT concentrations have been observed among the different groups.
**TABLE 3.5:** Alkaline phosphatase (ALP), SGPT, SGOT, and LDH activities in serum after two months supplementation with CrP for rats fed on a normal diet (chow+water). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>147.4 ±15.3</td>
<td>124.0 ±15.2</td>
<td>143.4 ±12.1</td>
</tr>
<tr>
<td>SGPT</td>
<td>64.4 ±6.6</td>
<td>45.9 ±4.2</td>
<td>53.8 ±6.2</td>
</tr>
<tr>
<td>SGOT</td>
<td>38.5 ±5.3</td>
<td>74.0 ±7.2</td>
<td>68.8 ±11.5</td>
</tr>
<tr>
<td>LDH</td>
<td>229 ±27.8</td>
<td>241 ±22</td>
<td>240 ±32.7</td>
</tr>
</tbody>
</table>

*Significant difference (p<0.05) with respect to the control

Low: Low dose CrP (0.286 µg/100g)

High: High dose CrP (1.43 µg/100g)
**TABLE 3.8:** Alkaline phosphatase(ALP), SGPT, SGOT, and LDH activities in serum after two months supplementation with Crp for rats fed on oil-rich diet (chow+5%w/w oil). Values denote mean ± SEM (n=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>135.7</td>
<td>189.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>192.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±13.9</td>
<td>±15.5</td>
<td>±21.8</td>
</tr>
<tr>
<td>SGPT</td>
<td>23.1</td>
<td>20.2</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>±3.2</td>
<td>±2.5</td>
<td>±2.2</td>
</tr>
<tr>
<td>SGOT</td>
<td>33.8</td>
<td>30.2</td>
<td>47.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±2.2</td>
<td>±3.8</td>
<td>±4.1</td>
</tr>
<tr>
<td>LDH</td>
<td>45.6</td>
<td>81.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±4.7</td>
<td>±9.4</td>
<td>±6.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant difference (p<0.05) with respect to the control

<sup>b</sup> Significant difference (p<0.05) between the low and high dose

Low: Low dose CrP (0.286 µg/100g)

High: High dose CrP (1.43 µg/100g)
3.4. Liver Fat Content Determination (Folch Method)

The effect of CrP upon liver lipid content of animals fed either a regular or a high fat diet was also studied. The lipid content as determined by the Folch method was not affected by the CrP treatment period regardless of the fat content in the diet. However, there was a positive correlation between liver fat content and the percentage fat in the diet (Fig. 3.4)

3.5 Stool Lipid content Determination

The concentrations of cholesterol and TAG in stools were determined during the last week of CrP study. Data showed no significant changes in both parameters when experimental groups were compared with the control group, indicating no effect of CrP on TAG and cholesterol excretion in the stools (fig. 3.5).

3.6. Glucose Tolerance Test

The glucose tolerance test was conducted on fasted animals previously fed a regular or a high fat diet along with or without CrP supplementation. Results have shown that serum glucose levels were relatively higher in both CrP groups with respect to the control group; however, no significant changes have been reached regardless of the fat content in the diet (fig 3.6)
Figure 3.4 Rat Liver fat content after 2 months ingestion of CrP as determined by Folch Method in rats fed either a normal or high fat diet.

Low: Low dose CrP (0.286 µg/100g)
High: High dose CrP (1.43 µg/100g)
**Figure 3.5** Concentrations of Cholesterol and TAG in Stools of rats fed a normal fat diet after 2 months supplementation of CrP. Values denote ±SEM(n=10).

Low: Low dose CrP (0.286 µg/100g)

High: High dose CrP (1.43 µg/100g)
**Glucose Tolerance Test**

![Bar chart showing glucose tolerance test results for normal and oil diets with control, low, and high CrP doses.](chart.png)

**Figure 3.6** Serum glucose concentrations in rats fed either a normal or high fat diet along with 2 months ingestion of CrP, after a GTT. Values denote ±SEM (n=10).

