EFFECT OF FISH OIL ON LIPID AND LIPOPROTEIN METABOLISM IN F1B HAMSTERS

LANDE BANDARAGE PUJITHA PRASAD DE SILVA

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Effect of Fish Oil on Lipid and Lipoprotein Metabolism in F1B Hamsters

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

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ABSTRACT

Fish oil, rich in omega-3 polyunsaturated fatty acids, is known to decrease plasma triacylglycerol concentrations mainly by decreasing plasma very-low density lipoprotein (VLDL) levels. However, the influence of fish oil supplementation on plasma low-density lipoprotein (LDL) cholesterol concentrations is controversial. Human populations with type-2 diabetes mellitus show a significant increase in LDL-cholesterol concentration when fed high fat fish oil diets. However, the mechanism for this increase is not known. We used F_1B hamsters, a model susceptible for high fat diet-induced type-2 diabetes mellitus. Hyperlipidemia was induced by feeding dietary cholesterol. The hamsters were fed diets rich in either fish oil or a MIX oil to investigate whether fish oil has beneficial effects in lowering plasma lipid and lipoprotein levels. We found that fish oil at high fat levels caused a significant increase in chylomicron like particles, even after 14 hrs of fasting. The plasma LDL- and VLDL-cholesterol concentrations were significantly higher where as HDL-cholesterol concentration was significantly lower in fish oil fed hamsters especially at high fat levels. The hepatic microsomal triglyceride transfer protein (MTTP) activity was not significantly altered by fish oil, suggesting that increased levels of plasma VLDL is not due to increased production in the liver. The plasma cholesteryl ester transfer protein (CETP) activity in fish oil fed hamsters was lower compared to the MIX diet fed hamsters, thus this increase in LDL-cholesterol concentration and decrease in HDL-cholesterol concentration in fish oil fed hamsters are not a result of increased CETP activity. It appears that the clearance of lipoproteins is inhibited in fish oil fed hamsters. In conclusion, fish oil feeding especially at high fat levels might not be beneficial in treating hyperlipidemia under certain pathophysiological conditions.

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ABBREVIATIONS

apo	apolipoprotein
CETP	cholesterol ester transfer protein
DHA	docosahexaenoic acid
DNA	deoxyribo nucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPA	eicosapentaenoic acid
HDL	high-density lipoprotein
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
LDL	low density lipoprotein
LDLr	low-density lipoprotein receptor
mRNA	messenger RNA
MTTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acids
NBD-CE	synthetic phospholipid and cholesterol esters linked to a fluorescent labeled nitrobenzooxadiazole-fluorophor
NBD-TG	synthetic triglycerols linked to a fluorescent labeled nitrobenzooxadiazole-fluorophor
n-3 PUFA	omega-3 polyunsaturated fatty acids
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation

SREBP	sterol-regulatory element binding protein
TLC	thin layer chromatography
LPL	lipoprotein lipase
VLDL	very low-density lipoprotein

Chapter 1: Introduction

1.1 Health effects of fish oil

Fish oil is well known to protect against coronary heart disease. The first report supporting the view that dietary fish oil prevents coronary heart disease was made in the 1950s by a Seattle cardiologist, Nelson who observed a 4-fold reduction of coronary heart disease following increased fish intake (Dyerberg, 1986). Epidemiological studies on the health effects of fish oil began with observational studies conducted in a coastal settlement of Greenland Eskimos in the 1970s by Bang and Dyerberg. It was observed that the polyunsaturated fatty acid content of the diet was very high among the Eskimo population compared to Danes (Bang *et al.* 1976; Dyerberg, 1986).

Eskimos had an incidence of heart disease that was estimated to be about 8% of that seen in a comparable population in Denmark (Kromann and Green, 1980). In Japan, where fish consumption is traditionally high, a concomitant shift in tissue lipid composition favoring n-3 PUFA has been interpreted as causative of a relatively low incidence of cardiovascular thrombotic disorders (Hirai, 1980). Recently, Kromhout *et al.* (1995) reported the protective effect of fish oil intake in relation to coronary heart disease in a sample of 272 elderly people who were followed for 17 years.

The beneficial effects of fish oil are reported to be due to the n-3 polyunsaturated fatty acid (n-3 PUFA) content of fish oil. The 1st double bond of n-3 PUFA is at the 3rd carbon atom of the carbon chain from the methyl end. The major n-3 PUFA found in fish oil are eicosapentaenoic acid (EPA, 20: 5) and docosahexaenoic acid (DHA, 22: 6). Pfeiffer *et al.* (1962) showed that defatted fish flesh had no effect on plasma cholesterol levels in rats, whereas oil that was extracted from the flesh lowered cholesterol levels, suggesting that the active component in fish is fish oil. When human subjects were given

the saturated plus monounsaturated and polyunsaturated fractions of sardine oil, plasma lipid lowering effect resided only in the latter (Imachi *et al.*, 1963). Furthermore, the effects of fish oil on lipid metabolism have been largely reproduced by purified n-3 PUFA (Nossen *et al.*, 1986). Considering the weight of this evidence it is fair to state that n-3 PUFA in fish oil are primarily responsible for fish oil induced changes in lipid metabolism.

Plasma lipid and lipoprotein levels were measured in representative group of Eskimos and compared to an age and sex matched group of Danes (Bang *et al.,* 1972). Total lipids, cholesterol and triacylglycerols, very-low density lipoproteins (VLDL) and LDL (low-density lipoproteins) levels of plasma were lower and HDL (high-density lipoproteins) levels were higher in Eskimos compared to the Danes. Although similar changes in lipids and lipoproteins have been observed by feeding fish oil in many other clinically controlled studies, there are many differences between Eskimos and people in industrialized countries in their behavior such as, exercise, cigarette smoking, genetic variability, alcohol use. Some of these factors may also contribute to the differences in incidence of coronary heart disease.

1.2 Possible mechanisms of amelioration of coronary heart disease by fish oil

1.2.1 Plasma triacylglycerol concentrations and coronary heart disease

Increased plasma triacylglycerol concentrations are known to be associated with increased risk of coronary heart disease, but the role of triacylglycerol levels as an independent risk factor remains unclear. For the first time Zilversmit (1979) proposed

that cholesterol ester-enriched chilomicron remnants, found in the postprandial phase are as atherogenic as LDL. Since then it has been shown that increased postprandial triacylglycerol concentrations are associated with the formation of small cholesterol ester-rich chylomicron remnants, which share the ability to mediate cholesterol influx into the arterial intima along with LDL (Shaikh *et al.*, 1991). In addition, there is a recent body of evidence to support the hypothesis that postprandial triacylglycerol metabolism plays a causal role in the pathogenesis and progression of coronary heart disease (Karpe and Hamsten, 1995; Bergeron and Havel, 1997).

1.2.2 Fish oil and fasting plasma triacylglycerol concentrations

It has been established that fish oil suppresses plasma triacylglycerol concentrations in both fasting (Wong *et al.*, 1984; Surette *et al.*, 1992b) and postprandial states (Ikeda *et al.*, 2001). The reduction in plasma triacylglycerol concentrations by fish oil in the fasting state is ascribed to the suppression of hepatic secretion of VLDL triacylglycerols (Nossen *et al.*, 1986; Wong and Paul, 1987). Several human studies have examined the mechanism of triacylglycerol-lowering effect of fish oil. Harris *et al.* (1984) found that n-3 polyunsaturated fatty acids prevented and rapidly reversed carbohydrate-induced hypertriglyceridemia in normolipidemic subjects. Since carbohydrate feeding is known to stimulate triacylglycerol synthesis and VLDL secretion (Mancini *et al.*, 1973), this finding suggested that fish oil inhibits hepatic triacylglycerol synthesis. Kinetics of VLDL apolipoprotein B (apoB) and triacylglycerols in normal human subjects and hypertriglyceridemic patients were examined by Nestel *et al.* (1984). They found that the triacylglycerol lowering effect was mainly attributed to hepatic

triacylglycerol production rather than increased removal. According to this study, the synthesis rates of VLDL apoB and triacylglycerol are reduced by more than 50%. Experiments on both human (Wong *et al.*, 1987) and animal (Wong *et al.* 1985; Nossen *et al.*, 1986,) hepatic cells have further shown that n-3 PUFA from fish oil inhibit hepatic triacylglycerol synthesis and secretion.

Low doses of fish oil (Sanders *et al.*, 1985) as well as high doses of fish oil (Connor *et al.*, 1986) inhibit VLDL triacylglycerol synthesis. Several human studies have shown that n-3 PUFA from fish oil reduce fasting plasma triacylglycerol concentrations dose dependently (Sanders and Roshanai, 1983; Blonk *et al.*, 1990; Schmidt *et al.*, 1990). When the data from these studies were pooled, it emerged that the change in fasting plasma triacylglycerol levels is correlated with the dose of n-3 PUFA intake (R^2=0.874) (Roche and Gibney, 2000). However, it would be interesting and beneficial to observe whether this dose dependent hypotriglyceridemic effect of fish oil would exist at very high dietary fat levels (using an animal model) in order to assure the safety of high therapeutic doses of fish oil.

1.2.2.1 Possible mechanisms for decreased triacylglycerol secretion by fish oil

Several studies have documented inhibition of hepatic triacylglycerol secretion by n-3 PUFA found in fish oil. Suppressed triacylglycerol synthesis as well as decreased apolipoprotein B (apoB) production can affect hepatic triacylglycerol secretion since apoB is an essential component of the VLDL particle. Nossen *et al.* (1986) observed that incubation of cultured rat hepatocytes with n-3 PUFA decreased hepatic triacylglycerol synthesis while there was no influence on apoB production, in comparison to cells

incubated with oleic acid. On the other hand, reduction of both triacylglycerol and apoB synthesis has been reported following incubation of cultured human hepatoma cells with n-3 PUFA (Wong *et al.*, 1986) and rat hepatocytes (Christopher *et al.*, 1990). A randomized controlled trial using obese men showed that fish oil effectively lowers the plasma concentrations of triacylglycerols chiefly by decreasing VLDL apoB production (Chan *et al.*, 2003).

Wong *et al.* (1984) demonstrated that the reduction in triacylglycerol synthesis was accompanied by increased hepatic fatty acid oxidation in perfused livers from rats fed a fish oil diet. In addition, feeding a fish oil diet for 2 weeks caused a significant increase in peroxisomal β oxidation activity of fatty acids in rats compared to the safflower oil diet fed counterparts (Yamazaki *et al.*, 1987). These data suggest that the fish oil induced increased fatty acid oxidation can result in lower hepatic fatty acid levels, hence decreased triacylglycerol synthesis. Interestingly, the peroxisomal proliferation seen with fish oil is also seen with clofibrate, a drug, which also lowers triacylglycerol levels. However, unlike clofibrate, fish oil does not reduce the activity of detoxifying enzymes and thus fish oil may be safer than the drug (Yamazaki *et al.*, 1987).

It has been observed that n-3 polyunsaturated fatty acids are less efficiently activated to acyl-CoA, or EPA/DHA-CoA and they are poor substrates for triacylglycerol synthesis as compared to saturated fatty acids and monounsaturated fatty acids (MUFA) (Nossen *et al.*, 1986). This observation is consistent with other studies indicating that saturated fatty acids are preferentially incorporated into triacylglycerol (Davis and Boogaerts, 1982). Diversion of n-3 polyunsaturated fatty acids from pathways of esterification as a major factor in triacylglycerol lowering effect of fish oil has also been

suggested (Wong *et al.*, 1985). In addition to the effects of n-3 PUFA on triacylglycerol formation, an inhibitory effect on *de novo* fatty acid synthesis and reduction in acetyl-CoA carboxylase activity in rat livers has been reported (Iritani *et al.*, 1980).

Wilkinson *et al.*, 1998 provided evidence to suggest that n-3 PUFA perturb intracellular events in VLDL assembly and hence decreased VLDL secretion. Feeding fish oil to rabbits caused an interruption of intracellular apoB transfer and hence decreased assembly of VLDL particles.

