

THE INTERRELATIONSHIP OF DIETARY CHOLESTEROL, COPPER AND ZINC ON PLASMA LIPIDS AND TISSUE COPPER AND ZINC LEVELS IN THE RAT

by

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PREFACE

The work presented in this thesis was carried out by the author from January 1990 to December 1992, in the Department of Physiology, University of Durban-Westville, under the supervision of Professor F.J. Burger.

I declare that these studies are my original work and have not been submitted in any form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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CHAPTER ONE

1. INTRODUCTION

Coronary heart disease (CHD) has become a major cause of death in Western countries, the etiology of which is multifactorial (Lau & Klevay, 1982). The positive correlation between high serum cholesterol and the incidences of CHD is well established (Dalen, 1988). Experimental and epidemiological evidence strongly implicates dietary factors to hypercholesterolemia (Bialkowscha, *et al.*, 1987).

Nutritional and metabolic imbalances in copper and zinc have been shown to affect cholesterol metabolism in humans and animals (Klevay, *et al.*, 1984; Carr & Lei, 1990). In addition to having a high fat content, the contemporary American diet provides less copper than the recommended daily intake of 2 mg. Hypercholesterolemia due to copper deficiency is widely accepted but the mechanisms remain unclear (Carr & Lei, 1990). Many mechanisms have nevertheless been proposed, these include changes in liver lipid metabolism, lipoprotein lipase action and lecithin : acyl transferase activity. In addition to causing hypercholesterolemia, copper deficiency has been shown to affect lysyl oxidase activity. This results in blood vessels been less pliable and prone to rupture (O'Dell, *et al.*, 1965).

High dietary copper on the other hand has been shown to cause free radical formation. These species are capable of oxidizing LDL thereby changing its physical characteristics for receptor intake. Oxidized LDL has been shown to accumulate in tissues since the scavenger receptors that bind them lack the sophisticated feedback system that is present in normal receptors. (See Kok, *et al.*, 1988; Kalant, *et al.*, 1991.) The discoveries of oxidized LDL and its uptake via special scavenger receptors appears to have shifted the emphasis of research in atherosclerosis.

Thus a U-shaped relationship of copper to the incidences of coronary artery diseases appears evident with marginal copper diets causing hypercholesterolemia and high dietary copper causing the formation of oxidized LDL via free radical activity (Kok, *et al.*, 1988).

Experimental and epidemiological evidence therefore strongly implicates copper in maintaining the integrity of the circulatory system.

1.1 STUDY PERSPECTIVE

The main purpose of this study was to investigate the effects of dietary cholesterol and varying copper levels on plasma lipoproteins in the rat while keeping dietary zinc levels constant. In this way the effect of high dietary copper intake on zinc absorption could be investigated. Due to this proposed antagonistic relationship between dietary copper and zinc, tissue levels of these

elements were investigated. Sprague Dawley rats were chosen for this study because hypercholesterolemia due to copper deficiency has been previously demonstrated in this strain (Lau & Klevay, 1981; Carr & Lei, 1990).

CHAPTER TWO

2. LITERATURE SURVEY

2.1 COPPER

Copper is an essential trace element that is needed in diverse biological processes. Copper is known to play a key role in haemoglobin formation, electron transport, myelin sheath integrity, elastin formation and melanin formation. Therefore due to this diverse role, nutritional imbalances in copper intake have been implicated in a wide variety of diseases such as anaemia, hypercholesterolemia, cardiac hypertrophy, weakening of arteries, jaundice and necrosis. (See Fields, 1983.)

Recommended daily dietary intake of copper is 2-3 mg/day for man and 6 mg/kg diet for rats (Schoenemann, 1990). The adult human body contains between 75-150 mg of copper but the concentration of copper varies greatly amongst the various organs. Although bone and muscle copper levels are not as high as those in kidney, liver and heart, 50% of the body copper occurs in these tissues because of their larger relative mass. This indicates varying functions of copper in the different tissues. Copper is also present in vascular fluids forming complexes with various organic molecules, enzymes and proteins such as ceruloplasmin, cytochrome oxidase, monoamine oxidase and super oxide dismutase (Paynter, *et al.*, 1979).

2.2 **ABSORPTION**

Absorption of copper preferentially occurs in an acidic medium hence absorption occurs at both the stomach and small intestine (Evans, 1973). The absorption is mediated both via an active energy dependant process and a passive process.

The energy dependant process involves copper being transported as a copper-amino complex whereas the passive process involves the intestinal protein metallothionein. This process and the related antagonist relationship between copper and zinc is discussed in 2.4.

The absorbed copper is transported mainly by albumin to the liver for synthesis of ceruloplasmin. The ceruloplasmin is largely responsible for making copper available to body tissue (Evans, 1973).

The homeostatic balance of copper in the body is maintained by the liver and excretion of excess copper is done mainly via bile although negligible amounts are lost in urine (Evans, 1973).

2.3 **ZINC**

Zinc is widely distributed in the body and about 2-3 g is present in the adult body. Although the dietary need for zinc is high at 15 mg/day, it is available from a wide source of foods (Calhoun, *et al.*, 1974).

Zinc forms an integral part of at least 20 enzymes e.g. carbonic anhydrase, lactic dehydrogenase, alkaline phosphatase, carboxypeptidase, aminopeptidase and as a co-factor in DNA synthesis. Although zinc is essential for normal bodily functions, deficiency in developed countries are rare (Robertson, 1977). Deficiencies, however, cause stunted growth, slow healing of wounds and an alteration in taste and smell.

Absorption of zinc takes place in the duodenum and jejunum and involves the intestinal protein, metallothionein. This absorptive process and the antagonism between copper and zinc is discussed in 2.4. Zinc is excreted mainly by pancreatic and intestinal juices and only about 500 ug/day is lost in urine (Robertson, 1977).

In this study zinc was not used as an experimental variable, since dietary zinc was kept constant at 30 ug/g diet. The zinc status needs to be monitored though since copper and zinc do have an antagonistic relationship (Oestricher & Cousins, 1985).

2.4 ZINC AND COPPER INTERACTIONS

Antagonistic interactions between zinc and copper has been known to exist for over five decades, yet the amounts and ratios that are of practical significance are not well established (Oestricher & Cousins, 1985). This is partly due to the difficulty encountered in analysing most trace elements in biological systems

(Evans, 1973).

The American Institute of Nutrition (AIN) recommends a 30 mg/kg diet of zinc and a 6 mg/kg diet copper for rats. This amounts to a 5:1 ratio of zinc to copper (AIN report 1976). Recommended human dietary intake of copper and zinc are 2 and 15 mg per day respectively, a ratio of 7,5:1 (Bialkowsha, *et al.*, 1987).

The interaction between copper and zinc has been described as a mutually antagonistic one since high dietary intakes of either element affects the uptake of the other (Oestricher & Cousins, 1985). The average western diet, due to a high intake of animal fats, eggs and milk has a high zinc to copper ratio(Bialkowsha, *et al.*, 1987). This could significantly decrease the copper status in an individual that has a marginally low copper intake.

Metallothionein, a mucosal protein has been implicated in this relationship. Metallothionein binds the zinc and copper that is absorbed into the mucosal cell. This bound copper and zinc is available for serosal transfer. Copper is, however, more firmly bound than zinc and zinc can even be displaced in preference for copper (Oestricher & Cousins, 1985). The gene that codes for metallothionein in the intestine is receptive to the concentration of dietary zinc, increasing its transcription with increasing dietary zinc levels (Smith, 1978).

Therefore, at very high dietary levels of zinc mucosal metallothionein concentration reaches high levels. Therefore it has been observed that when dietary zinc levels are high (> 250 mg/kg diet) serum copper levels are low (Fisher, *et al.*, 1981).

This occurs because copper that is absorbed into the mucosal cell is strongly bound to the increased concentration of metallothionein. Less copper is therefore available for serosal transfer resulting in a decrease in copper made available to the body. Thus elevated dietary zinc leads to a low body copper status (Storey & Greger, 1987).

The reverse situation that of copper impeding zinc absorption also applies since the antagonism occurs at the intestinal level i.e. the competitive binding for metallothionein.

Studies on a 10 fold increase of dietary copper have resulted in a reduction in zinc absorption of only 20% (Oestricher & Cousins, 1985). Therefore the effect of increasing dietary copper on zinc absorption is not as severe as the effect of increased dietary zinc on copper absorption. This is due to the fact that the metallothionein gene is not receptive to dietary copper levels.

Table 1. **Metallothionein concentration in intestinal mucosa at various dietary levels of copper and zinc.**

DIETARY INTAKE (mg/kg diet)		Zn:Cu ratio	Methollothionein (pmol/g mucosa)
Cu	Zn		
1	5	5 : 1	400 ± 31
1	180	180 : 1	3 538 ± 169
6	30	5 : 7	2 092 ± 62
36	5	1 : 7	954 ± 31
36	180	5 : 1	4 323 ± 523

In this study, dietary copper in group D was administered at 100 mg/kg diet. Starcher *et al.*, (1980) have reported a decrease of 20% in zinc absorption when dietary copper was increased to 300 mg/kg diet. Oestrecher *et al.*, (1985) have found no decrease in zinc absorption for a copper intake of up to 36 mg/kg diet as illustrated in Table 1. The copper concentration of this study therefore falls within the range used by other researchers without causing a severe imbalance in the zinc absorption rate or zinc status.

2.5 CHOLESTEROL

Cholesterol is found in the body in two forms viz. as free cholesterol and as cholesterol esters.

Free cholesterol is required as a structural component of plasma membranes in mammalian cells and the ring structure of cholesterol is used as a precursor for the synthesis of bile acids and hormones (Goldstein & Brown, 1977).