- **Low**: Low dose CrP (0.286 µg/100g)
- **High**: High dose CrP (1.43 µg/100g)
CHAPTER 4

Discussion

Since the first demonstration of the role of chromium in normal glucose tolerance in rats (Schwarz & Mertz, 1959) there have been several studies that implicated chromium as a necessary factor for glucose and lipid metabolism. However, lots of controversies existed among the different reports, which necessitated further investigations. The present work was undertaken to investigate the effects of a 2-months period of Crp induced changes on blood lipid profile as major determinants of cardiovascular disease risk. The study, conducted on normolipidemic rats, was designed to cover Crp effects in concomitance with either a regular or a high fat diet. Two doses of Crp were used over the study period; the normal recommended supplemental dose (low dose) and an arbitrary high dose equivalent to 5 times the recommended supplemental dose (high dose). Such a high dose represents a possible toxic dose that can be used accidentally by certain subjects thinking of taking more supplements would be more beneficial. If, as suggested by many previous studies, chromium has a beneficial effect on atherosclerosis, then chronic intake of Crp as early as possible in life would result in maximum benefit regarding cardiovascular diseases. Our data, however, do not support the assertion that chromium supplementation will improve the atherogenic profile in normolipidemic rats, when either a regular or a high fat diet was followed by the animals.

In order to better understand the effect of Crp on blood lipid profile, in terms of synthesis, secretion and metabolism, the isolation of the VLDL (Sf 20-400) and LDL (Sf < 20) lipoprotein fractions was necessary. In the Sf>400 lipoprotein
fraction, only CMs of intestinal origin having apoB48 as a molecular marker are collected while small intestinal CMs and VLDLs newly secreted by the liver are collected in the Sf 20-400 lipoprotein fraction. In the human model, small CM and VLDL can be differentiated by their respective apoB48 and apoB100 markers. However, a liver secretion of VLDL with either apoB48 or apoB100 as a surface marker distinctively characterizes the rat species (Tennyson et al., 1989). Consequently, the assessment of the number of small CMs in the Sf 20-400 lipoprotein fraction is not feasible. Therefore, the apoB48 of the latter lipoprotein fraction was considered to be a VLDL constituent. Knowing that each VLDL particle contains one apo B molecule (Elovson et al., 1998), and rat liver secretes both apo B100 and apo B48 (Tennyson et al., 1989) measuring total VLDL apo B (apo B100 + apo B48) reflects the total number of newly secreted VLDL.

In the presence of a regular fat diet, chronic intake of the regular supplemental dose of Crp did not appear to affect serum concentrations of TAG, total cholesterol, HDL cholesterol, LDL cholesterol, total apo B, glucose and insulin. Similarly, the same dose did not affect VLDL and LDL cholesterol, TAG, phospholipid, apo B48 and apo B100 concentrations. Such a finding indicates that the low dose of Crp has no impact on lipoprotein synthesis, secretion and metabolism, hence confirming the no effect observed in the serum lipid profile. Chromium deficiency has been associated with atherosclerosis. When compared with patients without coronary artery disease, a decreased concentration of chromium has been demonstrated in sera (Newman, et al. 1978; Simonoff, et al. 1984) and aortic tissue (Schroeder, et al. 1962) of patients dying of coronary artery disease. Also, a chronic daily injection of chromium has been shown to reverse established atherosclerosis in the aorta of cholesterol-fed rabbits (Abraham, et al. 1991). However, in a double-blind randomized study conducted by Wilson and Gondy (1995) on young, non-obese human subjects without evidence of diabetes mellitus, hyperlipidemia or established atherosclerosis, chromium supplementation failed to improve atherosclerosis since no effect on serum lipids or glucose
tolerance has been observed. Similarly, Besong et al. (2001) found no effect of Crp on blood glucose, insulin, cholesterol and TAG concentrations when chromium was supplemented to laboratory rats. Also, Amato et al. (2000) described similar non-significant difference of serum HDL, LDL and apo B levels in either chromium treated and control groups. A favorable effect of chromium supplementation upon insulin action and lipid levels has been shown in obese insulin resistant rats (Cefalu et al., 2002). Although the results appear somehow to be contradictory and inconsistent, it seems that Crp supplementation may be beneficial only in subjects at high risk of atherosclerosis rather than normolipidemic and non-diabetic subjects. Also, one should not exclude that the benefit of Crp supplementation on atherosclerosis may be through mechanisms other than lipid profile.