Although there is no clear biochemical mechanism, the studies cited above have suggested a potential role of decreased fatty acid and apoB synthesis, increased fatty acid oxidation, decreased acylCoA formation and perturbed intracellular events in VLDL assembly, in fish oil-induced decreased triacylglycerol secretion from the liver.

1.2.2.2 Microsomal Triglyceride Transfer Protein (MTTP) regulation by fish oil

Triacylglycerol is concentrated within the hydrophobic core of apoB during the assembly of VLDL. MTTP is implicated in the acquisition of triacylglycerol by nascent apoB for assembly and secretion of VLDL (Kulinski *et al.*, 2002). Studies done on hamsters have shown that the amount and type of dietary fat can affect hepatic MTTP mRNA expression. Incremental increase of dietary fat level increased hepatic MTTP mRNA expression significantly in a dose dependent manner in hamsters fed a diet rich in saturated fatty acids and MUFA (Bennett *et al.*, 1995). The nature of the dietary fatty acids also influenced MTTP mRNA expression; diets rich in saturated fatty acids elevated MTTP mRNA levels significantly compared to the diets rich in MUFA or n-6 PUFA in hamsters (Bennett *et al.*, 1995). However, mRNA levels for MTTP were not

affected by either fish oil or corn oil, when rat hepatocytes were incubated with fish oil or corn oil chylomicron remnants (Botham *et al.*, 2003). However, when cells were shifted to a pro-oxidizing state by pretreatment with $CuSo_4$ for 24 hr, levels of mRNA for MTTP were increased by 2 fold by fish oil chylomicron remnants, whereas corn oil remnants had no significant effect. These changes would be expected to lead to an increase in VLDL secretion rather than decrease by n-3 PUFA. Thus, the role of MTTP in fish oil-induced suppression of VLDL secretion is questionable. On the other hand, the possibility of variation of fish oil-induced changes in MTTP activity due to species or strain specific metabolism should also be considered.

1.2.3 Postprandial hypotriglyceridemic effect of fish oil

The postprandial triacylglycerolemic response refers to a series of metabolic events that occur after ingestion of a fat containing meal. Several clinical studies have shown that postprandial lipemia is an important factor in the pathogenesis and progression of coronary heart disease (Groot *et al.*, 1991). It has been further observed that the concentration of postprandial chylomicron remnant apoB 48 is directly related to the rate of progression of coronary heart disease (Karpe *et al.*, 1994).

In the postprandial state three lipoprotein species can be affected; chylomicrons, chylomicron remnants and VLDL. However it is not known whether chylomicrons, chylomicron remnants, and VLDL are all affected equally with fish oil intake. Harris and Connor (1980) reported that consuming a diet rich in salmon oil blunts the postprandial rise in plasma triacylglycerol levels, but this effect was only observed when the subjects were on a fish oil background diet. Inhibition of the postprandial response by n-3 PUFA

could be due to slower formation or faster removal of chylomicrons and VLDL or a combination of these factors.

1.2.3.1 Effect of fish oil on Intracellular chylomicron formation

The rate of chylomicron formation is affected by the digestion and absorption of fat and intracellular metabolic events. Only a few studies have investigated the digestion of n-3 PUFA -rich triacylglycerols. It was observed that, EPA and DHA of marine oil are relatively resistant to pancreatic lipase mediated hydrolysis (Bottino *et al.*, 1967). Decreased absorption of n-3 PUFA has been reported especially when these fatty acids are in esterified form. Chen *et al.* (1985) observed that enteral infusion of EPA is absorbed equally as oleic acid in animals. However when fish oil (which contain esterified EPA) was infused, it was absorbed more slowly than corn oil (Chen *et al.*, 1987). This observation suggests that digestion rather than absorption of these fatty acids has an impact on postprandial response. Furthermore, Herzberg *et al.* (1992) reported that, EPA and DHA from fish oil were effectively absorbed from the intestine compared to linoleic acid in rats previously adapted to diets containing fish oil. Thus, it is possible that duration of fish oil feeding may have an impact on digestion and absorption of fish oil.

Although the influence of dietary n-3 PUFA from fish oil on intracellular chylomicron formation has been less studied, the effect of n-3 PUFA on intracellular metabolic events in hepatocytes has been extensively studied. As mentioned in section 1.2.2.1, n-3 PUFA are known to inhibit triacylglycerol synthesis in hepatocytes. Since triacylglycerol synthesis takes place in enterocytes during fat absorption, it is possible

that this process is likewise inhibited by fish oil, but there is no consistent evidence for this hypothesis. Cartwright and Higgins (1999) observed that enterocytes were less stimulated to synthesize and secrete chylomicron lipids, when rabbits were fed a fish oil diet for 2 weeks compared to sunflower oil diet. They further observed that decreased chylomicron synthesis was due to slow differentiation of enterocytes in to mature cells, giving new insight to potential fish oil-induced suppression of chylomicron production.

1.2.3.2 Influence of fish oil intake on removal of chylomicron and VLDL lipids

Decreased postprandial triacylglycerol levels observed with fish oil feeding may result from an increase in lipolytic rate of chylomicrons and VLDL or increase hepatic removal of chylomicron remnants. Westphal *et al.* (2000) observed a significant reduction of chylomicrons and VLDL in the early hours of postprandial phase in n-3 PUFA treated hypertryglyceridemic patients. They further observed increased removal of chylomicron remnants, but only in the late postprandial phase. Furthermore, Lambert *et al.* (2001) reported an increase in hepatic uptake of chylomicron remnants that are rich in n-3 PUFA compared to chylomicron remnants rich in saturated fatty acids in chylomicron remnant perfused rats (Lambert *et al.* 2001). The increase in n-3 PUFA-enriched chylomicron remnant uptake was not influenced by hepatic lipase activity. These studies tell that the postprandial hypotriglyceridemic effect of fish oil might partly be due to increased removal of chylomicron and VLDL lipids and increased hepatic removal of chylomicron remnants. Furthermore, increased chylomicron remnant uptake is mediated through a mechanism that is independent of hepatic lipase activity and dependent on properties or composition of chylomicron remnants.

1.2.3.2.1 Effect of fish oil on intravascular chylomicron and VLDL lypolysis

Lypolysis of chylomicron and VLDL triacylglycerols at peripheral tissues is facilitated by lipoprotein lipase, which is attached to the luminal surface of blood vessels. Mature lipoprotein lipase is secreted to the vascular endothelium mainly from the parenchymal cells of adipose and muscle tissues. The influence of n-3 PUFA on the activity of lipoprotein lipase and the rate of fatty acid release from n-3 PUFA-enriched chylomicron emulsions has been extensively studied, however, the results are controversial as described below.

Increased lipoprotein lipase activity in adipose tissue (Anil *et al.*, 1992; Benhizia *et al.*, 1994; Yoshiba *et al.*, 1999) and muscle (Herzberg and Rogerson, 1989) has been reported in n-3 PUFA rich diet fed rats compared to rats fed a n-6 PUFA-enriched diet. However, Ikeda *et al.* (2001) observed a fish oil induced increase in adipose tissue lipoprotein lipase activity with no difference in post heparin lipoprotein lipase activity in rats. Since tissue lipoprotein lipase activity reflects the activity of both intravascular and intracellular (parenchymal) lipase pools, enzyme activity in adipose tissue is not an accurate indicator of VLDL and chylomicron lipolysis. Thus, post heparin lipoprotein lipase activity (which measures intravascular lipoprotein lipase activity) and adipose tissue lipoprotein lipase mRNA expression is more representative of intravascular lipoprotein lipolysis than adipose tissue or muscle lipoprotein lipase activity.

There is growing body of evidence for a decrease in post heparin lipoprotein lipase activity in fish oil fed swine (Groot *et al.*, 1989; Huff *et al.*, 1993) and rats (Haug and Hostmark, 1987). Furthermore, Raclot *et al.* (1997) observed a reduction of lipoprotein lipase mRNA expression in retroperitonial tissues in DHA fed rats. A

decrease in lipoprotein lipase activity might be an adaptive response for the triacylglycerol lowering effect of fish oil. A positive correlation of fasting plasma triacylglycerol concentrations and lipoprotein lipase mRNA expression was observed by Murphy *et al.* (1999) in human subjects, which further suggests the possibility of adaptive change in enzyme activity with changes in plasma triacylglycerol concentrations. However, post heparin lipoprotein lipase activity remained the same in human subjects who were treated with fish oil (Nozaki *et al.*, 1991; Desager *et al.*, 1989; Harris *et al.*, 1988). In contrast, Harris *et al.* (1997) and Zampelas *et al* (1994) observed an increase in post heparin lipoprotein lipase activity in n-3 PUFA treated healthy human subjects. Above controversial results might be due to absence of *in vivo* conditions for the enzyme activity because *in vivo* lipoprotein lipase activity is known to be modified by other factors like apoCI, apoCII and apoE.

Although lipoprotein lipase mediates the catabolism of triacylglycerol-rich lipoproteins, the activity of this enzyme is not a direct indicator of *in vivo* hydrolysis of chylomicrons and VLDL. Several investigations have been done to study the effect of chylomicron and VLDL composition on the rate of lipoprotein lipase mediated fatty acid release, which is rather more representative of intravascular lipolysis than lipoprotein lipase activity.

It has been observed that lipoprotein lipase selectively hydrolyses chylomicron triacylglycerols, which results in chylomicron remnants that are relatively enriched with EPA and DHA. Chylomicrons derived from animals and human subjects fed a fish oil diet have shown decreased lipolysis by lipoprotein lipase (Botham *et al.*, 1997; Levy and Herzberg, 1999). Furthermore EPA and arachidonic acid-rich chylomicrons exhibit a

relative resistance to lipoprotein lipase compared to fatty acid esters with the chain length of C14-C18 (Melin *et al.*, 1991; Ekstrom *et al.*, 1989). *In vitro* hydrolysis of n-3 PUFA enriched emulsions by lipoprotein lipase and hepatic lipase showed relatively high resistance compared to n-6 PUFA enriched emulsions (Oliveira *et al.*, 1997). Kasim-Karakas *et al.*, 1995 observed a reduction of the absolute rate of triacylglycerol lipolysis in hypertriglyceridemic patients following n-3 PUFA supplementation. All these observations suggest that chylomicrons enriched with n-3 PUFA are resistant to lipoprotein lipase mediated lipolysis.

1.2.4 Coronary heart disease, LDL and fish oil

The association between high levels of LDL cholesterol and increased incidence of coronary heart disease is firmly established (Kinsella *et al.*, 1990). However, decreased LDL cholesterol concentrations by fish oil, as a causative factor for lower incidence of coronary heart disease remains questionable. While there are no direct data from which to answer the question, the reported reduction in atherosclerosis in swine fed fish oil (which occurred without lowering of LDL) (Weiner *et al.*, 1986) would suggest that modification of LDL levels by fish oil may not play an important role in reduction of atherogenesis and hence, coronary heart disease. The growing body of evidence for an increase in LDL concentrations by fish oil diet also argues against the anti-atherogenic effect of fish oil in terms of LDL concentrations and its relation to increased incidence of coronary heart disease. It is likely that, in addition to the LDL concentrations, changes in chemical and physical properties of LDL particles may also affect atherogenesis. Monkeys fed a fish oil diet incorporated large amounts of EPA into LDL cholesterol

esters, which led to lowering of phase transition temperature of whole LDL particle (Parks and Gebre, 1987). A decrease in transition temperature of the LDL particles is known to be associated with reduced atherogenesity, thus, this observation suggests that n-3 PUFA induced changes in the LDL particle itself may inhibit atherogenesity.