Due to its insolubility in an aqueous medium cholesterol is esterified with long chain fatty acids and packaged within the hydrophobic cores of plasma lipoproteins (Brown & Goldstein, 1986) ensuring its transport in blood. The four major lipoprotein fractions are very low density lipoprotein cholesterol (VLDL), intermediate density lipoprotein cholesterol (IDL), low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL).

The cholesterol esters in these lipoproteins are made available to body cells by strategically placed receptors. These receptors bind lipoproteins and cholesterol enters the cell by receptor mediated endocytosis (Brown & Goldstein, 1986). After intercellular hydrolysis, the cholesterol is available to the cell for either synthesis of plasma membranes, bile acids and steroid hormones or stored as cytoplasmic cholesteryl ester droplets. VLDL and IDL are not taken up to be used in a similar manner, rather they are metabolic intermediates or are involved in reverse cholesterol transport as in the case of HDL.

Elevated serum LDL levels are positively correlated with an increased risk of atherosclerosis (Humphries, *et al.*, 1990). Elevated serum LDL levels are either caused by an increase in lipoprotein production or a decrease in the rate of clearance from plasma (Dietschy, 1990).

2.5.1 LIPOPROTEIN FRACTIONS

i. **VLDL**

The VLDL fraction is rich in triglyceride and is synthesised and secreted in the liver (Miller, 1990). Apolipoprotein B100 is present on the surface and the particle has a diameter of 30-80 nm. After the action of lipoprotein lipase (LPL) VLDL gives rise to IDL. Drugs such as Gemfibrozil that lower LDL concentration, act to prevent lipase action on VLDL so as to prevent formation of LDL.

It is interesting to note that the concentration of VLDL is low in the rat compared to man (Oschry & Eisenberg, 1982). It has been postulated that the rat has an efficient system for the clearance of VLDL. This is also one of the reasons why rats could be relatively resistant to hypercholesterolemia (Oschry & Eisenberg, 1982).

ii. **IDL**

IDL is an intermediate in the catabolism of VLDL to LDL and has either of two fates:

1. It can be taken up and catabolized by the liver due to its apo B100 and apo E content or
2. It can be converted to LDL by lipoprotein lipase.

iii. **LDL**

About 50% of the LDL particle contains cholesterol and has a diameter of approximately 22 nm (Carol & Fieldman, 1989). Most of circulating LDL is obtained from the metabolism of VLDL and has only one apolipoprotein viz. apo B100. Its removal by liver or peripheral tissues is almost entirely dependant on receptors that recognise apo B100. In the genetic disorder of lipid metabolism, familial hypercholesterolemia, homozygous individuals lack the ability to synthesize receptors that recognise apo B100. These individuals have elevated levels of LDL and develop atherosclerosis early in life (Dietschry, 1990). LDL levels beyond 160 mg/dl are considered to be high risk factor.

In normal individuals the receptor feedback system to prevent over-accumulation of LDL is extremely sophisticated, atherosclerosis, however, develops in vessels only after an injury or insult has occurred while hypercholesterolemia is persistent (Dietschry, 1990).

iv. **HDL**

This is the smallest lipoprotein with diameters varying between 7.5 - 10.0 nm. The HDL class of lipoproteins has 3 subclasses designated HDL₁, HDL₂ and HDL₃. Classification is dependant on density taking into account protein and cholesterol content of the particle (Carol & Fieldman, 1989). Apolipoprotein A-1, A-11, C and E are present on HDL.

The role of HDL in reverse cholesterol transport and the inverse relationship between coronary arteries diseases and high serum HDL concentration has been well documented (Steinmetz & Kaffarnik, 1988; Gotto 1990). There are, however, fundamental differences of the origins and metabolism of the HDL particle between man and rat that have not yet been explained (Oschry & Eisenberg, 1982).

2.5.2 **LIPOPROTEINS OF RAT**

The rat is used extensively in animal research on lipoprotein studies. Although data is often extrapolated to man, the lipoprotein metabolism in rat differs from that of humans (Oschry & Eisenberg, 1982).

The differences in the lipoprotein fractions of rat and human can be attributed to the remnant removal pathway and the cholesterol ester transfer reaction.

The rat has an efficient ability to clear chylomicrons and VLDL via receptors in the liver (Oschry & Eisenberg, 1982). This results in a lower LDL level than in humans yet the LDL profiles are similar.

The second difference involves the absence of the cholesteryl ester transfer protein in the rat plasma. This protein is responsible for the transfer of cholesteryl esters (from the lecithin cholesterol acyl transferase reaction) to lipoproteins of lower density. Its absence in rat plasma results in appreciable amounts of larger less dense cholesteryl ester rich HDL₁ particles and the absence of HDL₂. (See Oschry & Eisenberg, 1982.)

The researcher therefore needs to be aware of these differences and the resultant shifts in the lipoprotein metabolic pathway when analysing data from rat serum.

2.6 LIPOPROTEIN METABOLISM

Knowledge of lipoprotein metabolism and the mechanism of interparticle lipid exchange makes it possible to understand the underlying causes of hypercholesterolemia. This also helps the researcher to understand the effect, if any, of the experimental variables on various fractions of lipoprotein.

Although this metabolic pathway is interrelated there are 3 distinct pathways that are present. They are discussed below.

i. **Exogenous pathways**

This involves the fate of the absorbed chylomicrons after it has acquired apolipoprotein apo B48 (from the liver) and apo E and C11 (in the lymph). Apo C11 activates lipoprotein lipase (LPL) that catalyses the hydrolysis of triglyceride from the lipid core to yield chylomicron remnants. Apo E present on these remnants, ensures recognition by the hepatic receptors for their uptake.

The interaction of the triglyceride with the high density lipoprotein (HDL) follows either of two pathways:

- a) The remnants after being taken up the liver form HDL.
- b) During the action of LPL, surface components from the chylomicrons are incorporated in nascent HDL increasing its mass.

The efficiency of removal of the chylomicron fraction is dependant on LPL activity and the presence of apo E. In fact the ratio of lipoprotein/lipase activity has been suggested as an indicator of atherosclerotic risk (Shepherd & Krauss, 1991).

The interrelationship between hypertriglyceridemia and elevated low density lipoprotein (LDL) levels can be explained at this level. When the triglyceride level is high, a greater concentration of remnants are absorbed by the liver increasing hepatic cholesterol concentration. This down regulates LDL receptors and thus causes an increase in circulating LDL levels (Gotto, 1990).

ii. **ENDOGENOUS PATHWAY**

This pathway begins by secretion of very low density lipoprotein (VLDL) with apo B100, C11 and E by the liver. In the process of being converted to LDL by LPL, apo C11 and E are lost to nascent HDL. The LDL with apo B100 is taken up by the hepatic tissues (65%) or extra-hepatic tissues (35%) (Gotto, 1990).

The key determining factor for removal of LDL for circulation is the presence of apo B100 or the B-receptors.

2.7 COPPER, HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS

A number of studies have demonstrated the relationship between a copper deficient diet and hypercholesterolemia (Allen & Klevay, 1978; Carr & Lei, 1990). Elevated serum cholesterol levels are regarded as a risk factor in the etiology of cardiovascular diseases (Fischer, *et al.*, 1980). A number of

mechanisms have been put forth to explain the relationship of copper, hypercholesterolemia and atherosclerosis (Lefevre, *et al.*, 1985).

2.7.1 COPPER DEFICIENCY AND CHOLESTEROL METABOLISM

The characteristic changes in lipoprotein composition and concentration in copper deficient animals and humans, has been attributed to a possible role of copper in cholesterol metabolism (Carr & Lei, 1990). Hypercholesterolemia is due to either an increase in production of cholesterol or a decrease in the rate of removal from serum (Dietschy, 1990).

The hypercholesterolemia observed in copper deficient rats by Lefevre *et al.* was due largely to an increase in the HDL₂ and HDL₁, fractions (Lefevre, *et al.*, 1985). This increase of HDL₁ and HDL₂, subfractions in rats is an indication of an increase in cholesteryl esters synthesis. Due to the absence of cholesteryl ester transfer protein the cholesteryl esters are incorporated into HDL₂ and then to HDL₁. In humans that lack the cholesteryl ester transfer protein gene, HDL₂ and the larger HDL₁ are produced in larger quantities than normal (Shepherd, 1991). Similar results with elevated HDL level, in copper deficient rats are reported in other studies (Lei, 1983). In addition to an elevated HDL level, elevated LDL levels are also reported in copper deficient rats (Lei, 1983). The HDL subfraction in humans is associated with reverse cholesterol transport, carrying extrahepatic cholesterol to the liver for degradation (Gotto, 1990). Therefore, elevated HDL levels would imply a greater quantity of cholesterol is available for degradation. This would be expected since the elevated HDL

levels in copper deficient rats also have a corresponding increase in apo E for hepatic receptor uptake (Lei, 1983). In humans that genetically lack the cholesteryl ester transfer protein, HDL₂ and HDL₁ are produced at much higher rates than average yet due to the high apo E in these fractions, hepatic uptake is increased (Shepherd, 1991). Despite these expectations high serum cholesterol levels and a prolonged half life of HDL are evident in copper deficient rats.

This would imply that copper deficiency in rats either down regulates receptor uptake of HDL in the liver or decreases the quantity of hepatic receptors that recognise apo E. Initial studies on receptor binding data revealed that there was a reduction in receptor binding rather than a decrease in receptor number (Lefevre, *et al.*, 1986).

More recently, however, it has been shown that in addition to an increase in plasma pool size, there is a differential clearance of HDL cholesteryl ester and protein moieties in copper deficient rats (Carr & Lei, 1990). Reports of a three-fold increase in cholesteryl ester clearance compared to HDL protein have been shown in livers of copper deficient rats (Carr & Lei, 1990). Therefore, a preferential clearance of HDL cholesteryl esters is apparent in copper deficiency.