The chronic effect of a high dose of Crp (1.43 μg/100 g body weight) on blood lipid profile was also studied. In the presence of a regular fat diet, the high dose of Crp did not affect the secretion of lipoprotein from the liver since VLDL cholesterol, TAG, phospholipid, apo B48 and apo B100 concentrations were not affected. However, a significant increase in LDL cholesterol, TAG, apo B48 and apo B100 concentrations was observed, indicating that the high dose of Crp is interfering with the clearance and metabolism of the lipoprotein particles from the blood. The mechanism of such an interference remains to be elucidated. Measurement of the plasma total apo B concentration also showed significant increases with the high dose of Crp. Apolipoprotein B is a better indicator of potential myocardial infarction than total cholesterol or LDL cholesterol (Waldius et al., 2001) especially in individuals with low or normal LDL-cholesterol (Sniderman et al., 2001). Consequently, the present study, being undertaken on normolipidemic rats, reveals that chronic intake of high doses of Crp is harmful rather than beneficial to atherosclerosis and the community willing to use supplemental Crp should be aware of not exceeding the recommended supplemental dose.
In the present study, the effect of increasing fat content in the diet, in concomitance with chronic Crp supplementation, upon blood lipid profile was also investigated. The increase in fat content in the diet was achieved by the addition of olive oil to the diet as 5% w/w. Olive oil is an excellent source of oleic acid, a monounsaturated fatty acid. The olive oil rich diet resulted in much improved lipid profile in the control group with respect to that observed in Crp groups. Surprisingly, the serum concentrations of TAG, total cholesterol and total apo B were significantly higher in both Crp groups with respect to the control group. Such a finding raises the possibility of fat interference with the effect of Crp on blood lipid profile. Consequently, chromium supplementation even in recommended doses seems to be inappropriate when the percentage fat content in the diet is high. This observation may in part explain the discrimination in results observed among the different studies in the literature, where the amount of fat in the diet seems to be a crucial factor. In addition, other conditions like species differences, the state of being normo- or hyperlipidemic, and the possibility of being insulin resistant or not are all factors that should be taken into consideration while conducting such studies. Most of the conducted studies evaluated the effect of Cr supplementation on a limited number of subjects, or on heterogeneous populations as regards to age, cigarette smoking, hypertension, diabetes and other confounding factors such as antihypertensive medications which induce insulin resistance (Wilson & Gondy, 1995). Further complication to this issue is the difficulty of estimating Cr stores and the lack of standards for assessment of Cr tissue levels prior to supplementation (Lee & Reasner, 1994).

In order to investigate whether the drug has resulted in drug-induced hepatotoxicity and to assert the cardioprotective role of Crp the activities of SGOT (AST), SGPT (ALT), LDH and ALP were determined. In the present study, when rats were fed a regular fat diet, it was shown that there were no Crp-related changes in the serum activities of these enzymes except with SGOT. Increased concentration of SGOT was observed with both doses of Crp used. In the presence of a high fat diet, an increase in serum LDH and ALP activities were
also observed with both doses. SGOT and LDH mostly found in the liver, are released in the bloodstream in high levels as a result of liver injury, therefore serving as fairly specific indicators of the liver status (Braunwald et al., 2001). Thus, after eight weeks of Crp intake there have been some kind of liver tissue damage when the diet was rich in fat.

Earlier studies did not reveal any acute or sub-acute adverse effects or abnormalities with short term ingestion of Crp typically less than 3 month with a dose of less than 300 µg per day (Wasser et al., 1997). However, some clinical case reported acute nausea and vomiting, skin disorders and allergies, hypertension, renal failure (red urine and stool), liver failure, anemia, neuropsychiatric effects/ sleep disorders, coma at doses of 1200-2400 µg/day for more than 5 months (Cerulli et al., 1998). In the present study the use of the high dose (1.43µg/100g body weight) for a period of 2 months increased SGOT and LDH activities and resulted in a somehow improper lipid profile. Although this does not indicate necessarily a liver failure condition as observed with higher doses and longer durations (Cerulli et al., 1998) of Cr supplementation, the present results confirm, with no doubt, that there is a relationship between large doses of Crp supplementation and minor liver function impairment. Consequently, in the present study one may partially attribute the negative lipid profile observed when the high dose of Crp is used to an impairment of liver function caused by the high concentration of Crp. This is supported by the fact that animals receiving Crp along with a high fat diet, which imposes an additional burden on liver cells, showed the worst scenario in term of lipid profile when compared with the control group receiving the same high fat diet. On the other hand, Crp supplementation as used in the present study showed no effect on serum iron level and possibly on anemia. Although, cholesterol and TAG excretion in the stools were highest in animals receiving the high dose of Crp, no significant effect has been observed. Data, then, suggest that Crp, as used, did not significantly interfere with the absorption of lipid by the digestive system. However, it is possible that with increasing the dose further more significant effects may be observed. It would be interesting to
measure Cr concentration in tissues and stool but the problem is its scarcity and scientists did not have until recently sensitive equipments to accurately determine Cr level, and they have also found large errors when trying to repeat their experiments, due to chromium volatility and its potential to assume disguises in other refractory forms (Quillin, 2003).