Although the effect of fish oil on plasma triacylglycerol and VLDL concentrations is well known, the effect on the regulation of LDL and HDL cholesterol concentrations is inconsistent. This may be attributed to several factors, i.e. individual variations, lack of control of confounding factors, pre-existing dislipidemias etc. Normolipidemic subjects show reduction in plasma LDL-cholesterol concentrations following intake of fish oil diet (Nestel *et al.*, 1986, Illingworth *et al.*, 1984). However, fish oil supplementation to hyperlipidemic subjects causes an increase in LDL cholesterol concentrations (Sullivan *et al.*, 1986, Hsu *et al.*, 2000).

Animal experiments also show varied effects of increased consumption of fish oil on plasma LDL-cholesterol concentrations. Fish oil feeding reduced LDL-cholesterol concentrations in African green monkeys (Parks and Crouse, 1992) but had no effect on LDL-cholesterol concentrations in Cynomolgus monkeys (Parks and Gebre, 1991). On the contrary, fish oil feeding to rabbits increased the LDL-cholesterol levels (Wilkinson *et al.* 1998). This inconsistency might be attributed to changes in lipoprotein metabolism in different animal species. Thus, we should be careful when interpreting and comparing the results of an animal model with human and other animal models.

1.2.4.1 Potential mechanisms

Several mechanisms have been proposed for both a reduction and an increase in plasma LDL concentrations by n-3 PUFA. Kinetic studies of ^{125}I-LDL metabolism in normal human subjects disclosed a significantly lower rate of synthesis of LDL apoB by n-3 PUFA-enriched diet compared to a control diet containing predominantly saturated fatty acids and MUFA (Illingworth *et al.*, 1984). Therefore, a concomitant increase or no change in LDL removal can result in low levels of LDL. Rats fed an n-3 PUFA-enriched diet showed an enhanced LDL-receptor (LDLr) activity with lower plasma LDL levels compared to n-6 PUFA-enriched diet fed rats (Ventura *et al.*, 1989; Spady *et al.*, 1993; Spady *et al.*, 1995). On the other hand, an increased binding of LDL to liver membranes, hence possibility of increased LDL clearance, has been reported in mice (Tripodi *et al.* (1991). Furthermore, Vasandani *et al.* (2002) reported that increased LDL uptake in n-3 PUFA-enriched diet fed mice is not dependent on LDLr activity. These studies suggest that n-3 PUFA decrease LDL synthesis as well as increase LDL clearance resulting in decreased plasma LDL levels, although the mechanism is not clear.

An increase in LDL concentrations can be explained by either increased LDL synthesis or decreased removal rate. LDL is synthesized from VLDL following hydrolysis and removal of VLDL triacylglycerols. Although it might seem unlikely that lower VLDL levels can be associated with raised LDL levels, there is a possibility of independent secretion of LDL particles (i.e. LDL that do not arise from VLDL) (Nestel *et al.*, 1984). In addition, Harris (1989) hypothesized, considering the investigations done on metabolic differences between VLDL particles of different sizes, that there is a fish oil-induced increased production of smaller VLDL particles (although the total VLDL

level is low), which are known to be readily converted to LDL by lipoprotein lipase compared to larger particles. The possibility of decreased LDL removal can be explained by either decreased ability of LDL to bind to LDLr or decreased synthesis of LDLr. Monkeys which were injected with LDL obtained from fish oil fed animals showed decreased LDL clearance compared to their counterparts injected with LDL obtained from olive oil fed animals (Schectman *et al.* 1996). This effect may be due to alteration of LDL particle size or composition, which reduces the affinity of LDL for its receptor. On the other hand, fish oil induced decreased expression of hepatic LDLr mRNA that may lead to decreased LDLr mediated LDL uptake, has been reported in animal (Wilkinson *et al.,* 1998) and human cell culture studies (Lindsey *et al.*, 1992).

LDL particle size and composition may be important determinants of LDL clearance because changes in particle size are known to be associated with alterations in affinity to LDLr (Nigon *et al.*, 1991). Increased LDL particle size has been observed in hypertensive human subjects who were treated with fish oil compared to controls that were treated with corn oil (Suzukawa *et al.,* 1995) and in normal human subjects who were treated with EPA and DHA compared to base line particle size (Lindsey *et al.*, 1992). Sanchez-Muniz *et al.* (1999) reported an increase in the LDL cholesterol /LDL apoB ratio, which reflects a probable increase in LDL particle size in normal subjects following fish oil supplementation compared to saturated fatty acids and MUFA treated counterparts. In contrast, Cynomolgus monkeys who were fed a fish oil diet had smaller LDL particles, compared to lard fed monkeys (Parks *et al.*, 1991, Linga *et al.*, 1993). The inconsistency in fish oil-induced changes in LDL particle size might be attributed to a lack of control for confounding factors, like MUFA, PUFA, saturated fatty acid and

cholesterol content in the diet fed to the control group or lack of control of the pre-interventional diet.

In addition, the correlation of LDL particle size with LDL binding affinity has also been studied. A proportional increase of fibroblast LDL uptake with increase in LDL particle size was observed in Cynomolgus monkeys by Linga *et al.* (1993). Furthermore, the binding affinity of LDL from monkeys was correlated with LDL particle size (Hannah *et al.*, 1997). However, Nigon *et al.* (1991) demonstrated that larger or smaller LDL particles from humans had a lower LDLr binding affinity than medium-sized LDL particles, hence slower turn over rate. These findings imply that alteration of particle size might have an impact on LDLr mediated LDL uptake, hence fish oil-induced changes in plasma LDL levels might partly be due to alteration in LDL particle size.

1.2.5 Effect of fish oil on HDL

The influence of fish oil on plasma HDL cholesterol concentration is also inconsistent. The HDL cholesterol concentrations increased upon intake of fish oil in human trials done on normal subjects, but no effect of fish oil was observed in hyperlipidemic patients (Harris, 1996). In contrast, HDL cholesterol concentrations decreased in response to n-3 PUFA-enriched diet in studies done on hamsters (Surette *et al.* 1992a) and nonhuman primates (Ward and Clarkson, 1985).

The HDL fraction comprises mainly two sub fractions, namely HDL_2 and HDL_3. Whether the protective effect of HDL on coronary heart disease can be attributed to one or both HDL sub fractions remains unclear. The effect of fish oil supplementation on

HDL_2 and HDL_3 was reported in a few studies and the results of these studies are inconsistent. The HDL_2 sub fraction is found to be lower in patients on hemodialysis who were treated with MaxEPA, a concentrate of n-3 PUFA (Rylance *et al.*, 1986). However there were no controls for fat intake in this study. In contrast, a significant increase in HDL_2/HDL_3 ratio has been observed in hypercholesterolemic patients following fish oil diet (Abbey *et al.*, 1990). Harris *et al.* (1988) observed no changes in HDL sub fractions in hyperlipidemic patients following fish oil supplementation compared to vegetable oil. The inconsistency of the results of fish oil induced changes in total HDL cholesterol and HDL subfraction concentrations might be due to lack of control of confounding factors like pre-existing dislipidemic state.

1.2.6 Effect of fish oil on reverse cholesterol transport pathway

The reverse cholesterol transport pathway, which removes cholesterol from perepheral tissues and returns it to the liver, is known to be one of the main determinants of the LDL and HDL lipid composition. Cholesteryl ester transfer protein (CETP) plays the major role in this pathway transferring cholesterol ester from HDL to VLDL and LDL in exchange for triacylglycerols (Quinet *et al.*, 1991). While the effect of n-3 PUFA on plasma lipoprotein levels has been extensively studied, less is known about the impact on reverse cholesterol ester transport.

Bagdade *et al.* (1996) observed a dramatic fall in cholesterol ester transfer from HDL to LDL and VLDL in normolipidemic insulin dependent diabetic (IDDM) subjects who were treated with n-3 PUFA compared to pre-treatment levels, although, there was a paradoxical increase in mass of CETP. Furthermore treating hypercholesterolemic men

with n-3 PUFA showed normalization of plasma cholesterol ester transfer compared to accelerated pre-treatment levels (Bagdade *et al.*, 1992). Furthermore, HepG2 cells, incubated with EPA and DHA show a significant reduction in hepatic CETP mRNA expression (Hirano *et al.*, 2001). Since the findings of above studies conducted on human subjects and a human cell line are consistent with decreased cholesterol ester transfer following dietary supplementation of n-3 PUFA, these fatty acids may have a beneficial role in the nutritional therapy of hyperlipidemia and IDDM as, accelerated cholesterol ester transfer is known to be atherogenic in those patients. In addition, the above studies suggest that, plasma CETP mass and/or CETP activity might not reflect actual cholesterol ester transfer from HDL to LDL and VLDL.

1.2.6.1 Effect of fish oil on lecithin cholesterol acyl transferase (LCAT) activity

LCAT plays an important role in the cholesterol ester transport pathway by catalyzing the esterification of free cholesterol in HDL particles. Using reconstituted HDL particles made of egg yolk lecithin-[^{14}C] cholesterol-apoA1 as exogenous substrate, it was found that there was no difference in plasma LCAT activity in fish oil diet fed monkeys compared to lard fed monkeys (Parks *et al.*, 1989). However, n-3 PUFA are found to be less utilized by LCAT for formation of cholesterol esters; rather, LCAT preferentially utilizes linoleic acid (n-6 PUFA) for cholesterol ester formation (Parks *et al.*, 1989; Parks *et al.*, 1997). These data suggest that resistance of n-3 PUFA for esterification may lead to decreased reverse cholesterol transport.

1.2.7 Pro-atherogenic effect of fish oil

Decreased HDL concentration and increased LDL concentration is known to be proatherogenic. A Fish oil induced increase in LDL concentrations was consistently shown in studies done on patients with hyperlipidemia and type-2 diabetes mellitus. Therefore, fish oil might be proatherogenic at least in this part of human population. In addition, Hayes (1990) reported an elevation of the apoB to apoA1 ratio in Syrian Golden hamsters fed a fish oil diet. The increase in apoB/apoA1 ratio on fish oil feeding also point towards a pro-atherogenic effect of fish oil.

Besides the possibility of a pro-atherogenic effect of fish oil, several studies have reported a hyperlipidemic effect of fish oil, which was more obvious in animals fed fish oil diet supplemented with cholesterol (Kubow *et al.*, 1996, Lu *et al.*, 1996, Lin *et al.* 1995, Surette *et al.*, 1992a). Although a similar effect was observed with a n-6 PUFA-enriched diet supplemented with cholesterol, the effect of fish oil diet given along with cholesterol was significantly higher compared to n-6 PUFA-enriched diet (Lu *et al.*, 1996). This hyperlipidemic effect is mainly due to an increase in plasma VLDL and LDL cholesterol concentrations rather than an increase in triacylglycerol concentrations. Lin *et al.* (1995) observed an increase in total plasma VLDL and LDL cholesterol concentrations in Syrian hamsters who were fed a fish oil diet supplemented with 0.5% w/w cholesterol compared to hamsters fed a coconut oil diet or soybean oil diet supplemented with cholesterol. They further observed an attenuation of the hypotriglyceridemic effect of fish oil when the fish oil diet was supplemented with 0.5% w/w cholesterol. The hyperlipidemic effect of fish oil was dependent on the level of dietary cholesterol and dietary n-3 PUFA content (Surette *et al.*, 1992). Syrian golden

hamsters consumed diets containing incremental increase in dietary n-3 PUFA from fish oil with 0.1% w/w cholesterol showed an increase in VLDL and LDL cholesterol concentrations with increase dietary fat level. However, with low dietary cholesterol (0.015% w/w), this dietary fat level dependent hypercholesterolemic effect of fish oil was not seen (Surette *at al.*, 1992a). These observations suggest that, in spite of the consistent demonstration of hypotriglyceridemic effect of fish oil in human subjects, the hamster model behaves differently giving a fish oil-induced, dose dependent hyperlipidemic effect when there are moderate to high levels of dietary cholesterol. Furthermore, unlike human subjects, who show a fish oil-induced attenuation of the hypercholesterolemic effect of dietary cholesterol (Nestel et al., 1986), there is a dose dependent hypercholesterolemic effect of dietary cholesterol in hamsters fed a fish oil diet.