Since biliary cholesterol excretion and liver cholesterol levels remain virtually unchanged in copper deficient rats, it is postulated that the HDL cholesteryl

esters that are cleared by up to a threefold increase are repackaged into HDL and re-secreted into intravascular circulation. Further evidence of the latter is an increase in hepatic hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase activity during copper deficiency (Carr & Lei, 1990).

The apolipoprotein of LDL are mainly apo B. Besides the possibility of being an intrinsic component of apo B, copper is needed to solubilize apo B in an aqueous solution (Lau & Klevay, 1982). This would imply that copper deficiency could alter the structure of apo B resulting in the impairment of LDL binding in extrahepatic receptor sites. The increase in LDL levels in copper deficient rats could be due to this relationship of copper and apo B.

Copper deficiency via these mechanisms thus causes a shift in the cholesterol pool from the liver to the plasma effectively resulting in hypercholesterolemia. Due to the decrease in receptor dependant activity in copper deficiency, cholesterol uptake depends heavily on the receptor-independent process. This is a non-saturable process that occurs in many of the organs and normally accounts for only 20-40% cholesterol uptake hence the hypercholesterolemia persists (Dietschy, 1990).

Since a similar mechanism exists in humans, i.e. an increase in cholesteryl esters with a copper deficient diet (Klevay, *et al.*, 1984). The increase in cholesteryl esters are incorporated into lower density lipoproteins due to the presence of the cholesteryl ester transfer protein (Oschry & Eisenberg, 1982).

Due to the interparticle exchange mechanisms (Fig. 1) especially with lower density lipoproteins in humans, the hypercholesterolemia would be largely due to LDL. High LDL levels in humans have been positively correlated with coronary artery disease (Steinmetz & Kaffarnik, 1988). Therefore in humans with a prolonged marginally low copper intake the resultant hypercholesterolemia could lead to atherosclerosis. In a study quoted by Klevay, *et al.*, (1984) plasma cholesterol levels of a healthy male volunteer on a diet containing 0.8 mg of copper was increased significantly.

There was a significant increase in LDL concentration during the copper depletion phase of this study. Plasma LDL levels returned to pre-experimental levels after repletion of copper. This indicates that hypercholesterolemia in this study was due to a marginally low dietary copper intake and the resultant hypercholesterolemia was due mainly to an increase in the LDL subfraction (Klevay, *et al.*, 1984).

2.7.2 COPPER AND PLASMA LIPOPROTEIN LIPASE

A significant positive correlation has been shown to exist between copper status and plasma lipoprotein lipase (LPL). LPL is involved in the lipolysis of triglyceride rich VLDL fractions into remnants. These remnants are converted by hepatic lipase from IDL to LDL (Shepherd, 1991).

In humans that lack LPL, VLDL levels are high and HDL low. Copper deficient rats do display lower LPL activity and higher VLDL and LDL levels (Lau & Klevay, 1982). The higher VLDL in rats can be explained using present

knowledge of lipoprotein metabolism (Shepherd, 1991). If VLDL is not lipolysed by LPL then VLDL levels should rise. But this does not explain the elevation in LDL levels since the products of lipolysis contribute to the formation of LDL (Shepherd, 1991). Lau & Klevay (1982) have attributed the elevation to a lower hepatic uptake of VLDL and LDL.

These differences between human and rat could therefore be due to a difference in lipoprotein metabolism that is presently not understood. Since a parallel study has not been carried out on humans, it may therefore not be possible to compare humans that genetically lack LPL to copper deficient rats that have lower LPL activity per se.

The lower LPL activity in copper deficient animals is attributed to the possible role of copper in enzyme activation since metals are an integral part of at least 27% of known enzymes. The exact mechanism is, however, not understood (Lau & Klevay, 1982).

2.7.3 COPPER AND LECITHIN : CHOLESTEROL ACYL TRANSFERASE (LCAT)

The cholesterol of peripheral tissues has to be esterified before being incorporated into HDL₃, converting it finally into HDL₁, a heavier particle that is involved in reverse cholesterol transport (Gotto, 1990). The plasma enzyme LCAT catalyses the transfer of fatty acids from the C-2 position of lecithin to the hydroxyl group of unesterified cholesterol (Lau & Klevay, 1982).

This enzyme is regarded as either a metallo-enzyme or as enzyme that is dependant on copper for its synthesis. This is due to a decrease in its activity during copper deficient states (Lau & Klevay, 1982) LCAT activity is increased in both animals and humans that exercise.

In humans a portion of esterified cholesteryl esters from the LCAT reaction are eventually located in LDL increasing its cholesterol content (Vega, *et al.*, 1991). Although LDL is not a direct substrate for the LCAT reaction, this is possible via the transfer protein activity which transfer the esters from HDL to LDL. Only 10% of this ester fraction can be transferred in this manner due to an equilibrium that exists between the surface of the lipoprotein and the central core of cholesteryl esters in the lipoprotein (Fielding & Fielding, 1981).

In copper deficiency, decreased LCAT activity would mean greater accumulation of cholesterol in the tissues. During prolonged inadequate copper nutriture, this could result in excessive buildup of cholesterol in peripheral tissues resulting in foam cell formation and eventual growth of atheromatous plaques.

In rats due to the absence of cholesteryl ester transfer protein, an increase in HDL₁ subfraction does not involve in any interchange with LDL. This implies that any esterification by LCAT is carried only on the HDL subfraction (Oschry & Eisenberg, 1982).

In rats that are copper deficient there is an increase in the HDL₁, a similar

pattern that is apparent in cholesterol fed animals (Oschry & Eisenberg, 1982). In humans an increase in HDL₁ can only occur if there is an increase in LCAT activity. Since LCAT is regarded as an enzyme that is copper dependent it would be expected that there would be a decrease in LCAT activity in copper deficient states (Lau & Klevay, 1982). This therefore demonstrates a fundamental difference in the origin of HDL₁ in humans and rats. Therefore rat HDL₁ that is rich in cholesteryl ester is often regarded as a fraction of unknown origin. Although the levels of HDL₁ rise during copper deficient states, this could be due to factors other than LCAT activity (Lefevre, *et al.*, 1985).

2.7.4 COPPER AND THYROID FUNCTION

The positive correlation between hypothyroidism and hypercholesterolemia is well accepted (Allen, *et al.*, 1982; Lefevre, *et al.*, 1985). In the rat thyroid hormones have been implicated in hypercholesterolemia by stimulating hepatic cholesterol biosynthesis, decreasing intestinal cholesterol absorption and increasing bile formation and cholesterol excretion (Allen, *et al.*, 1982).

In copper deficient rats, serum thyroxine levels are significantly lower than control rats and the resultant hypercholesterolemia has been attributed to a decrease in LDL degradation (Allen, *et al.*, 1982). The degradation of LDL can be increased in such a deficient condition by intravenous thyroxine administration (Allen, *et al.*, 1982). This indicates that the hypercholesterolemia could partly be due to the thyroxine deficiency that is encountered in copper deficient animals (Lefevre, *et al.*, 1985).

The mechanism of this action and the effect of thyroxine deficiency on apolipoprotein is still unknown (Lefevre, *et al.*, 1985). The decrease in serum thyroxine levels is due to a decrease in thyroxine synthesis or secretion in copper deficiency. The functioning of the pituitary gland is, however, not affected in copper deficiency since thyrotropin-releasing hormone levels are normal (Allen, *et al.* 1982).

2.8 SUPEROXIDE DISMUTASE AND LDL OXIDATION

The copper containing superoxide dismutase (CuSOD) enzyme that is present in eukaryotic cells (including aortic and pulmonary artery cells) prevents lipid peroxidation by O_2^- radicals. These radicals are formed by cellular reactions and are known to be involved in peroxidation of LDL (Paynter, *et al.*, 1979) this results in a number of structural changes to LDL viz. fragmentation of apo B and covalent binding of lipid oxidation products to apo B.

Aortic and liver endothelial cells clear oxidized LDL more rapidly than native LDL (Steinbrecher, *et al.*, 1990). A recently characterized scavenger receptor has been implicated in being very receptive to oxidized LDL (Masana, *et al.*, 1991). The scavenger receptor uptake can thus result in a massive accumulation of intracellular cholesterol esters that can lead to foam cell formation (Masana, *et al.*, 1991). Foam cell formation is one of the steps in the pathogenesis of atherosclerosis.

The body copper status is positively correlated with CuSOD activity (Paynter,

et al., 1979). Since the copper concentration in the tissues vary (Evans, 1973) the CuSOD activity varies accordingly. The liver, kidney and red blood cell have greater activity than heart, brain, lung and muscle respectively (Paynter, *et al.*, 1979). A copper deficient diet can therefore lead to a greater concentration of O_2^- radicals in tissues. In fact it has been shown that the liver can be damaged by lipid peroxidation in copper deficient rats (Paynter, *et al.*, 1979). Extracts of LDL from human atherosclerotic plaques have also shown changes in apo B100 structure and electrophoretic mobility; changes that are compatible with oxidation of LDL. Probucol, an antiatherotic drug that inhibits oxidation of LDL has been shown to be very effective in the prevention of LDL oxidation and as well as its uptake in various tissues (Steinbrecher, 1990).

It would be reasonable therefore to speculate that CuSOD activity has a protective effect of minimizing the generation of O_2^- radicals that ultimately ends in cholesterol deposition in peripheral tissues.

2.9 COPPER AND LYSYL OXIDASE

Spontaneous rupture of blood vessels and death have been reported in many experimental animals that are deficient in copper (Starcher, 1964; Prohaska, *et al.*, 1982). These ruptures have been associated with a depressed lysyl oxidase activity. Lysyl oxidase is a cuproenzyme that is responsible for cross linking of select peptidyl lysine residues to peptide aldehyde in elastin and collagen (Opsahl, *et al.*, 1982). These cross links enable structures with collagen or elastin to be pliable. The absence of cross links as in copper

deficiency renders these tissues fragile and prone to rupture (Opsahl, *et al.*, 1982).