Previous studies conducted on subjects at high risk for the development of type 2 diabetes showed that an 8 months period of Cr supplementation (1000μg/day) did not affect body weight, abdominal fat distribution, and body mass index (Cefalu et al. 1999). Similar studies have shown that CrP supplementation exhibited a positive change in body composition with (Hasten et al., 1992) and without statistical significance (Hallmark et al., 1993) while other studies failed to show any positive change (Clancey S. et al., 1994). Chronic Cr supplementation in the present study failed to modify liver fat content when the diet was either regular or rich in fat. However, increasing fat content in the diet was associated with an increase in liver fat content regardless of CrP supplementation.

Impaired glucose tolerance can be a consequence of Cr deficiency. Therefore, glucose tolerance can be improved by Cr supplementation or can be maintained inspite of a reduced insulin output in malnourished children (Hopkins et al., 1968, Gurson & Saner, 1971), in patients with Cr deficiency associated with total parenteral nutrition (Brown, et al., 1986, Anderson et al., 1983), and in middle age individuals with mild glucose intolerance (Anderson et al., 1983 and 1991). But those with normal glucose tolerance no further improvement will happen with Cr supplementation (Mertz, 1993). In a study done by Wilson & Gondy on 26 non-obese young adults, there was no statistically significant difference in the percentage change of fasting glucose or lipids between the Cr and the placebo groups after 90 days of supplementation with 220μg/day of Cr. However, they (Wilson et al., 1995) showed that immunoreactive insulin of Cr treated group
decreased significantly only in subjects with high initial fasting immunoreactive insulin. This decrease was attributed to possible subclinical Cr deficiency which produced mild insulin resistance. In the present study, the serum insulin concentrations in the Crp groups were slightly lower than the control when the diet was regular in fat. However, in the presence of a high fat diet, the serum insulin concentration was positively correlated with the Crp dose used and significance was reached between the high dose and the control group. Again, such a finding brings up the fact that a high fat diet along with a high dose of Crp supplementation interferes not only with lipid metabolism but also with the serum concentration of insulin resulting in hyperinsulinemia. Harbis et al. (2001) have shown that, in the absence of insulin-resistance syndrome, hyperinsulinemia delays and exacerbates postprandial accumulation of TAG-rich lipoprotein by having a role in the metabolism of these particles. Consequently, the observed effect of Crp supplementation along with a high fat diet upon blood lipid profile might in part be attributed to the hyperinsulemia that happened in animals receiving Crp supplement. Conducting the glucose tolerance test on all groups did not show any improvement, even with the hyperinsulinemic groups. This will also support the fact that with normal glucose tolerance no further improvement will happen with Cr supplementation (Mertz, 1993).

In conclusion, the present investigation is in favor of what Liu & Morris (1978) have suggested: Cr is accepted as an essential nutrient, not as a drug; its apparent effect like that of any essential nutrient, depends on the nutritional status of the test subjects; it improves an impaired function or restores it to normal, if that impairment developed because of Cr deficiency. In concomitance with a regular fat diet, chronic Crp supplementation in normolipidemic rats with the recommended dose, may not be harmful but also not beneficial. Increasing the the recommended Crp dose 5 times, however, is not recommended because of delayed clearance and metabolism of lipoprotein particles. On the other hand, when a high fat diet is followed, Crp supplementation, in either the low or the high
dose, appeared to have a negative impact on blood lipid profile and to cause hyperinsulinemia.

Further similar studies on human subjects may be necessary to confirm the observed effects on rats because of possible species differences.

Suggested further studies:
- Perfusion of the pancreas with different doses of Crp in the presence and absence of different concentrations of fat emulsion in order to assess changes in insulin secretion accordingly.
References