The mechanism behind the hyperlipidemic effect of fish oil, supplemented with cholesterol is not clear. Lu *et al.* (1996) and Lin *et al.* (1995) have measured the activity of acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme which controls the rate of intracellular cholesterol esterification and 3 hydroxy-3-methyl glutaryl-CoA (HMG-CoA), the enzyme that control the cholesterol synthesis, in Syrian golden hamsters fed a fish oil diet supplemented with cholesterol. The hepatic ACAT activity was significantly increased in hamsters fed a cholesterol supplemented fish oil diet compared to coconut oil and soybean oil counterparts. However, HMG-CoA activity was reduced in all the diet groups supplemented with cholesterol. Increased formation of cholesterol esters by increased ACAT activity might be a potential explanation for increased VLDL cholesterol concentrations by the cholesterol supplemented fish oil diet, although there is reduction in endogenous cholesterol synthesis due to decreased HMG-CoA activity. On

the other hand, the effect of the cholesterol supplemented fish oil diet on hepatic secretion of VLDL in hamsters has not been studied. Thus, further investigations need to be carried out to understand whether increased cholesterol esterification is associated with increased hepatic secretion of VLDL.

Another possible mechanism for this hyperlipidemic effect is decreased catabolism of VLDL and/or chylomicrons. McAteer *et al.* (2003) demonstrated dietary cholesterol-induced increase in VLDL and chylomicron levels in F_1B hamsters due to suppression of plasma lipoprotein lipase activity. Thus, the possibility of decreased lipolysis as a cause of this hyperlipidemic effect of cholesterol supplemented fish oil has to be explored.

A possible role of oxidative stress in the hyperlipidemic effect of fish oil has also been studied. Dietary supplementation of vitamin E could prevent the fish oil-induced hyperlipidemic effect in hamsters (Kubow *et al.*, 1996), indicating the reduced antioxidant capacity as a contributing factor. However, the mechanism behind oxidative stress related hyperlipidemia in fish oil fed hamsters is yet to be studied.

1.3 Hamster as an animal model to study the diet induced regulation of lipid metabolism

The hamster is widely used as an animal model to study the effects of diet on lipoprotein metabolism and atherosclerosis since the lipoprotein profile of hamster resembles closely that of humans. Moreover, the hamster is highly susceptible to atherosclerosis (Nistor *et al.*, 1987). Increases in plasma lipid concentrations could be easily induced in hamsters by adding only small, physiological amounts of cholesterol to

the diet (Kowala *et al.*, 1991). In addition, the hamster has similar plasma CETP activity to that seen in humans whereas, other animal models, like the pig, rat and mouse have virtually no plasma CETP activity (Ha and Barter, 1982).

The F_1B hamster is a hybrid offspring of Bio 87.2 and Bio 1.5 parent strains and it is an excellent animal model for hyperlipidemia and atherosclerosis. Feeding an atherogenic diet to F_1B hamsters results in elevated plasma cholesterol levels, but this increase is, in contrast to other strains of hamsters, more pronounced in the VLDL and LDL fraction than in the HDL fraction (Kowala *et al.*, 1991). In addition, compared to the DSNI (dominant spot normal inbred) strain, F_1B hamster showed more susceptibility to hyperlipidemia and atherosclerosis due to increase in triacylglycerol rich lipoproteins i.e., chylomicrons and VLDL, following dietary supplementation of cholesterol (McAteer *et al.*, 2003). As a consequence, the HDL to total plasma cholesterol ratio is lower in the hyperlipidemic F_1B hamster than in other hyperlipidemic hamster strains. This makes the hyperlipidemic F_1B model more comparable to the human situation, as hyperlipidemic human subjects also show lower HDL to total plasma cholesterol ratio. In addition, the F_1B hamster is suggested to develop type-2 diabetes mellitus when fed high fat diets, which makes this model comparable to the human population with this dyslipidemic disorder.

1.4 Justification of this study

Data from previous studies suggest that the amount of fish oil might have varied effects on LDL- and HDL-cholesterol concentrations under normal or hyperlipidemic conditions. This study was designed to investigate the regulation of lipid and lipoprotein

metabolism by fish oil, at various fat levels, under normal and hyperlipidemic conditions. We used the F_1B hamster, a model suggested to develop type-2 diabetes mellitus when fed high fat diets. Hyperlipidemia was induced by feeding high cholesterol diets. The animals were fed either a diet rich in fish oil or a mixed oil (MIX) diet to investigate whether fish oil has beneficial effects in lowering plasma lipid levels.

Chapter 2: Methodology

2.1 Animals and diets

The F1B hamsters (7 weeks old) were obtained from Bio Breeders Inc. (Water Town, MA) and fed a chow diet for one week prior to feeding specific diets. After this equilibration period, hamsters were divided into 8 groups (n=12) and each group was fed with one of the specified diets. The specified diets consisted of fat free semipurified diet (ICN Biomedical Inc., OH) that was supplemented with either fish oil (menhaden oil, Sigma Chemical Co., St. Louis, MO) or a mixture of lard and safflower oil in 1.5:1 ratio (MIX diet) from local supermarket. The composition of the semipurified diets with fat and cholesterol is given in Table 1. The fat content of the diets was either 5% w/w (low fat) or 20% w/w (high fat). Due to the presence of cholesterol in fish oil, the low fat fish oil diet contained 0.025% w/w of cholesterol, and the high fat fish oil diet contained 0.1% w/w of cholesterol. Thus, the same amount of cholesterol was added to the low fat and the high fat MIX diets to keep cholesterol content similar. To get high cholesterol contents the fish oil and MIX diets were supplemented with additional cholesterol to bring the final concentration of cholesterol to 0.25% w/w. Highest purity grade finely powdered cholesterol (98% pure, Sigma-Aldrich, St. Louis, MO) was dissolved in fat and added to the diets. Lipids were extracted from the diets and the fatty acid composition was analyzed using gas liquid chromatography (Keough and Davis, 1979). The fatty acid composition of the diets is given in Table 2. All diets were stored at -20^0 C.

All animals were housed in individual cages in a single room with enriched environment. The room was lit from 07:00 to 19:00 hours, with temperature maintained at 21^0 C and humidity at 35±5 %. The animals were given fresh diets every day for two weeks. The diet consumption was checked every day. The animals were weighed at the

Table 2.1 Composition (g/kg) of the semipurified diets designed for a 5% (w/w) or a 20% (w/w) fat level.

Ingredient*	Diets			
	Low fat [3] FO	Low fat [3] MIX	High fat[4] FO	High fat[4] MIX
	(g/kg)			
Casein	201	201	200	200
DL-Methionine	3	3	3	3
Sucrose	500	500	306	306
Corn starch	150	150	200	200
Vitamin Mix[1]	11	11	11	11
Mineral Mix[1]	35	35	40	40
Fiber[2]	50	50	50	50
Fat	50	50	200	200
Cholesterol	0.25	0.25	1	1

*Ingredients were from ICN Biomedicals, Cleveland, OH, USA.
[1]Supplied in quantities adequate to meet NRC requirements (National Research Council, 1995)
[2]Cellulose was supplied as Alphacel non-nutritive bulk (ICN Biomedicals Inc., Aurora, OH).
[3]Semipurified diet designed for a 5% (w/w) fat level.
[4]Semipurified diet designed for a 20% (w/w) fat level.
Abbreviations used; FO, fish oil diet

Table 2.2 Fatty acid composition of the diets. Lipids were extracted from the diets and fatty acid composition was determined by gas chromatography. Abbreviations used: ΣSFA, sum of saturated fatty acids, ΣMUFA, sum of monounsaturated fatty acids, ΣPUFA, sum of polyunsaturated fatty acids, ND, not detected.

Fatty acids	Fish oil	MIX
	% w/w	
14:0	9.6	1.0
16:0	19.3	19.3
16:1 n7	13.1	2.0
18:0	3.8	10.0
18:1	13.8	31.0
18:2 n6	2.7	34.0
18:3 n3	4.5	3.0
18:4 n3	3.4	ND
20:1 n9	1.6	ND
20:4 n6	1.0	ND
20:5 n3	12.9	ND
22:5 n3	2.4	ND
22:6 n3	12.0	ND
ΣSFA	32.0	30.0
ΣMUFA	28.0	32.0
ΣPUFA	38.0	37.0
Σn-3 PUFA	35.0	3.0
Σn-6 PUFA	3.0	34.0
n-6/n-3 ratio	0.09	11.3

beginning of the study period, one week later and at the conclusion of the study. After two weeks on specified diets the animals were sacrificed after 14 hrs of fasting. Blood was collected by cardiac puncture into tubes containing EDTA (1.2 mg/ml, pH 7.4) and centrifuged immediately at 3000 x *g*, 4^0 C for 15 minutes (Fless, 1991) to separate plasma. 10% NaN_3 was added (1µl/ml) to each plasma sample to prevent bacterial and fungal growth. Plasma samples were stored on ice at 4^0 C. Liver and adipose tissue were removed and quick-frozen in liquid nitrogen. The tissues were stored at -70^0 C until further use.

2.2 Plasma lipoprotein isolation

2.2.1 Separation of chylomicrons

Plasma from high fat fish oil diet (20% w/w) fed hamsters was milky and contained chylomicron-like particles. Plasma was centrifuged at 15,500 *g* for 20 min at 12^0 C (McAteer *et al.*, 2003) to separate chylomicrons that were stored at 4^0 C for further analysis. The infranatant (remaining plasma which contain other lipoproteins) was separated and used for isolation of other lipoproteins.

2.2.2 Micropreparative lipoprotein isolation

For serial isolation of individual lipoprotein fractions, solutions containing increasing amounts of NaCl or NaBr were used to sequentially adjust the density of plasma (Bauer, 1991, Salter *et al.*, 1998). For this procedure, 0.5 ml of plasma was mixed with d=1.0063 NaCl (0.5 ml) containing 0.01% w/w EDTA in 1 ml tubes to adjust the final density of the mixture to d=1.006. Tubes ware centrifuged at 100,000 RPM for

2.5 hrs at 16^0 C; acceleration, 5; deceleration, 7 using TL 100 fixed angle rotor (Beckman instruments, Inc., CA). After centrifugation, the top 0.5 ml (d< 1.006) was aspirated and labeled as VLDL. The bottom layer (0.5 ml) was mixed with d= 1.12 NaCl (0.5ml) containing 0.01% w/w EDTA to adjust the final density to d=1.060 and centrifuged under conditions described above. The top layer (0.5 ml, d=1.006-1.060) was aspirated and labeled as LDL (LDL fraction contained both IDL and LDL). Remaining bottom layer (0.5 ml) was mixed with d=1.36 NaBr containing 0.01% w/w EDTA to adjust the final density to d=1.20 and centrifugation repeated under above conditions for 3.5 hrs. After centrifugation, the top layer (0.5 ml, d=1.060-1.20) was removed and labeled as HDL. Isolated individual lipoprotein fractions i.e. VLDL, LDL and HDL were stored at 4^0 C for further analysis.

2.3 Plasma, lipoprotein and liver lipid analysis

Lipids were extracted from liver samples by homogenizing 50 mg of liver in 2 ml chloroform/methanol (2:1) (Folch *et al.*, 1957). The lower organic phase was dried under nitrogen, and resuspended in 100 µl of isopropanol.

Total cholesterol concentration was assayed in plasma, chylomicron like particles,VLDL, LDL, HDL and liver lipid extracts using cholesterol assay kit # 402 (Sigma Diagnostics Inc, St. Louis, MO). Total triacylglycerol concentration was assayed in plasma, VLDL, LDL, HDL and liver lipid extracts using triglyceride assay kit # 344 (Sigma Diagnostics Inc, St. Louis, MO). Free cholesterol was assayed in plasma, individual lipoprotein fractions and liver lipid extracts using free cholesterol assay kit (Wako Chemicals, VA). Cholesterol ester concentration was determined by subtracting

the free cholesterol concentration from total cholesterol concentration. Phospholipid concentrations of plasma lipoprotein fractions were analyzed using the method of Bartlett *et al.* (1959). Surface lipid content and core lipid content of LDL particles represent the sum of free cholesterol and phospholipid concentrations (surface lipid) and the sum of cholesterol ester and triacylglycerol concentrations (core lipid) of LDL respectively.