Copper plays an integral part in lysyl oxidase activity by binding to the enzyme, increasing its stability and decreasing its degradation. Copper is delivered to the site by ceruloplasmin (Harris, 1976). Therefore low copper levels as verified by low ceruloplasmin levels renders lysyl oxidase less stable and prone to proteolysis.

Although a decrease in dietary copper does decrease lysyl oxidase function, low levels of lysyl oxidase do not cause hypercholesterolemia. Since low lysyl oxidase activity can be corrected with copper supplementation, low copper intake over a period of time early in life could result in lesion formation (Klevay, *et al.*, 1977). Therefore hypercholesterolemia later in life could result in an atherosclerotic plaque forming at the point of the initial lesion. This suggests a role of copper in the initial and subsequent steps in atherosclerosis (Klevay, *et al.*, 1977). This could be a reasonable speculation since the average American diet is marginally deficient in copper at ± 1 mg/day (Lau & Klevay, 1981). This does not even compensate for urinary and faecal losses estimated to be 1.30 mg/day (Lau & Klevay, 1981).

2.10 HIGH DIETARY COPPER

More animal studies on copper have been limited to the effects of copper deficiency rather than an excessive copper intake (Liu, *et al.*, 1986).

Detailed studies on rats using dietary copper as high as 100 mg/kg diet as used in this study, have however been reported (Liu, *et al.*, 1986). Significant increases in systolic blood pressure and blood haemoglobin levels were the only differences from control animals. There were no significant differences in serum total cholesterol, triglycerides or liver copper concentration. Liver copper concentrations are in fact not altered significantly even with a dietary copper intake of 500 mg/kg diet (Liu, *et al.*, 1986).

This is due to the recently discovered ability of hepatocytes to accumulate excess copper in lysosomes. These lysosomes promptly secrete their contents directly into bile thus alleviating copper overload (Gross, *et al.*, 1989). Direct secretion of copper into bile has also been suggested as a mechanism of biliary excretion of copper, but this mechanism remains speculative (Gross, *et al.*, 1989).

The increase in blood haemoglobin concentration in animals on very high dietary copper intakes is due to the greater availability of iron for haemoglobin synthesis. Since copper plays a role in mobilizing iron for heme synthesis. Higher haemoglobin concentrations increase blood viscosity resulting in an increase in systolic blood pressure. This could explain the higher systolic blood pressures observed in rats fed a high copper diet (Liu, *et al.*, 1986).

Human epidemiological studies have shown a strong positive correlation between high serum copper and the risk of developing coronary artery diseases

(Kok, *et al.*, 1988). Although positive correlations between hypercholesterolemia (due to high LDL fraction) and low serum copper levels have been shown in humans, results are nevertheless inconsistent (Kok, *et al.*, 1988). Animal studies have however shown that marginal copper intakes results in hypercholesterolemia (Field, *et al.*, 1983; Carr & Lei, 1990). These data suggest that there is a U-shaped relationship between dietary copper and the risk of developing coronary artery disease (Kok, *et al.*, 1988).

A high dietary copper intake results in elevated levels of non-specifically bound copper in tissues and serum (Kok, *et al.*, 1988). These species could catalyse free radical formation. In vitro studies have shown that oxidation of LDL takes place only in the presence of Cu^{+2} (Kalant, *et al.*, 1991). Oxidized LDL is readily taken up by peripheral tissues including major blood vessels via recently characterized scavenger receptors (Masana, *et al.*, 1991). This mechanism has been implicated as being one of the major steps in the pathogenesis of atherosclerosis (Kalant, *et al.*, 1991).

Elevated dietary copper could thus be involved in the pathogenesis of atherosclerosis even without corresponding hypercholesterolemia. This could be the underlying reason for the inconsistent positive correlation between coronary artery diseases and total cholesterol in epidemiological studies (Kok, *et al.*, 1988).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. ANIMALS AND HOUSING

Forty eight weanling male Sprague Dawley rats, weighing 60-100 g were used for the study.

The rats were assigned to four groups viz:

A Control - standard AIN-76 diet

B Normal copper + 1 % cholesterol

C Deficient copper + 1 % cholesterol

D Excess copper + 1 % cholesterol

Each rat was housed individually in polyethylene cages with suspended stainless steel grid flooring (Techniplast, Italy). Access to polyethylene food trays was via a small suspended stainless steel tunnel. All wasted diet was collected in a separate compartment. Polyethylene water bottles with stainless steel nozzles were used. The room was kept at constant temperatures with a constant 12 hour day/night cycle.

All cages, water bottles and food trays were washed weekly in Teepol and rinsed in deionised water.

3.2 DIET

The rats were fed a purified diet formulated by the American Institute of Nutrition(AIN-76). The diet details of four groups are as follows:

- A. Control, basal diet formulated on the AIN-76 diet.
- B. Normal copper, 6mg/kg diet, 1% cholesterol(Merck)
- C. Deficient copper, no copper; 1% cholesterol.
- D. Excess copper, 100 mg/kg; 1% cholesterol.

Note: In all groups zinc was kept constant at 30 mg/kg diet. The supplementation of the copper and cholesterol were followed as recommended by the American Institute of Nutrition.

The composition of the AIN-76 purified diet is as follows:

<u>Diet</u>	<u>%</u>
Casein	20.0
DL Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fibre	5.0
Corn oil*	5.0
AIN Mineral mix	3.5
AIN Vitamin	1.0
Choline bitartrate	<u>0.2</u>
	100.0

* In the cholesterol supplemented groups(B,C and D) 1% cholesterol was

added and 4% corn oil was used(Koo & Ramlet,1983)

AIN-76 Vitamin Mixture

<u>VITAMIN</u>	<u>per kg mixture</u>
Thiamin HCL	600 mg
Riboflavin	600 mg
Pyridoxine HCL	700 mg
Nicotinic acid	3.0 g
D-Calcium pantothenate	1.6 g
Folic acid	200 mg
D-Biotin	20 mg
Cyanocobalamin	1 mg
Retinyl acetate	400 000 IU
dl- α -Tocopheryl acetate	5 000 IU
Cholecalciferol	2.5 mg
Menaquinone	5.0 mg
Sucrose(finely powered)	to make 1000.0 g

AIN-76 Mineral Mixture

<u>Ingredient</u>	<u>g/kg mixture</u>
Calcium phosphate, dibasic	500.0
Sodium chloride	74.0
Potassium citrate monohydrate	220.0
Potassium sulphate	52.0
Magnesium oxide	24.0
Magnesium carbonate (43-48%)	3.5
Ferric citrate (16-17%)	6.0
Zinc carbonate (70%)	1.6
Cupric carbonate (53-55%)	0.3
Potassium iodate (KIO ₃)	0.01
Sodium selenite (Na ₂ SeO ₃ .SH ₂ O)	0.01
Chromium potassium sulphate	
[CrK(SO ₄) ₂ . 12 H ₂ O]	0.55
Sucrose, finely powered to make	1000.0 g

3.2.1 DIET PREPARATION AND STORAGE

All diet ingredients were weighed in plastic weighing boats on an electronic balance(Mettler BB 300). Plastic inspection gloves were always worn when handling diet ingredients or diet. All crystal/salt components were ground to a similar consistency mortar and pestle.

Final mixing of the salt mix commenced with the compounds of lowest mass and gradually via a series of trituated steps ingredients of increasingly greater mass were finally included. Thorough mixing was carried out upon each addition in a mortar and pestle. The salt mixture was finally mixed in a mechanical food mixer(Foster Equipment, Co, England) for 1 hour.

Two salt mixtures were prepared, one standard AIN-76 mixture and the other having no copper (cupric carbonate). The standard AIN-76 salt mixture was used in the high copper diet. The diet was, however, supplemented with cupric carbonate to produce a final diet containing 100 mg/kg copper. The salt mixtures were stored in air tight, light resistant containers.

The vitamin mix was prepared in a similar manner but the fat soluble vitamins were mixed with either sucrose or another vitamin(powdered form) of comparable mass. The vitamin mix was stored in a refrigerator.

The final diet was prepared in 2 kg batches. Mixing commenced with the mortar and pestle and final mixing was carried out in the mechanical mixer. The fibre was mixed with either the corn oil or cholesterol crystals. This helped to stabilize the fibre and prevent any loss during mixing. Since the corn oil (Sigma chemicals USA) did not contain anti oxidants the diet was stored in plastic bags in a refrigerator. Storage of large quantities of diet was therefore avoided as recommended by AIN in its second report on nutritional standards (AIN-1980).

3.2 DIET ADMINISTRATION AND MONITORING

All four groups were fed their respective diets *ad libitum*. Recordings of diet consumption were done at approximately the same time each day.

Deionised water was obtained using an Elgastat B114 deionising unit. Water was also made available *ad libitum*. All animals were weighed weekly and examined for any irregularities. The faeces and urine were also examined for any change in colour; consistency and traces of any blood or cloudiness.

3.3 SAMPLE COLLECTION

The day before sample collection, the animals were weighed and were fasted overnight.

The animals were anaesthetized in a 1.7l closed circuit anaesthetizing chamber with 0.2 ml Fluothane(active ingredient 2 Bromo; 2 chloro 1,1,1 trichloroethane.)

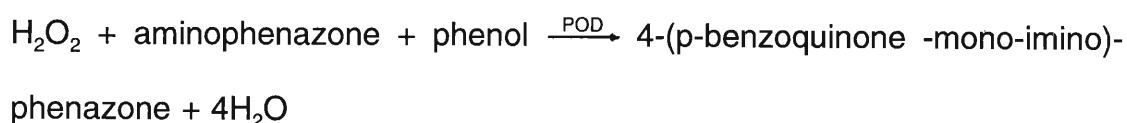
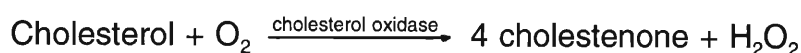
Blood was collected by the cardiac puncture method into syringes with 19 gauge needles. One ml of blood was dispensed into a heparinized microvessel which is used for routine blood analysis. The remaining blood was transferred into glass centrifuge tubes and allowed to stand for 10 minutes at 3000 rpm in a bench top centrifuge. The serum was removed using disposable Liqui-pipettes and frozen in polyethylene flip top vials at -10°C.

After the blood was removed the rat was dissected to expose the thoracic cavity. The liver was removed without any fat and stored in polyethylene tubes in deep ice. Both hind limbs were also removed after being skinned. The tibia was cleared of excess musculature and stored in polyethylene tubes in ice. After sample collection, the liver and bones were stored at -10°C.

3.4 ANALYSIS

3.4.1 CHOLESTEROL

Total cholesterol was determined with Boehringer Mannheim cholesterol monotest kits using the Chod-Pap method. The end product of the test kit reaction, benzoquinone was determined photometrically. The reaction on which the test principle is based is as follows:

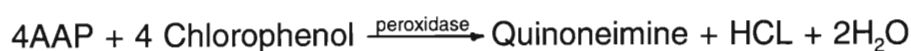
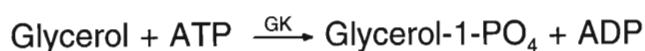


The end result was determined photometrically at 500 nm (Pye Unicam). The reaction mixture was increased by a factor of 1.5. All instructions were followed as per Boehringer instruction sheet.

3.4.2 TRIGLYCERIDES

The triglyceride levels were determined by using the Beckman Dri-Stat^R reagent kit. The end product of the 4 step reaction, a Quinoneimine dye which is directly proportional to the concentration of triglycerides is determined photometrically at 500 nm.

The enzymatic steps in this reaction are:



The instructions were followed as per Beckman instruction booklet.

3.4.3 HIGH DENSITY LIPOPROTEIN (HDL) CHOLESTEROL

Boehringer HDL precipitant was used to precipitate very low density lipoprotein (VLDL), chylomicrons and low density lipoproteins (LDL). The HDL is therefore left in the supernatant. The test kit contains phosphotungstic acid and magnesium ions that cause the precipitation reaction.

The HDL concentration was determined by Boehringer Monotest Cholesterol kits as outlined in 3.5.1.

During all reactions, Merck Qualitrol control sera were used. All pipetting were

ge with Gilson micropipettes. The reactions were carried out in matched quartz cuvettes at $37^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$.

3.5 LIPOPROTEIN ELECTROPHORESIS

Electrophoretic separation of lipoproteins were done on agarose gel. Beckman Paragon lipoprotein electrophoresis kits were used as per Beckman instruction. The lipograms were scanned qualitatively on a densitometer(Gelman ACD 18)

3.6 TRACE ELEMENT ANALYSIS

Wet ashing in 65% Nitric acid (Spectrosol: Merck) was chosen to solubilize the tissues (bone and liver) for atomic absorption analysis. Wet ashing has been found to yield better results for copper and zinc than dry ashing(Clegg *et al.*, 1981). The results obtained by Clegg *et al.*(1981) with wet ashing differed by less than 1.5% from that of values given by the American National bureau of Standards (NBS)

The procedure that was followed to wet ash liver and bone are as follows.

3.6.1. LIVER

The frozen liver was thawed and a section was cut from the median lobe of the liver. The section was blotted on ashless filter paper and approximately 0.5g of tissue was used. The liver slice was added to a 50ml Erlenmeyer flask together with 5ml nitric acid and three glass beads. The flask was sealed with parafilm. The liver was allowed to predigest for 12 hours after which the flasks

were mounted on an electric digester fitted with a constant draught manifold. All extracted fumes eventually mixed with water that ran down the sink of the fume cupboard.

The temperature of the flasks were carefully controlled so that the mixture boiled slightly for 4 hrs (Clegg, *et al.*, 1981). During each digestion at least one standard and a blank were included. The end product was a light yellow clear digest that was diluted to 25 ml with deionised water.

3.6.2 BONE

Bones were removed from freezer and cleared of any adhering tissue. The cartilage was removed from both the epiphysis, weighed and placed in a beaker in a drying oven at 100°C. After 12 hrs, the mass of bones remained constant. The bones were weighed for final mass and put into a desiccator for 1 hr.

The desiccated bones were put into a 50ml volumetric flask that contained 5.00 ml nitric acid and three glass beads. The digestion procedure followed was as described for that of liver.

The final acid digest was diluted to 10 ml in an Erlenmeyer flask with deionised water.

During the handling of both tissues, plastic gloves were worn and plastic spatulas used. All glassware were acid washed for 24 hrs in 20% nitric acid

and rinsed thoroughly in deionised water. Both copper and zinc standard solutions at a concentration of 1.0 mg/ml were used (BDH, Poole, England).

3.6.3 TRACE ELEMENT ANALYSIS

A Perkin Elmer A 2380 (Perkin Elmer, Norwalk, CT) atomic absorption spectrophotometer with an air acetylene flame was used. It was programmed to take an average of three readings, utilizing a 3 s integration time. All readings were obtained in the concentration mode.

3.7 STATISTICAL ANALYSIS

All tabulated data was statistically analysed. The Kruskal - Wallis test was used for intergroup comparison. If significant differences were observed, the Wilcoxon 2 sample test was performed using Bonferroni's adjustment. Differences in the latter test were considered significant if $p < 0.0125$.

This statistical procedure was adopted due to the sample size ($n = 12/\text{group}$) and group number (4 groups) that was used in this study.

The student t test was applied to analyse the densitometric results.

CHAPTER FOUR

4. RESULTS

4.1 BLOOD ANALYSIS

Table 4.1 Cholesterol concentration (mg/dl)

Group	Mean	S.D.
A	96.46	10.5
B	86.88	15.43
C	108.07	11.64
D	88.04	11.04

The Kruskal-Wallis showed a significant difference in cholesterol concentrations. The Wilcoxon test, however, showed significant differences between groups B and C ($p = 0.0014$) and groups C and D ($p = 0.0005$). Group C having a higher level than B and D. There was no significant difference between groups A and B ($p = 0.1486$) and groups A and C ($p = 0,0178$).

Table 4.2 Triglyceride concentration (mg/dl)

Group	Mean	S.D.
A	49.12	13.68
B	47.64	26.68
C	45.00	26.66
D	41.00	12.84

No significant difference in triglyceride concentrations were observed between any of the groups ($p = 0.5935$).

Table 4.3 HDL concentration (mg/dl)

Group	Mean	S.D.
A	29.90	5.63
B	27.61	3.51
C	32.49	3.72
D	28.52	6.86

The Kruskal Wallis showed a significant difference between the groups ($p = 0.0470$).

A significant difference was found only between groups B and C ($p = 0.0101$) using the Wilcoxon test.

Table 4.4 LDL concentration (mg/dl)

Group	Mean	S.D.
A	57.10	11.2
B	48.28	18.12
C	66.28	10.08
D	51.20	8.74

The Kruskal Wallis test showed a significant difference between the groups ($p = 0.0036$), but the significant difference was, however, found only between groups C and D ($p = 0.0036$) using the Wilcoxon test.

Table 4.5 Haemoglobin concentration (g/dl)

Group	Mean	S.D.
A	12.72	1.14
B	12.72	1.03
C	12.02	1.65
D	13.57	1.07

A significant difference was only observed between groups C and D ($p = 0.0051$). Group D having the higher value and Group C the lowest value of the groups.

Table 4.6 Haematocrit (%)

Group	Mean	S.D.
A	44.00	2.48
B	43.50	3.60
C	41.37	4.52
D	45.21	2.58

No significant differences were found between the groups ($P = 0.0664$).

4.2 TRACE ELEMENT ANALYSIS

4.2.1 COPPER ANALYSIS

Table 4.7 Liver copper concentration (ug/g)

Group	Mean	S.D.
A	5.38	1.40
B	6.10	1.26
C	3.37	1.05
D	6.70	1.11

A significant difference was found between the 4 groups using the Kruskal-Wallis test (p = 0.0001).

Table 4.8 Bone copper concentration (ug/g)

Group	Mean	S.D.
A	3.26	1.25
B	2.82	0.72
C	2.17	0.70
D	3.13	0.74

No significant differences were found between the groups using the Kruskal-Wallis test ($p = 0.034$).

Table 4.9 Liver zinc concentration (ug/g)

Group	Mean	S.D.
A	26.91	0.56
B	28.12	3.97
C	23.25	2.35
D	26.29	2.18

A significant difference was observed between the groups using the Kruskal-Wallis test ($p = 0.0027$).

Table 4.10 Bone zinc concentration (ug/g)

Group	Mean	S.D.
A	20.20	1.73
B	21.50	2.20
C	20.86	2.68
D	20.89	2.21

No significant differences between the groups were observed using the Kruskal-Wallis test ($p = 0.553$).

4.3 CORRELATION ANALYSIS

The antagonistic relationship between dietary zinc and copper creates the possibility of an association between tissue zinc and copper levels. The Spearman Correlation Test was carried out to check for any association between tissue zinc and copper level.

Table 4.11a

Group A

	Zinc : Bone	Zinc : Liver
Copper : Bone	0.263	
Copper : Liver		-0.249

Table 4.11b

Group B

	Zinc : Bone	Zinc : Liver
Copper : Bone	-0.166	
Copper : Liver		0.028

Table 4.11c

Group C

	Zinc : Bone	Zinc : Liver
Copper : Bone	-0.329	
Copper : Liver		0.246

Table 4.11d

Group D

	Zinc : Bone	Zinc : Liver
Copper : Bone	-0.486	
Copper : Liver		0.221

INTERPRETATION OF CORRELATION

- $r \geq 0.7$ strong correlation
- $0.3 \leq r < 0.7$ moderate correlation
- $r < 0.3$ weak correlation

negative r value denotes a negative correlation.