2.4 Lipoprotein lipase activity

2.4.1 Extraction of lipoprotein lipase from adipose tissue

Lipoprotein lipase extraction, preparation of apoCII source and lipoprotein lipase enzyme assay was performed using a previously published method (Nilsson and Schotz , 1976). White adipose tissue of hamsters (500 mg) was chopped and homogenized in 2.5 ml of ice-cold water containing 20 μg/ml heparin. The homogenate was placed on ice for 30 min to separate aqueous phase and lipid phase. The lower aqueous phase (1 ml) was taken and mixed with ice-cold acetone (5 ml) and 100 μg/ml BSA (40 μl). The mixture was placed on ice for 1 hr and then centrifuged at 2300 RPM for 10 min at 0^0 C. The pellet was washed with ice-cold acetone (5 ml) and recentrifuged under above conditions. The pellet was resuspended in ice-cold diethylether (5 ml) and centrifuged under similar conditions. The diethylether was poured off and pellet was dried under nitrogen. The dried pellet was stored at -70^0 C for analysis of lipoprotein lipase activity.

2.4.2 Preparation of apoCII source

Hamster blood was collected by cardiac puncture into a glass tube and incubated at 37^0 C for 1hr. Blood was centrifuged at 1,100 rpm for 20 min to separate serum. Serum was collected into a fresh glass tube using a pasteur pipette and heated at 56^0 C in an oven for 1 hr to inactivate lipoprotein lipase enzyme. Serum was stored at -20^0 C as the source of apoCII.

2.4.3 Purification of Tri [9,10-3H] oleoyl glycerol and preparation of stock glycerol emulsion

150 µl (750 µci) of tri [9,10-3H] oleoyl glycerol was obtained from Amersham Pharmacia Biosciences (QB) and purified by running on a TLC plate developed in a solvent system containing hexane: diethylether: acetic acid (80:20:1). Triolein was used as the standard. Tri [9,10-3H] oleoyl glycerol band was cut and extracted with chloroform: methanol (2:1) to prepare the stock glycerol emulsion. Triolein (414 mg) and phosphatidylcholine (20 mg) were added to a vial containing purified tri [9,10-3H] oleoyl glycerol (750 µci) and mixed. The mixture was dried under N_2, glycerol (30 ml) was added to the tube and resulting mixture was sonicated (at 45 amplitude) for 4 times at 1min intervals. The stock glycerol emulsion was stored at room temperature.

2.4.4 Lipoprotein lipase (LPL) enzyme assay

The LPL pellet (section 2.4.1) was resuspended in 600 µl of 20 mM Tris/HCl containing 20 µg/µl heparin and left on ice for 30 min. The assay was standardized for the optimum volume of LPL extract. To stay within the linear range, 50 µl of LPL

extract was used for all the assays. LPL extract (50 µl) and 0.15 M NaCl (25 µl) were added to a glass tube and preincubated at 30^0 C for 15 min. Boiled LPL extract (50 µl) was used as the blank. The reaction was started by adding diluted glycerol emulsion (50 µl). Diluted glycerol emulsion consisted of 0.62 M Tris/HCl containing 5% BSA at pH 8.6 made up in 0.15 M NaCl: serum (apoCII source): stock glycerol emulsion at the ratio of 1: 0.5: 1. Incubation was continued at 30^0C for 60 min in a shaking water bath. Reaction was stopped by adding 1.625 ml of methanol: chloroform: heptane (1.45: 1.25: 1) and 0.525 ml of 0.1 M potassium bicarbonate-potassium tetrabotrate buffer (pH 10.5). Each tube was vortexed for 30 seconds and then centrifuged at 1,100 rpm for 10 min at 25^0 C. Upper phase (0.5 ml) counted in a scintillation counter and enzyme activity was expressed as pmol/hr/mg adipose tissue.

2.5 Microsomal triglyceride transfer protein (MTTP) assay

Liver (100 mg) was homogenized in 1 ml of buffer containing 10 mM Tris, 150 mM NaCl and 1 mM EDTA at pH 7.4. The homogenate was sonicated at 45 amplitude (x 3) at 1 min intervals and stored at -70^0C. MTTP activity in the liver homogenate was assayed using the MTTP Activity kit (Roar Biomedical Inc., Columbia University, NY). The assay was standardized to determine the optimum amount of protein in the linear range. To stay within the linear range, 1.5 mg of protein was used for all the assays. The homogenate (1.5 mg protein) was combined with 5 µl of "acceptor" (VLDL), 5 µl of "donor" (synthetic triacylglycerols linked to a fluorescent labeled nitrobenzooxadiazole-fluorophor) (NBD-TG) in 250 µl total volume of buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA at pH 7.4). Boiled homogenate (1.5 mg protein) was used as the blank. The

reaction mixture was incubated at 37^0 C for 3 hrs and transferred to a 96-well black microplate. The fluorescence was measured using Spectra Max Gemini fluorescence spectrophotometer, at an excitation wavelength of 465 nm and an emission wavelength of 535 nm. The fluorescent neutral lipid is present in a self-quenched state when contained within the core of the "donor". The MTTP-mediated transfer is determined by the increase in fluorescence intensity as the fluorescent lipid is removed from the self-quenched "donor" to the "acceptor". The fluorescence readings were standardized to pmol triacylglycerol transferred/hr by plotting a standard curve according to the manufacturer's instructions to derive the relation between fluorescence intensity and mass transfer generated by dispersing the "donor" in isopropanol. The unquenched fluorescence intensity of the fluorescent neutral lipid contained within the core of the "donor" was determined by dispersing 5 µl of "donor" in 2 ml of 100% isopropanol. Serial dilutions of the dispersion were made to generate a standard curve of fluorescence intensity (excitation 465 nm/ emission 535 nm) versus mass of fluorescent cholesterol ester. MTTP activity was expressed as pmol/hr/mg.

2.6 Cholesteryl ester transport protein (CETP) activity assay

CETP activity in the plasma samples was assayed by a previously published radioactive method (Tall *et al.*, 1987). Lipoproteins (LDL; density 1.024-1.063 and HDL; density>1.125) were isolated from human plasma (Murdoch and Breckenridge, 1994) and the HDL fraction was radiolabled, using ^{14}C-cholesterol oleate purchased from Amersham Pharmacia Biosciences (QB) (Tall *et al.*, 1987). The assay was standardized to determine the optimum volume of plasma to be in the linear range. For further

experiments 5 µl of plasma was used to stay in the linear range. The assay was also standardized to determine the optimum incubation time. To stay within the linear range, 1 hr of incubation time was used for all assays. Plasma (5 µl) was combined with radiolabeled HDL (5 µg), LDL (50 µg) and 115 µl of incubation buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA) and incubated at 37^0C for 1 hr. Incubation buffer (5 µl) was used as the blank. The LDL fraction was precipitated using 1 M heparin (25 µl) containing 1 mg $MnCl_2$ and kept on ice for 30 min. The radioactivity in the supernatant and the precipitate was counted and the results were expressed as percent cholesterol ester transferred/hour.

2.7 CETP mass

CETP mass in plasma samples was quantitatively assayed using CETP ELISA-DAIICHI kit (Daiichi Pure Chemicals Co., LTD, Tokyo). For the *in vitro* quantitative assay of CETP mass, plasma was diluted 120 times with dilution buffer (citrate buffer, pH 5.5). Sample (50 µl) was added to the center of each microplate test well, coated with anti-CETP antibodies (anti-CETP MoAb). Dilution buffer (50 µl) was used as the blank. After 2 hrs of incubation at room temperature, the solution was removed from the wells, and the wells were thoroughly washed with the wash buffer (350 µl of phosphate buffer, pH 7.2). Anti CETP antibody conjugated with horse raddish peroxidase (anti-CETP MoAb HRP) (50 µl) was then added to each well and incubated for 1 hr at room temperature. After incubation, the solution was removed from the wells and the wells were washed thoroughly. Substrate solution (50 µl) containing o-phenylenediamine was then added to each well and incubated for 15 min at room temperature. Reaction was

stopped by adding stop regent (50 µl of 7.7% H_2SO_4). The intensity of the color that develops was read by Spectra Max 190 microplate reader at 492 nm. Concentration of the samples was calculated using a standard curve.

2.8 LDL-receptor (LDLr) mRNA abundance

All reagents for RNA isolation and RT-PCR were purchased from Invitrogen (Toronto, ON) unless otherwise stated. Total RNA from hamster livers was isolated using the FastRNA Kit, GREEN and the FastPrep FP120 instrument (Qbiogene, Carlsbad, CA) and stored at -20^0 C. The integrity of the RNA was checked by running the samples on a 1% agarose gel in borate buffer. Hepatic LDLr mRNA abundance was determined by reverse transcription and *in vitro* PCR amplification. Complementary DNA was synthesized from total liver RNA (2 µg) using Superscript™ II reverse transcriptase and used as templates for *in vitro* DNA amplification. LDLr and β-actin mRNA sequences were amplified using published sequences of specific primers (Bennett *et al.*, 1995) (hamster LDLr sense 5'-TCGGTGACAA-TGTCGCACCAAG-3', antisense 5'- GCTTCTGGTACACTGGTTGTC-3'; hamster β-actin sense 5'-CATCGTACTCCTGCTTGCTG-3', antisense 5'- GCTACAGCTTCAC-CACCACA-3'). The PCR conditions were as follows; Initial heating up to 94^0 C, then 94^0 C for 1min, 55^0 C for 2 min and 72^0 C for 3 min for a total number of 30 cycles. The total number of cycles (30) for each PCR reaction was chosen to remain within the exponential phase of the reaction. The products were resolved on a 1.5% agarose gel (Figure 2.1). The representative bands were quantified using ChemiImager™ 4400 gel documentation

system (Alpha Inotech Corporation, San Leandro, CA). LDLr m-RNA expression was normalized against β-actin m-RNA expression and expressed as relative units.

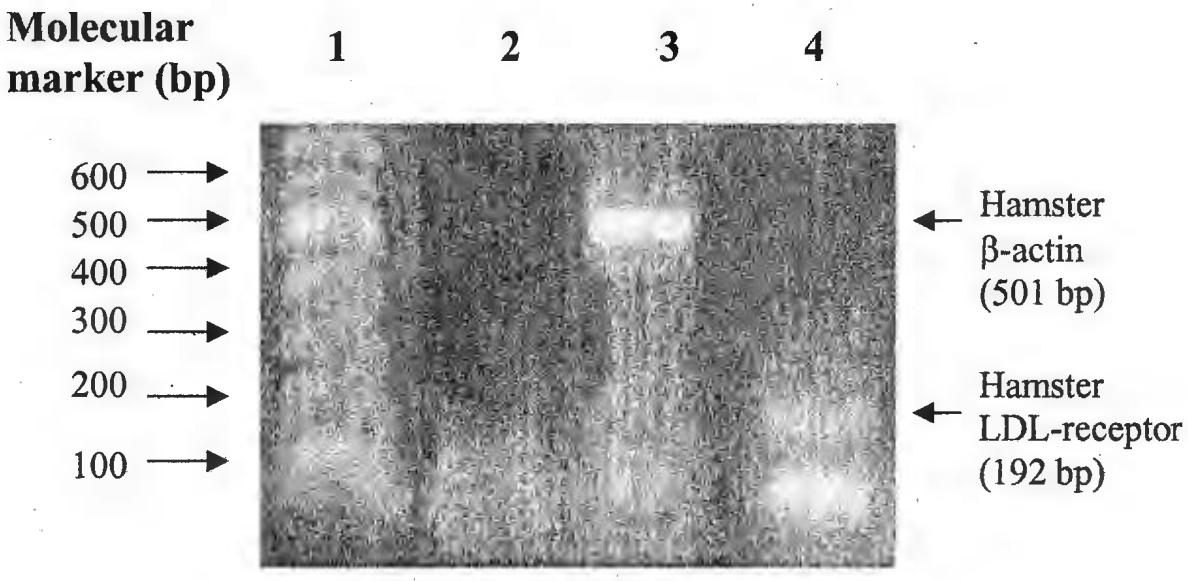
2.9 Electron microcopy

Electron microscopy was performed on LDL and VLDL fractions in order to assess the lipoprotein particle size. The lipoprotein fraction was applied to the carbon surface of the carbon coated copper grids (400 mesh, Marivac Ltd., QC) and incubated at room temperature for 5 minutes. The excess fluid was removed and samples were negatively stained using 2% phosphotungstic acid, pH 6.4 for 5 minutes (Anderson *et al.* 1989). Grids were dried in air and lipoprotein particles were visualized using electron microscope type JEOL-JEM 1200 EX, at 30,000 magnification.