4.4 FOOD CONSUMPTION

Table 4.12 Average weekly food consumption (g)

Wk	A	B	C	D
1	100.7	96.1	96.9	92.4
2	109.2	109.6	107.5	109.2
3	120.8	119.3	118.9	119.7
4	119.2	120.9	116.4	120.4
5	117.8	120.0	116.5	123.0
6	116.9	117.9	120.0	123.6
7	119.9	109.6	114.3	121.1
8	116.0	102.0	111.9	110.7
Σ	920.5	895.4	902.4	920.1

No significant difference in food consumption was observed between the groups using the Kruskal-Wallis test ($p = 0.544$).

Table 4.13a. Increase in mass (g)

Wk	A	B	C	D
1	78.3	75.7	79.2	76.0
2	135.8	131.4	136.1	128.5
3	183.7	179.0	183.8	176.7
4	264.4	259.4	257.2	261.0
5	294.6	289.3	284.8	286.1
6	318.8	313.7	307.3	312.4
7	338.1	327.8	320.1	334.0
8	350.5	323.0	326.0	342.0

Table 4.13b. Percentage gain in mass(g)

Wk	A	B	C	D
0	0	0	0	0
1	73.4	73.6	71.8	69.1
2	134.6	136.5	132.1	132.5
3	237.7	242.7	224.7	243.4
4	276.2	282.2	259.6	276.4
5	307.2	314.4	288.0	311.1
6	331.8	333.0	304.2	339.5
7	347.6	339.0	311.6	351.3
8	348.7	342.7	312.6	351.3

Table 4.14 Densitometric analysis of lipoproteins

Fraction	A	B	C	D
Beta (LDL)	81.32	80.66	81.13	70.93
S.D. ±	13.11	10.74	9.97	13.86
Alpha (HDL)	18.68	20.24	17.97	28.07
S.D. ±	12.90	9.78	18.03	12.82

The student t test showed no significant difference in the alpha and beta fractions between Groups A-B; A-C and B-C. A significant difference in the Alpha and Beta fractions was ,however, shown to exist between A-D and hence the other two experimental groups. The differences were: alpha band $p < 0.05$ beta band $p < 0.05$.

Both the chylomicron and pre-beta bands were absent.

FIG.2 FOOD CONSUMPTION(g)

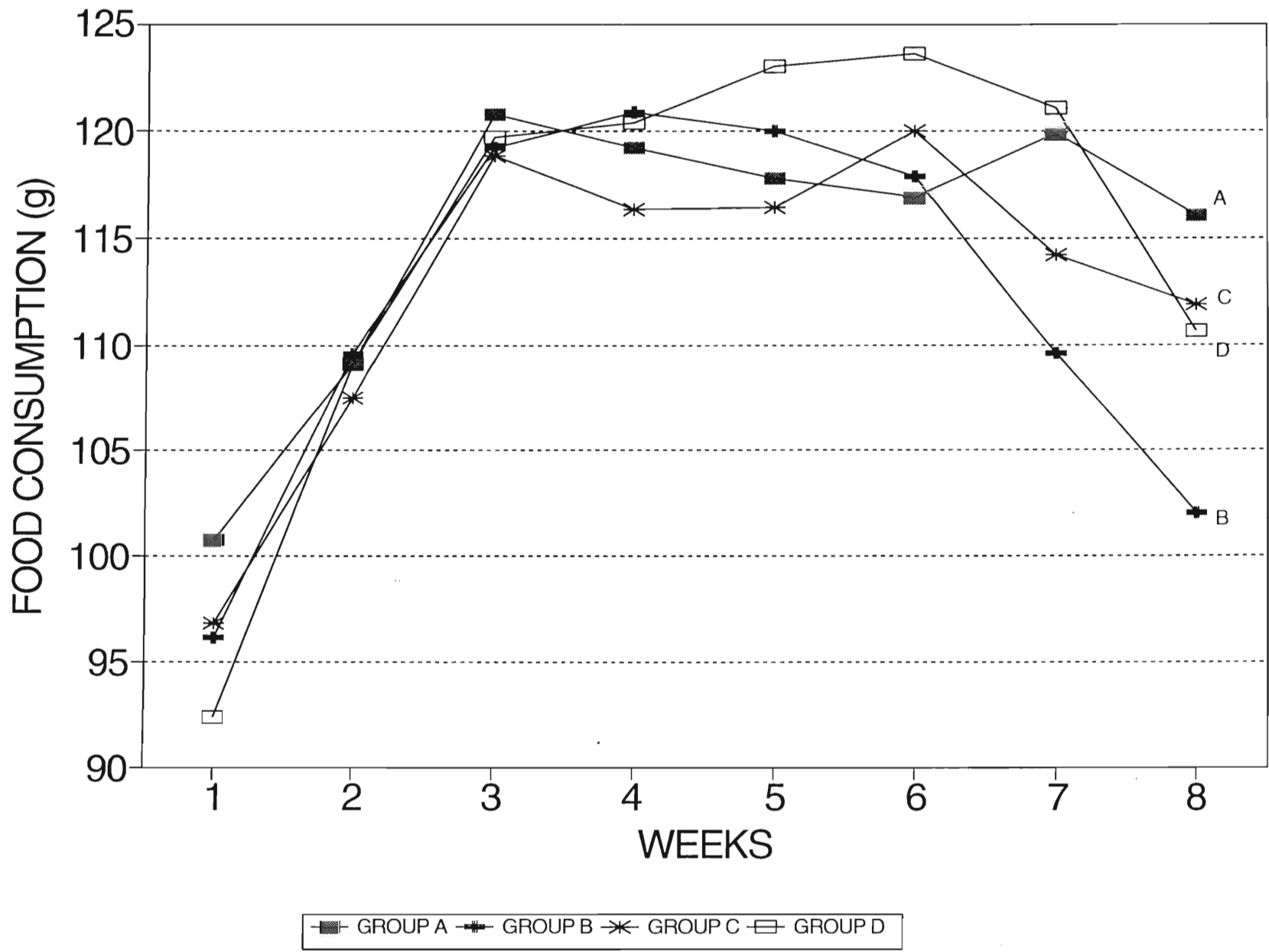
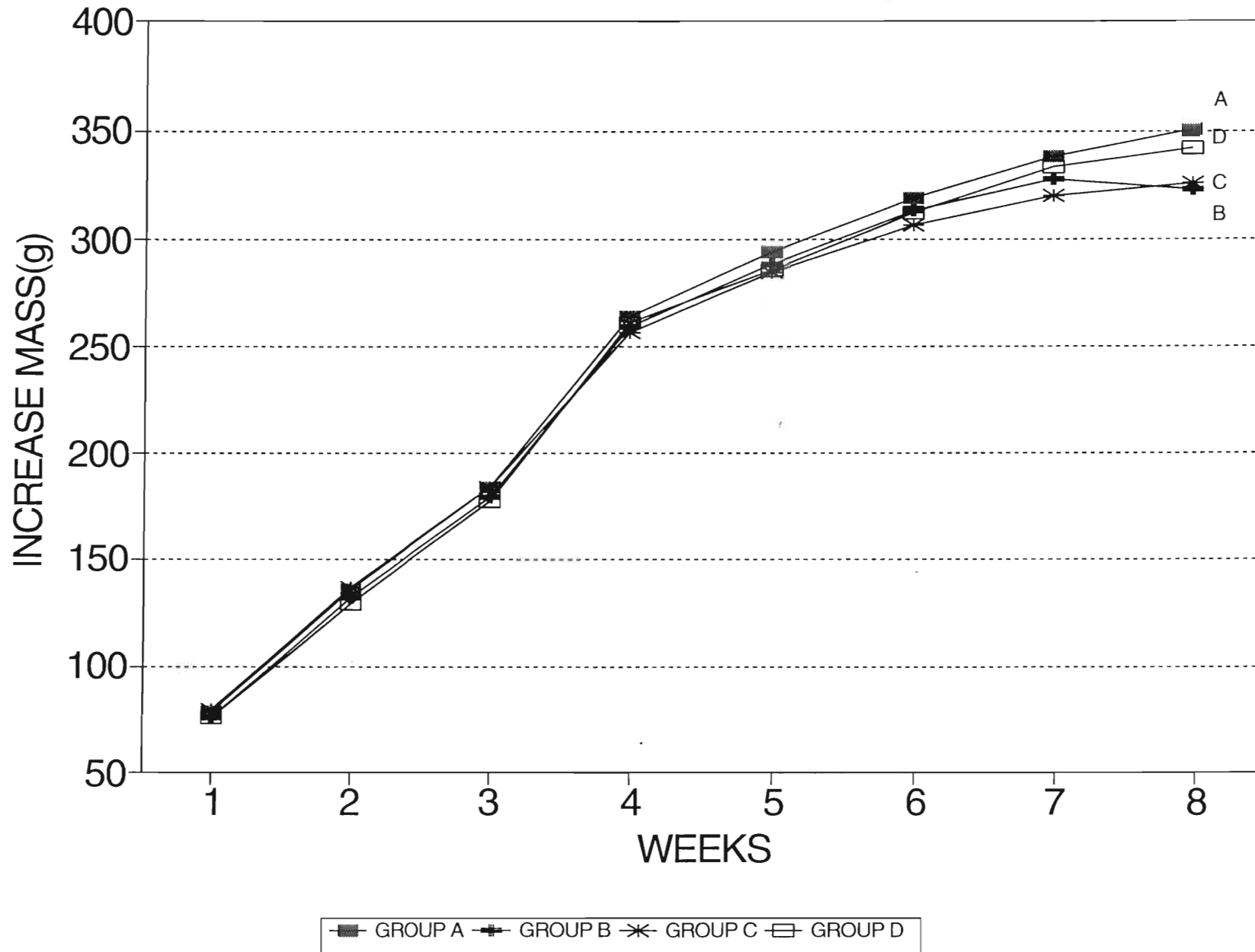


FIG.3 INCREASE IN MASS(g)



CHAPTER FIVE

5. DISCUSSION

5.1 GROWTH AND FOOD CONSUMPTION

In this study no differences in final body mass or growth was observed between the control and experimental groups. The difference between the control and experimental groups were as follows:

- Group A : Control AIN-76 diet (AIN-76 & AIN 1980)
- Group B : Control diet + 1% cholesterol
- Group C : Copper deficient + 1% cholesterol
- Group D : High copper intake (100 mg/kg diet) + 1% cholesterol.

N.B. Dietary zinc was constant at 30 mg/kg diet as recommended by AIN (AIN-76).

These results can be attributed to the food consumption which showed no statistical difference between the groups. These results imply that a diet either deficient or excessive in copper does not affect body mass or growth of rats during an eight week experimental period.

These results are in agreement with previous studies (Fischer, *et al.*, 1980) but differ from that of Lei(1983) who found significant reduction in growth and body mass in copper deficient rats. This discrepancy could partly be attributed to the nature of the carbohydrates used in the diets of these studies or the animals could have been in a greater deficiency state.

Sucrose and glucose as used in the latter study, magnifies the effects of copper deficiency compared to starch (Fields, *et al.*, 1983). The AIN-76 diet used in this study provides a better balance between starch and sucrose than in the diet used by Lei(1983).

5.2 TRACE ELEMENT ANALYSIS

The animals in experimental Group C fed a copper deficient diet were in fact copper deficient. This was confirmed by lower hepatic copper levels than the control (Table 4.7). Although liver copper levels were significantly lower than the control group ($p = 0.0001$) levels lower than $3 \mu\text{g/g}$ liver have been previously reported in copper deficient states (Allan *et al.*(1982) Lei, 1983). Hepatic copper levels are nevertheless considered a good index of body copper status (Allen *et al.*, 1982).

Excess dietary copper at a 100 mg/kg diet failed to produce an increase in hepatic copper concentration since no significant difference was observed between Groups A-D and B-D. These results also imply that dietary cholesterol fed at 1% had no effect on copper status in the rat. These

results are in agreement with other studies (Lei, 1986).

The ability to resist a change in copper status during high dietary copper intakes has been attributed to effective biliary excretion in rats. Excess hepatic copper produces an increase in hepatocyte lysosomes that sequester the copper. The lysosomal contents are thereafter excreted into the liver canaliculi via exocytosis. Since biliary copper reabsorption is negligible, this is regarded as the major excretory pathway of copper. (See Gross, *et al.*, 1988)

Analysis of bone copper levels have shown inconsistent results in this study. Rats on copper deficient diets have shown to have bone copper concentrations similar to that of rats on copper adequate diets (Group B). Excess dietary copper has also failed to produce a significant increase in bone copper levels since there is no difference between the control group and rats on excess copper diet (Group D). From this data it appears that bone copper levels are not an accurate index of copper status in rats. Hepatic copper levels are, however, considered to be a very good index of copper status (Allen, *et al.*, 1982) and is therefore used routinely in studies (Fischer, *et al.*, 1980; Field, *et al.*, 1983; Liu, *et al.*, 1986).

Analysis of tibia zinc levels have revealed no significant differences in zinc levels between the groups. This is of particular interest to this study since bone zinc concentrations are an important index of zinc status (Calhoun, *et*

al., 1974). This implies that dietary copper as high as 100 mg/kg diet (Group D) did not antagonise the uptake of zinc so as to affect zinc status. This appears as reasonable assumption since bone zinc levels are regarded as an indicator of dietary zinc levels and punch biopsies of bone are used to assess zinc status (Calhoun, *et al.*, 1974). Correlation analysis has, however, shown a weak negative correlation between bone copper and zinc levels in Groups B and D ($p = 0.245$ and 0.295 respectively). Since the dietary intake of Group B had levels of copper and zinc recommended by the American Institute of Nutrition (AIN) as in the control group, the correlation of Group B should be similar to Group A. Group A has a weak positive correlation ($p = 0.166$). It therefore appears that the correlation between bone copper and zinc levels are inconsistent.

Zinc liver levels showed a significant difference between the groups ($p = 0.027$). Animals fed the copper deficient diet (Group C) showed a significant decrease in zinc liver levels compared to the other groups. The currently accepted absorptive process of zinc and copper via metallothionein cannot be used to explain these results since low dietary copper has not been shown, affect zinc uptake (Oestricher & Cousins, 1985).

Human hair biopsy of survivors of myocardial infarctions have shown a consistently high ratio of zinc to copper (Bialkowska, *et al.*, 1987). Although chemical analysis of hair is controversial with respect to trace element status, high ratios of zinc:copper in human hair could be regarded as a

potential risk factor for coronary artery diseases (Bialkowska, *et al.*, 1987). If liver zinc to copper ratios can also be used in a similar manner, copper deficient animals in this study have the highest zinc:copper ratio of all groups. This could have implications in liver cholesterol metabolism since copper deficient animals also display hypercholesterolemia (Lau, 1982; Carr & Lei, 1990). No literature is, however, available on studies that specifically compare tissue zinc:copper ratios to hypercholesterolemia.

In a study by Lefevre, *et al.*, (1986). on copper deficiency and hypercholesterolemia, reported liver copper and zinc levels when expressed as a ratio yield similar trends as in this study (Lefevre, *et al.*, 1986).

5.3 SERUM CHOLESTEROL

The Wilcoxon test between groups showed significant differences in serum total cholesterol between groups B-C ($p = 0.0014$) and Groups C-D ($p = 0.0005$). No significant differences were observed between Groups A-B, A-D and B-D.

Trace element analysis of liver confirmed that rats on a deficient diet were in fact copper deficient. The resultant hypercholesterolemia observed in this group of animals could therefore be attributed to the copper deficiency.

Hypercholesterolemia in copper deficient animals and humans is well accepted (Klevay, *et al.*, 1984; Carr & Lei, 1990).

The hypercholesterolemia due only to the copper deficiency would be a reasonable assumption since dietary cholesterol fed at 1% had no significant effect on serum cholesterol. This was evident in the Wilcoxon A-B test comparison ($p = 0.1486$). Studies have, however, demonstrated a specific increase in serum HDL levels in rats fed cholesterol at the same concentration as in this study (Koo & Ramlet, 1983; Lefevre, *et al.*, 1986).

The exact mechanism that causes hypercholesterolemia in copper deficient rats is unknown (Carr & Lei, 1989). It has been postulated however, that the increase in cholesteryl ester synthesis by the liver could be due to a greater need for cholesterol during copper deficiency (Carr & Lei, 1990). An increase in cholesteryl ester synthesis is however confirmed by the increased rate at which labelled mevalonate ($[^{14}\text{C}]$ -mevalonate) is converted to cholesterol (Shao & Lei, 1980).

A shift in cholesterol from the liver pool to the serum pool has been proposed to explain the hypercholesterolemia evident in copper deficient rats (Shao & Lei, 1980).

Although high serum total cholesterol levels are often used as a risk factor against coronary artery diseases, analysis of the lipoprotein fractions provides a better insight into the possibility of an imbalance in lipid metabolism.

The hypercholesterolemia evident in copper deficient rats of this study was due to an increase in both HDL and LDL levels. This has been previously observed, in copper deficient Sprague-Dawley rats (Carr & Lei, 1990; Lei, 1983). Most studies have, however, found a significant increase in HDL fraction only (Lefevre, *et al.*, 1986).

The increase in HDL was only observed when groups B-C were compared ($p = 0.0101$). There were no differences between the other groups. This means that the HDL concentration of Group C did not change significantly from the control group. The mean HDL concentration of Group C is, however, slightly higher than that of the control group. Reports of other copper deficient studies have, however, found statistically significant differences between control and deficient groups (Carr & Lei, 1990). The diet of Group B can, however, be considered a normal balanced equivalent of the deficient diet. Therefore the increase in HDL concentration of the deficient group with respect to Group B indicates the trend that is generally observed in copper deficient rats (Lefevre, *et al.*, 1986).

The increase in the HDL concentration and subsequent increase in cholesterol pool size in copper deficient rats has been attributed specifically to a selective increase in the HDL₁ subfraction (Carr & Lei, 1990). This is due firstly to an increase in cholesteryl ester synthesis in the liver of copper deficient rats, the exact mechanism of which is unknown (Lefevre, *et al.*, 1986).

Secondly, due to the recently discovered disproportionate clearance of HDL cholesteryl ester compared to protein, larger quantities of cholesteryl esters are available in the liver of copper deficient rats (Carr & Lei, 1990).

Due to the absence of the cholesterol ester transfer protein in rats, the cholesterol esters are incorporated into the HDL fraction (Oschry & Eisenberg, 1982). The continued synthesis of cholesterol esters in copper deficient rats results in the constant enrichment of HDL₂ resulting in the formation of the heavier more dense HDL₁ subfraction (Lefevre, *et al.*, 1986).

Therefore copper deficiency in the rat has been shown to selectively affect HDL metabolism which in turn has obvious implication in cholesterol homeostasis.

The Wilcoxon test between groups showed a significant difference in serum LDL levels between Groups C-D ($p = 0.0004$). No significant differences were observed between any of the other groups. This implies that the copper deficient diet did not produce an increase in LDL levels relative to the control group. High dietary copper has, however, not been shown to affect serum lipid levels in previous studies (Liu, *et al.*, 1986).