2.10 Statistical analysis

The effect of diet type (DT), dietary fat level (DL) and dietary cholesterol (CHOL) was determined using 3-way analysis of variance and a Tukey's post hoc test was used to test significant differences revealed by the ANOVA. Values are group means ± SD, n=12; Differences were considered to be statistically significant if the associated P value was <0.05 (Steel and Torrie, 1980).

Figure 2.1 RT-PCR of LDL-receptor and β-actin. Total RNA was reverse transcribed and cDNA amplified by PCR for LDL-receptor and β-actin. Lane 1 shows 100 bp ladder (molecular marker); lane 2 shows the amplification in the absence of reverse transcriptase; lane 3 shows the amplification of hamster β-actin in the presence of reverse transcriptase and lane 4 shows the amplification of hamster LDL-receptor in the presence of reverse transcriptase.


Molecular marker (bp)
1
2
3
4
600
500
400
300
200
100
Hamster β-actin (501 bp)
Hamster LDL-receptor (192 bp)

Chapter 3: Results

3.1 Body weights and Liver weights of hamsters

The body weight and liver weight of hamsters fed low fat and high fat fish oil and MIX diets are given in table 3.1. There was no significant effect of dietary fat level or diet type on body weight gain. Diet type had no significant effect on liver weights both at low fat or high fat levels. There was a significant increase in liver weights in high fat diet fed hamsters compared to low fat diet fed hamsters irrespective of the diet type. However, food consumption measurements indicate no change in food intake in hamsters fed various diets

3.2 Red Blood Cell (RBC) fatty acid composition

RBC fatty acid composition was analyzed to confirm the incorporation of dietary fatty acids and is given in Table 3.2. RBC from fish oil diet fed hamsters showed increased levels of EPA and DHA at the expense of linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) compared to MIX diet fed hamsters. Thus, the length of the experimental feeding period was sufficient to induce significant changes in the fatty acid composition of the tissues and these changes reflect dietary lipid intake. The extent of incorporation of n-3 PUFA into RBC lipids was equivalent in both low fat diet groups and high fat diet groups irrespective of diet type.

Table 3.1: Body weight and liver weight of hamsters fed various diets. Hamsters were weighed at the beginning and conclusion of the experiment. The livers were collected and weighed at the conclusion of the experiment. Values shown are mean ± SD, n= 12 for each diet group. [a, b]Mean values for liver weight with unlike superscript letters are significantly different.

Diet type	Fat level % w/w	Original body weight	Final body weight	Liver weight
			(g)	
FO	5	101.18±3.67	95.6±5.45	3.40±0.24[a]
FO+Chol	5	99.95±7.33	91.68±5.65	3.80±0.34[a]
MIX	5	103.45±4.85	97.43±4.53	3.28±0.13[a]
MIX+Chol	5	103.70±6.45	96.53±6.87	3.85±0.40[a]
FO	20	105.42±5.65	100.57±7.25	4.14±0.29[b]
FO+Chol	20	104.47±3.37	97.97±3.87	4.44±0.47[b]
MIX	20	108.10±5.98	108.43±4.95	4.35±0.48[b]
MIX+Chol	20	107.27±4.73	107.55±4.01	5.12±0.84[b]

3.2 Red Blood Cell (RBC) fatty acid composition of hamsters fed various diets. Blood was centrifuged at 4^0 C at 3000 x *g* for 15 minutes. RBCs were collected (Bottom layer) and lipids were extracted using the method of Folch *et al.* (1957). Fatty acid composition was determined by gas liquid chromatography. n= 12 for each diet group. Abbreviations used: FO. Fish oil diet; MIX, MIX diet; ND, not detected.

Fatty Acid	Dietary Groups			
	FO 5%	MIX 5%	FO 20%	MIX 20%
14:0	0.7	0.4	1.7	0.4
16:0	24.3	25.0	24.0	25.0
16:1n7	3.1	1.6	5.4	ND
18:0	9.4	10.26	9.5	11.3
18:1	20.0	19.0	19.2	18.2
18:2n6	8.06	17.2	5.2	22.8
20:4n6	8.6	10.3	6.0	9.1
20:5n3	6.5	1.0	10.9	0.9
22:4n6	0.7	1.1	0.5	0.8
22:5n3	2.6	2.1	2.0	0.9
22:6n3	12.7	5.5	12.3	6.8

3.3 Effect of dietary fats on plasma lipid profile

3.3.1 Effect of diet type on plasma lipids

The plasma lipid composition (total cholesterol, free cholesterol, cholesterol ester and triacylglycerol) of hamsters fed various diets is shown in Figure 3.1-3.4. The hamsters fed fish oil diet had significantly higher levels of plasma total cholesterol, free cholesterol, cholesterol ester and triacylglycerol compared to hamsters fed a MIX diet.

3.3.2 Effect of dietary fat level on plasma lipid profile

Irrespective of the diet type, hamsters fed the high fat diets had significantly higher levels of total cholesterol, free cholesterol, cholesterol ester and triacylglycerol concentrations compared to hamsters fed the low fat diets. However, this hyperlipidemic effect of dietary fat level was greater in hamsters fed the fish oil diet (DT X DL interaction, $p= 0.0001$).

3.3.3 Effect of addition of cholesterol to diets on plasma lipid profile

Addition of cholesterol to the diets showed an increase in plasma total cholesterol, free cholesterol, cholesterol ester and triacylglycerol concentrations in both fish oil and MIX diet fed hamsters (Figure 3.1-3.4). However, this increase is prominent in fish oil diet fed hamsters (DT X CHOL interaction, $p= 0.0003$, < 0.03, < 0.002, $p< 0.03$ respectively). Furthermore, the effect of dietary cholesterol on plasma total cholesterol, cholesterol ester and triacylglycerol concentrations was greater in high fat diet fed hamsters compared to low fat diet fed hamsters (DL X CHOL interaction, $p< 0.02$).

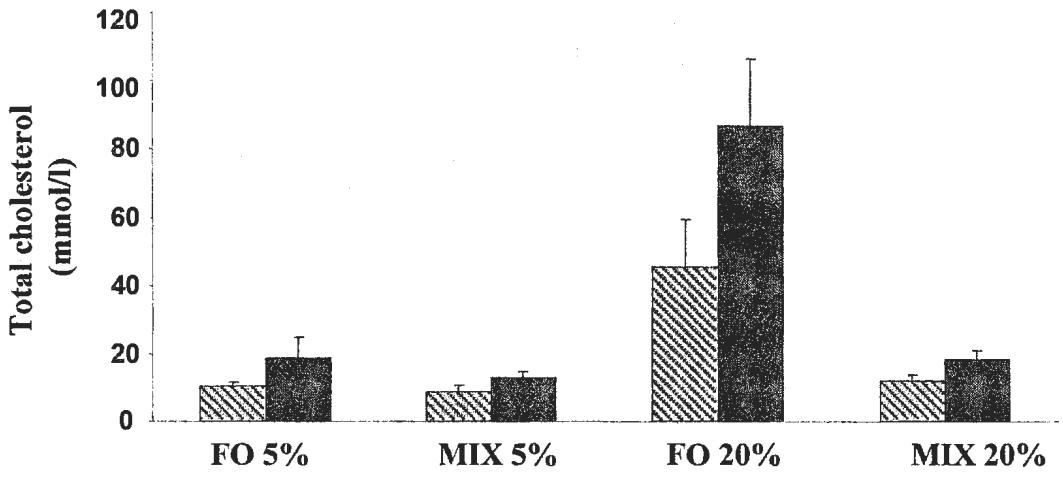


Figure 3.1: Plasma free cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet, (FO) or a MIX diet at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. The free cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.3: 3-way ANOVA results for plasma total cholesterol concentrations.

Source	F-Value	P-Value
Diet type	121.177	0.0001
Diet level	125.992	0.0001
Cholesterol	36.258	0.0001
Diet type * diet level	90.587	0.0001
Diet type * cholesterol	16.160	0.0003
Diet level * cholesterol	12.556	0.0012
Diet type * diet level * cholesterol	9.406	0.0042

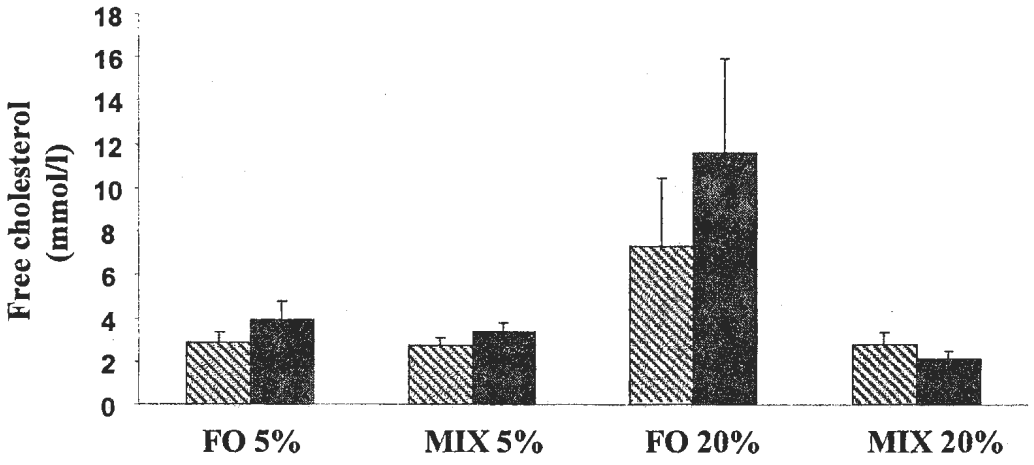


Figure 3.2: Plasma free cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. The free cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.4: 3-way ANOVA results for plasma free cholesterol concentrations.

Source	F-Value	P-Value
Diet type	42.047	0.0001
Diet level	23.896	0.0001
Cholesterol	5.535	0.0244
Diet type * diet level	34.686	0.0001
Diet type * cholesterol	5.638	0.0232
Diet level * cholesterol	0.769	0.3866
Diet type * diet level * cholesterol	4.180	0.0485

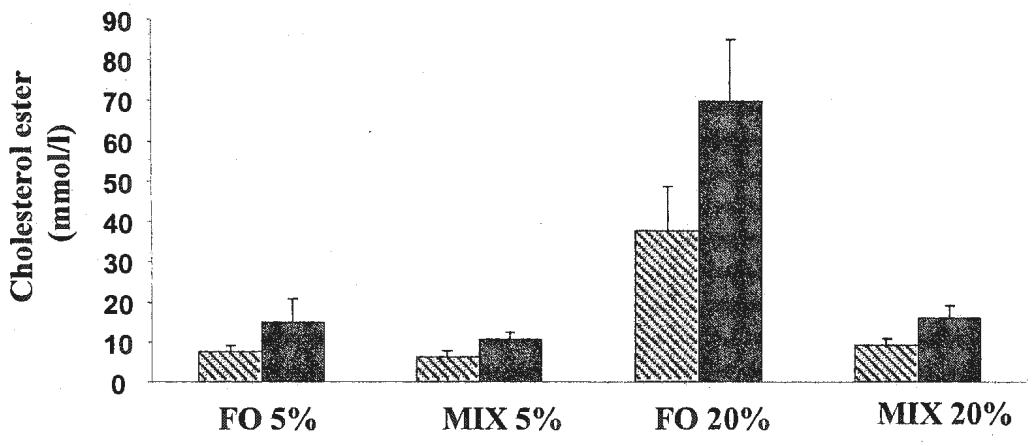


Figure 3.3: Plasma cholesterol ester concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Cholesterol ester concentration was calculated as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.5: 3-way ANOVA results for plasma cholesterol ester concentrations.

Source	F-Value	P-Value
Diet type	120.991	0.0001
Diet level	137.628	0.0001
Cholesterol	41.483	0.0001
Diet type * diet level	93.455	0.0001
Diet type * cholesterol	12.368	0.0012
Diet level * cholesterol	11.176	0.0020
Diet type * diet level * cholesterol	7.776	0.0085

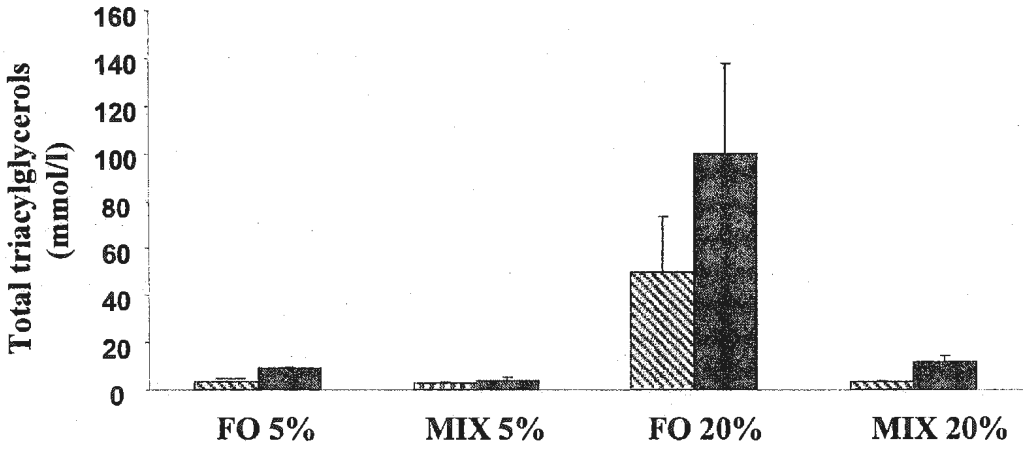


Figure 3.4: Plasma triacylglycerol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Triacylglycerol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.6: 3-way ANOVA results for plasma total triacylglycerol concentrations.

Source	F-Value	P-Value
Diet type	52.558	0.0001
Diet level	56.466	0.0001
Cholesterol	11.585	0.0019
Diet type * diet level	44.093	0.0001
Diet type * cholesterol	5.751	0.0227
Diet level * cholesterol	7.236	0.0114
Diet type * diet level * cholesterol	3.660	0.0650

3.4 Effect of dietary fats on lipoprotein lipid profile

The hamsters fed a high fat fish oil diet (20% w/w) had milky plasma which contained very high levels of chylomicron like particles, which were rich in cholesterol (Figure 3.5) and triacylglycerols (Figure 3.6), irrespective of absence or presence of cholesterol.

3.4.1 Effect of dietary fats on VLDL lipids

3.4.1.1 Effect of diet type on VLDL lipids

Marked differences in VLDL composition were seen in hamsters fed the different diets (Figure 3.7-3.11). There was a significant increase in VLDL total cholesterol, free cholesterol, cholesterol ester, triacylglycerol and phospholipid concentrations in fish oil diet fed hamsters compared to MIX diet fed hamsters.

3.4.1.2 Effect of dietary fat level on VLDL lipids

Increasing the dietary fat level from low fat to high fat resulted in a significant increase in VLDL total cholesterol, free cholesterol, cholesterol ester, triacylglycerol and phospholipid concentrations in both fish oil and MIX diet fed hamsters (Figure 3.7-3.11). This dietary fat level dependent increase was greater in fish oil diet fed hamsters compared to MIX diet fed hamsters (DT X DL interactions, p= 0.0001, 0.0001, 0.0001, 0.02 respectively).

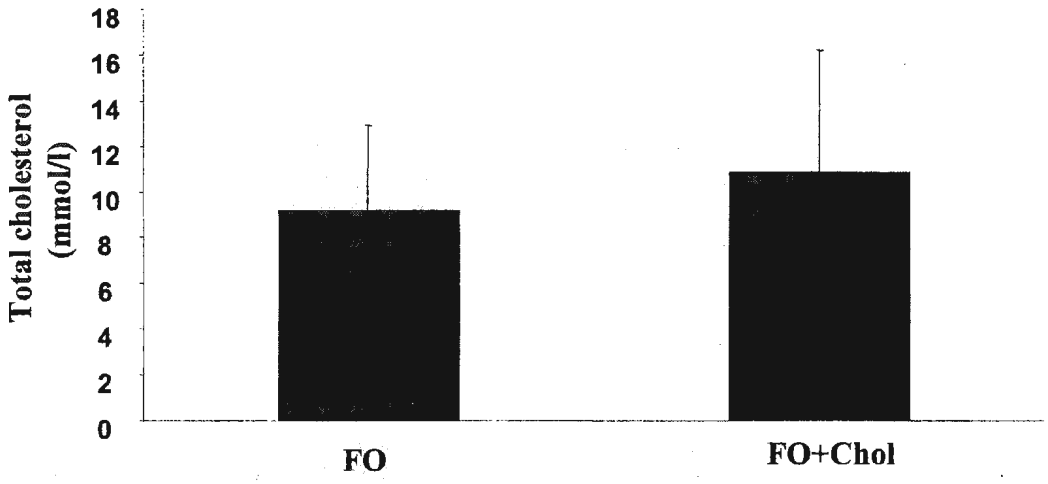


Figure 3.5: Chylomicron total cholesterol concentrations of hamsters fed high fat fish oil diets. Hamsters fed a high fat fish oil diet had milky plasma due to chylomicron like particles. This fraction was separated and total cholesterol concentration was analyzed as described in methods. Differences between groups were evaluated using Student's *t* test. Abbreviations used: FO, fish oil diet; Chol, cholesterol.

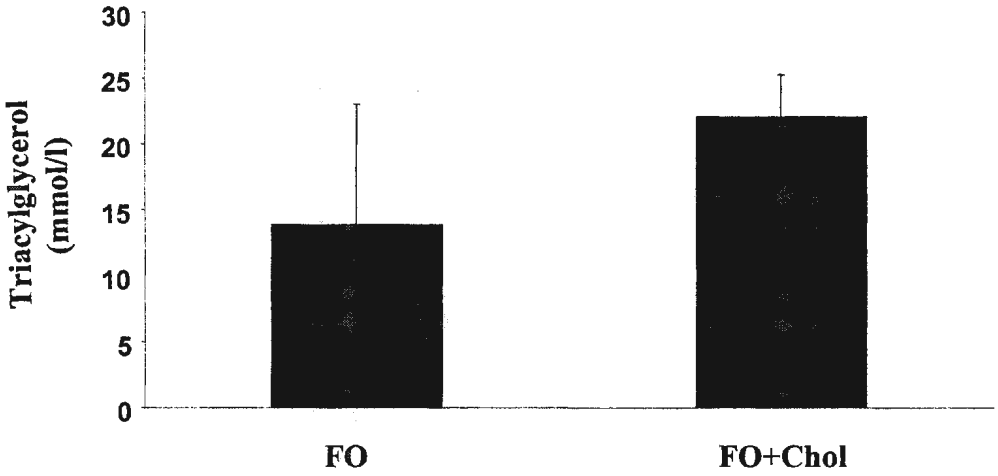


Figure 3.6: Chylomicron triacylglycerol concentrations of hamsters fed high fat fish oil diets. Hamsters fed a high fat fish oil diet had milky plasma due to chylomicron like particles. This fraction was separated and total cholesterol concentration was analyzed as described in methods. Differences between groups were evaluated using Student's *t* test. Abbreviations used: FO, fish oil diet; Chol, cholesterol.

3.4.1.3 Effect of addition of cholesterol to the diets on VLDL lipids

Dietary supplementation of cholesterol resulted in an increase in VLDL total cholesterol, cholesterol ester and triacylglycerol concentrations in both fish oil and MIX diet fed hamsters (Figure 3.7-3.10). Dietary-cholesterol induced increase in VLDL cholesterol ester concentration was prominent in fish oil diet fed hamsters compared to MIX diet fed hamster (DT X CHOL interaction, $p< 0.006$). There was no effect of dietary fat level on dietary cholesterol-induced changes in VLDL cholesterol or triacylglycerol concentrations.

3.4.2 Effect of dietary fats on LDL lipids

3.4.2.1 Effect of diet type on LDL lipids

The LDL lipid composition is shown in Figure 3.12-3.16. LDL from fish oil diet fed hamsters showed significantly higher concentrations of total cholesterol, cholesterol ester, triacylglycerol and phospholipid compared to MIX diet fed hamsters. However, the trend of increase in free cholesterol concentrations in fish oil diet fed hamsters was not significant.

3.4.2.2 Effect of dietary fat level on LDL lipids

Increasing the dietary fat level of fish oil from low fat to high fat caused a significant increase in LDL free cholesterol ($p< 0.0001$), triacylglycerol (DT X DL interaction, $p<0.0001$) and phospholipid concentrations ($p=0.0001$), however, a significant decrease in LDL total cholesterol and cholesterol ester concentrations was

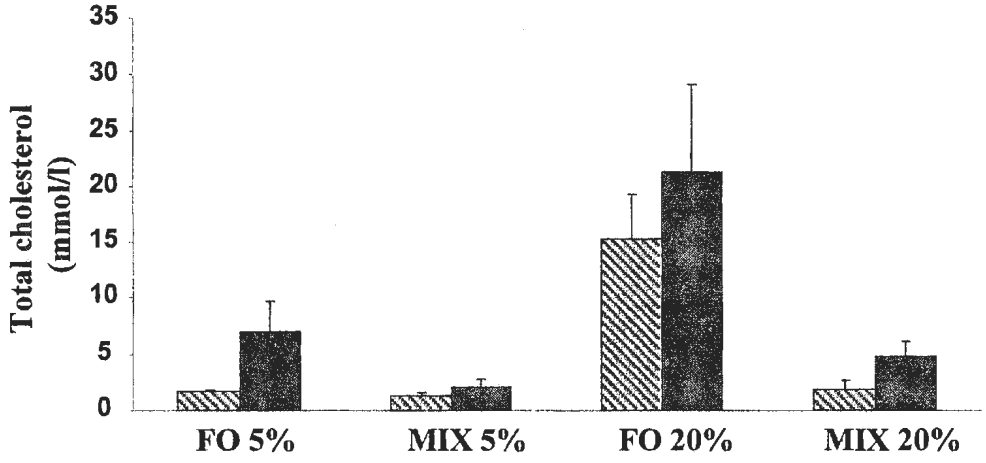


Figure 3.7: VLDL total cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat, level in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Total cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.7: 3-way ANOVA results for VLDL total cholesterol concentrations.