The LDL values of this study are much higher than values obtained in other studies with rats of comparable age and mass, fed a similar diet (Carr & Lei, 1989). The average serum LDL value of 16.2 mg/dl has been reported for

previous studies in copper deficient rats (Lefevre, *et al.*, 1986). A lack in significant increase of serum LDL levels has been attributed to a possible marginal copper deficiency (Lefevre, *et al.*, 1986). The rats in this study were probably marginally copper deficient due to the relatively short duration of the experiment (8 weeks). Lower liver copper concentration have confirmed that rats in other studies were in a greater stage of copper deficiency than this study (Lei, 1983).

Lau and Klevay(1982) have hypothesised that the increase in LDL concentration observed in rats that are more severely copper deficient could be due to the resultant decrease in lipoprotein lipase activity. In view of the most recently elucidated metabolic pathway, this hypothesis appears invalid (Shepherd & Krauss, 1991). A decrease in lipoprotein lipase activity should result in a significant increase in VLDL concentration and its associated protein, apo B (Refer, Fig. 1). This is not always apparent in copper deficient rats (Lefevre, *et al.*, 1986). But it has also been shown that rats clear plasma VLDL extremely efficiently (Oschry & Eisenberg, 1982). It therefore appears that present knowledge of lipoprotein metabolism cannot totally explain the increase in serum LDL levels that is sometimes evident in copper deficient rats.

It has been shown, however, that Cu^{+2} is necessary for the solubilization of apo B in aqueous solutions. This strongly implicates Cu^{+2} and apo B *in vivo* (Lefevre, *et al.*, 1986). Therefore in copper deficient states there could be

an impairment in the apo Breceptor interaction. Since apo-B is strongly associated with LDL, the slight elevation of LDL that is sometimes evident in copper deficiency in the rat could be due this impaired apo-Breceptor interaction (Lau & Klevay, 1982).

The possibility of increasing LDL levels being secondary to increased HDL levels in rats appears apparent when proportions of apo-B100 and apo-E are correlated (Lefevre, *et al.*, 1986). This could explain the increase in LDL levels during a more severe copper deficient state (Lefevre, *et al.*, 1986).

Hypercholesterolemia due to a marginally deficient copper diet has been demonstrated in a male vounteer (Klevay, *et al.*, 1984). The hypercholesterolemia was due largely to an increase in the LDL subfraction. In humans, due to the presence of the chlosteryl ester transfer protein, an increase in cholesteryl esters are encorporated into the lower density liproporteins viz. LDL (Oschry & Eisenberg, 1982).

Serum LDL levels have been positively correlated with coronary artery disease. LDL levels above 160 mg/dl have been considered as a high risk for coronary artery diseases by the American National cholesterol Education Program (Vega, *et al.*, 1991). Although these findings on a single volunteer strongly support the role of copper in the etiology of coronary artery disease, more research needs to be carried out to determine the exact mechanisms underlying these changes in humans (Klevay, *et al.*, 1984).

Therefore epidemiological and experimental evidence indicates that copper plays a role in cholesterol metabolism and homeostasis in both humans and animals (Klevay, *et al.*, 1984). The average American diet has been shown to be marginally deficient in copper (< 1.0 mg/day) thus further supporting this hypothesis (Klevay, *et al.*, 1984).

No significant difference was observed in serum total cholesterol between Groups A-D ($p = 0.0931$) and B-D ($p = 0.9770$). This implies that dietary copper at 100 mg/kg diet had no significant effect on serum total cholesterol. This has been previously observed in rats (Liu & Medeiros, 1986). The liver is known to play a central role in lipoprotein metabolism. The liver copper concentration of rats fed copper at 100 mg/kg diet did not significantly change. This implies that there was no trace element imbalance in the liver that could lead to an alteration in lipoprotein metabolism. The failure to increase copper status during copper loading could be attributed to the efficient biliary excretory mechanism discussed in 5.2.

Despite not inducing hypercholesterolemia, high dietary intakes of copper have been implicated in coronary artery diseases (Kok, *et al.*, 1988). This is due to the elevated levels of non-specifically bound copper in serum. These species have been known to catalyse free radical formation that is capable of oxidizing LDL *in vitro* (Kalant, 1991). Oxidized LDL has been shown to be rapidly taken up by specialized scavenger receptors located in many tissues including aortic endothelial cells (Steinbrecher, *et al.*, 1990). Densitometric

analysis of the beta-fraction of animals fed a high dietary copper in this study supports this hypothesis. The results of this study should be interpreted cautiously, however, since oxidized LDL was not included as part of the controls in the electrophoretic run. This is discussed further in 5.5.

This mechanism has been strongly implicated in the pathogenesis of atherosclerosis for two reasons. Firstly, the scavenger receptors lack the sophisticated feedback system that normally regulates cellular cholesterol synthesis and secondly oxidized LDL has been shown to accumulate in cells (Steinbrecher, *et al.*, 1990).

Evidence to support this hypothesis is present in findings of a study conducted in Finland. In this study high copper concentrations in drinking water have been positively correlated with an increase in mortality due to coronary artery diseases (Punsar, *et al.*, 1974). In addition, material from human atherosclerotic plaques have been shown to be derived from oxidized LDL (Hoff, *et al.*, 1990). These results thus implicate dietary copper in coronary artery diseases without a resultant hypercholesterolemia (Kok, *et al.*, 1988).

Oxidized LDL and Free radical species can only be detected accurately by HPLC (Steinbrecher, *et al.*, 1990). This technique despite being beyond the scope of this study would have specifically determined the effect of the high

dietary copper on oxidized LDL and free radical species. Although liver copper concentrations showed no increase with high dietary copper intakes, serum copper analysis should have been carried out since it has been positively correlated with free radical formation (Kok, *et al.*, 1988).

5.4 TRIGLYCERIDES

Serum triglyceride levels showed no significant difference between the groups using the Kruskal-Wallis test ($p = 0.5935$). The triglyceride concentration ranged from 21.3-29.0 mg/dl. This is below the expected range of 39-128 mg/dl (Carol & Fieldman, 1987).

Although semi synthetic diets, as used in this study, have been known to increase the serum lipoprotein values, this was not observed in this study. The rats at the end of this study were approximately 4 months old, whereas data for most reference ranges are obtained from rats that are over 9 months old. This could be a possible reason for the below average triglyceride values obtained in this study. (See Loeb & Quimby, 1989.)

Triglyceride rich particles in humans (such as VLDL and chylomicrons) have been shown to be involved in the atherogenic processes both directly and indirectly (Shepherd & Krauss, 1991). Their overall risk to coronary artery disease, however, is debatable (Assman, *et al.*, 1991). Their role in atherosclerosis in humans can be attributed to the cholesteryl ester transfer protein. This protein promotes the exchange of cholesteryl esters for triglycerides between all serum lipoprotein species resulting in an increase in VLDL levels. High VLDL levels result in high LDL levels. (See Shepherd & Krauss, 1991.) In rats on the other hand due to the absence of the cholesteryl ester transfer protein, triglycerides would lose this aforementioned significance.

This is evident since even when hypercholesterolemia was present, this had no effect on triglyceride values in the rats of this study. Therefore this study has shown that dietary copper ranging from 0-100 mg/kg and cholesterol at 1% had no significant effect on serum triglycerides.

5.5 DENSITOMETRIC ANALYSIS OF LIPOPROTEINS

Densitometric analysis has shown no significant differences in the beta and pre-beta fractions between Groups A-B, A-C and B-C. The Student t test has, however, shown a significant difference in the alpha and beta fractions between Group D and the other groups (A, B, C).

Oxidized LDL has been shown to acquire a greater negative charge than native LDL. An increase in net negative charge will result in an increase in electrophoretic mobility (Kalant, *et al.*, 1991). This would imply that the beta band that is characteristically associated with the LDL fraction changes. The LDL fraction of Group D in this study decreased significantly compared to the control group ($p < 0.05$). Since high dietary copper has been implicated in LDL oxidation via free radical formation (Kok, *et al.*, 1988), this decrease in the beta fraction could be attributed to the oxidized LDL that had acquired an altered electrophoretic mobility. This would be an appropriate assumption since enzymatic analysis failed to show a significant increase in LDL levels of Group D.

Therefore although the lipoprotein kits used in this study have been

standardized for human lipoprotein fractions, they have indicated a possible alteration in mobility of the LDL fraction when rats are exposed to a high dietary copper intake. This warrants further investigation and the outcome would be able to shed some light on the hypothesis linking a high dietary copper intake to atherosclerosis via oxidized LDL.

The chylomicron and pre-beta bands were absent. The absence of the chylomicrons is partly due to the fact that the animals were fasted for 12 hrs. The absence of pre-beta band (predominantly VLDL) could be attributed to the extremely efficient clearance of VLDL in rats resulting in low VLDL levels. In addition, the cholesteryl ester concentration of VLDL is extremely low in rats compared to humans (Oschry & Eisenberg, 1982).

CONCLUSION

A copper deficient diet with normal dietary zinc levels administered during an eight week experimental period induced copper deficiency in rats. This was confirmed by trace element levels of appropriate tissues. Despite being moderately copper deficient, the resultant hypercholestroemia could be attributed to the copper deficiency. The characteristic increase in the HDL fraction of copper deficient rats was, however, not pronounced.

There was also no evidence of an interrelationship between 1% dietary cholesterol and serum lipoproteins or tissue trace element levels.

A high dietary copper did not affect the serum lipoprotein levels and body copper and zinc status. An alteration in the electrophoretic mobility of the LDL fraction, however, appeared evident upon densitometric analysis. This provides some indication of the possible presence of oxidized LDL species when dietary copper levels are high.

Studies have shown that the change in the HDL fraction of the rat during copper deficiency parallels that of human LDL change during similar conditions. Since current knowledge of lipoprotein metabolic pathways and mechanisms of interparticle lipid exchange are reasonably well established, extrapolation from animal studies can now be done within narrow limits.

With the discoveries of oxidized LDL and its uptake via special scavenger receptors, a relatively new era of research has been reached. The rat as a model in this particular field ,however, needs further investigation.

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