Source	F-Value	P-Value
Diet type	73.689	0.0001
Diet level	54.352	0.0001
Cholesterol	12.409	0.0015
Diet type * diet level	36.298	0.0001
Diet type * cholesterol	3.618	0.0675
Diet level * cholesterol	0.396	0.5345
Diet type * diet level * cholesterol	0.057	0.8126

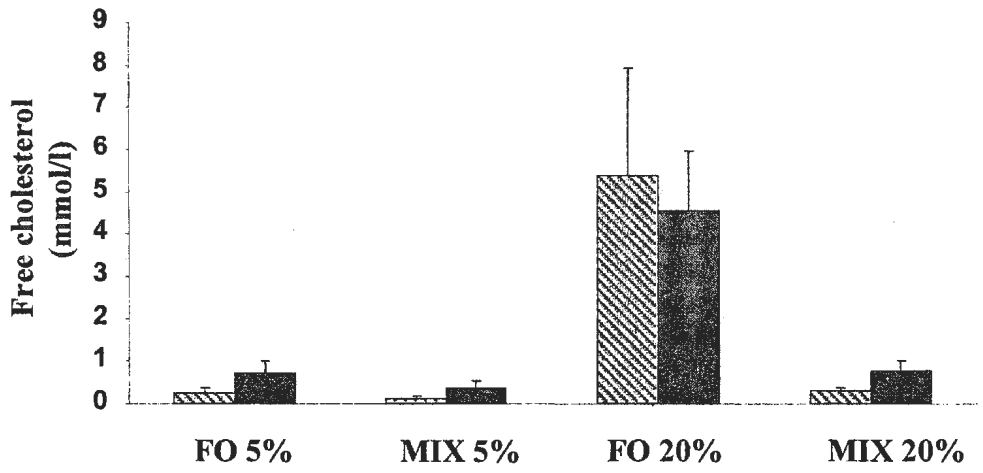


Figure 3.8: Changes in VLDL free cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. The free cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Only significantly different comparisons ($p< 0.05$) are indicated in the figure. Values are means for 12 animals with SD shown by vertical bars.

Table 3.8: 3-way ANOVA results for VLDL free cholesterol concentrations.

Source	F-Value	P-Value
Diet type	88.220	0.0001
Diet level	92.254	0.0001
Cholesterol	0.153	0.6986
Diet type * diet level	70.916	0.0001
Diet type * cholesterol	1.136	0.2944
Diet level * cholesterol	1.147	0.2923
Diet type * diet level * cholesterol	2.717	0.1091

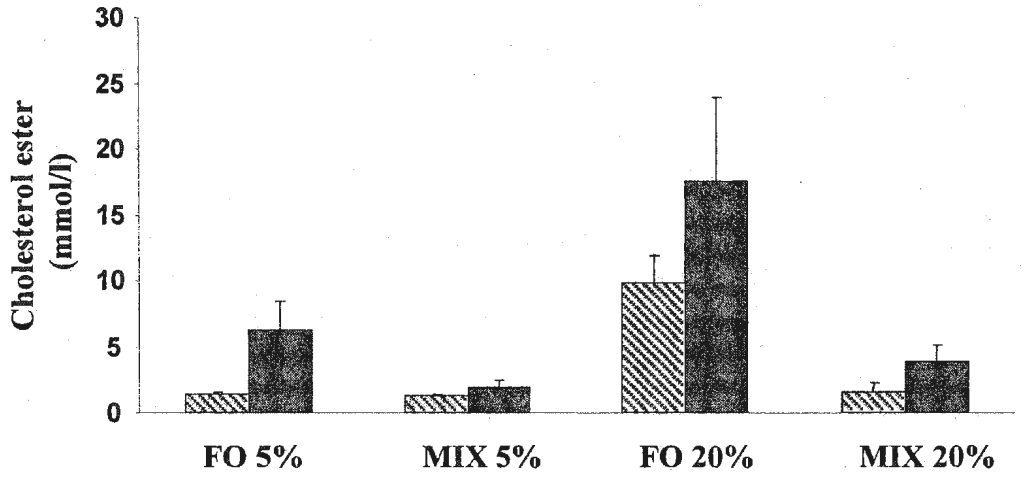


Figure 3.9: VLDL cholesterol ester concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Cholesterol ester concentration was calculated as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.9: 3-way ANOVA results for VLDL cholesterol ester concentrations.

Source	F-Value	P-Value
Diet type	66.882	0.0001
Diet level	44.269	0.0001
Cholesterol	21.548	0.0001
Diet type * diet level	29.317	0.0001
Diet type * cholesterol	9.144	0.0053
Diet level * cholesterol	1.774	0.1936
Diet type * diet level * cholesterol	0.168	0.6849

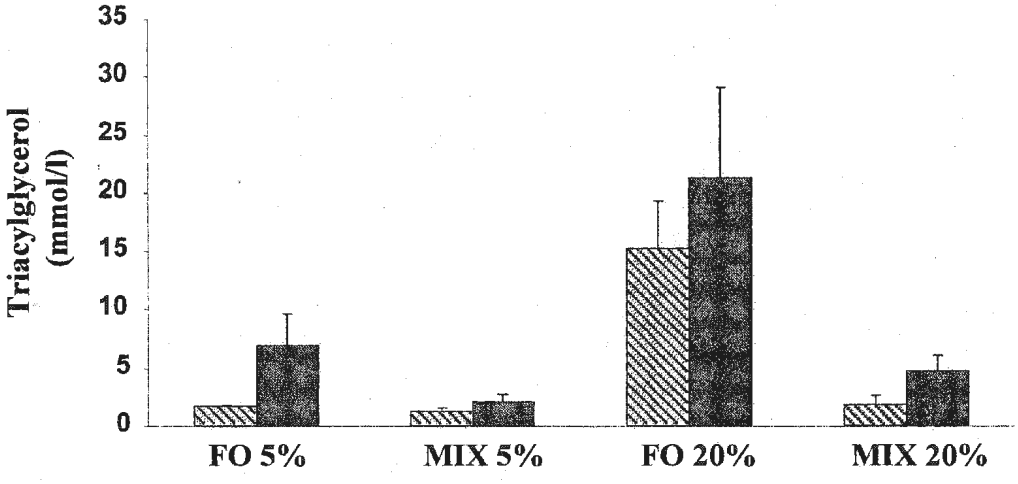


Figure 3.10: VLDL triacylglycerol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Triacylglycerol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.10: 3-way ANOVA results for VLDL triacylglycerol concentrations.

Source	F-Value	P-Value
Diet type	18.634	0.0002
Diet level	26.054	0.0001
Cholesterol	4.425	0.0457
Diet type * diet level	7.537	0.0110
Diet type * cholesterol	0.001	0.9707
Diet level * cholesterol	0.914	0.3481
Diet type * diet level * cholesterol	1.407	0.2467

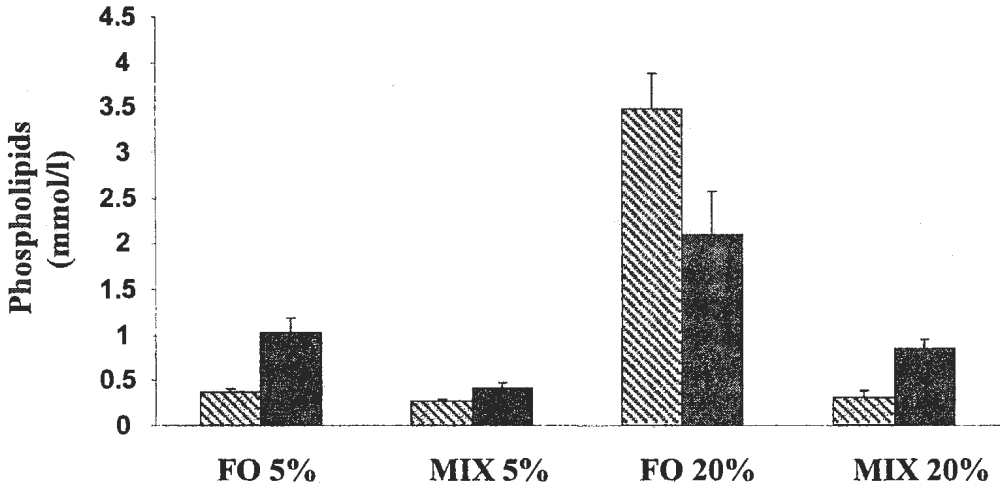


Figure 3.11: VLDL phospholipid concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Phospholipid concentration was assayed as described in the methods. Differences between groups were evaluated using 3-way ANOVA.

Values are means for 12 animals with SD shown by vertical bars.

Table 3.11: 3-way ANOVA results for VLDL phospholipid concentrations.

Source	F-Value	P-Value
Diet type	282.180	0.0001
Diet level	230.102	0.0001
Cholesterol	0.017	0.8985
Diet type * diet level	146.681	0.0001
Diet type * cholesterol	20.899	0.0001
Diet level * cholesterol	27.800	0.0001
Diet type * diet level * cholesterol	61.734	0.0001

observed (DT X DL interaction, $p < 0.002$, 0.03 respectively). There was no effect of dietary fat level on LDL total cholesterol, cholesterol ester or triacylglycerol concentrations in MIX diet fed hamsters.

3.4.2.3 Effect of addition of cholesterol to the diets on LDL lipids

Cholesterol supplementation to fish oil diet resulted in an increase in LDL total cholesterol (DT X CHOL interaction, $p = 0.0007$), cholesterol ester ($p = 0.0001$) and phospholipid (DT X CHOL interaction, $p = 0.009$) concentrations (Figure 3.12-3.16). Dietary cholesterol-induced increase in LDL triacylglycerol concentration was observed only in low fat fish oil diet fed hamsters (DL X CHOL interaction, $p < 0.02$). Furthermore, the effect of dietary cholesterol was greater in low fat fish oil diet fed hamsters with respect of total cholesterol (DT X DL X CHOL interaction, $p < 0.004$) and cholesterol ester (DL X CHOL interaction, $p = 0.0004$) concentrations. There was no effect of dietary cholesterol amount on LDL lipids in MIX diet fed hamsters.

3.4.3 Effect of dietary fats on HDL lipids

3.4.3.1 Effect of diet type on HDL lipids

Changes in HDL lipid composition are given in (Figure 3.17-3.21). Fish oil diet fed hamsters had significantly lower concentrations of HDL total cholesterol, free cholesterol, cholesterol ester and phospholipid concentrations compared to MIX diet fed hamsters. However, there was no significant effect of fish oil on HDL triacylglycerol concentrations.

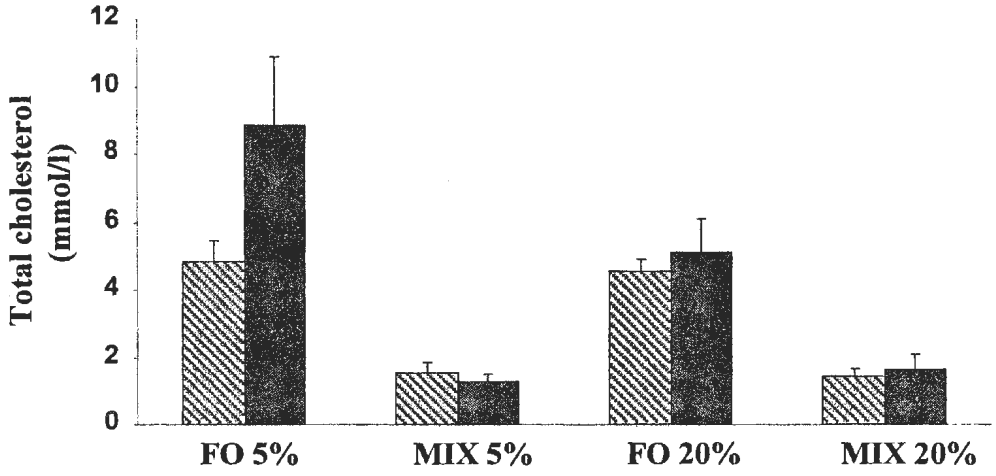


Figure 3.12: LDL total cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Total cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.12: 3-way ANOVA results for LDL total cholesterol concentrations.

Source	F-Value	P-Value
Diet type	205.346	0.0001
Diet level	9.300	0.0052
Cholesterol	13.125	0.0012
Diet type * diet level	12.652	0.0015
Diet type * cholesterol	14.797	0.0007
Diet level * cholesterol	6.335	0.0183
Diet type * diet level * cholesterol	10.184	0.0037

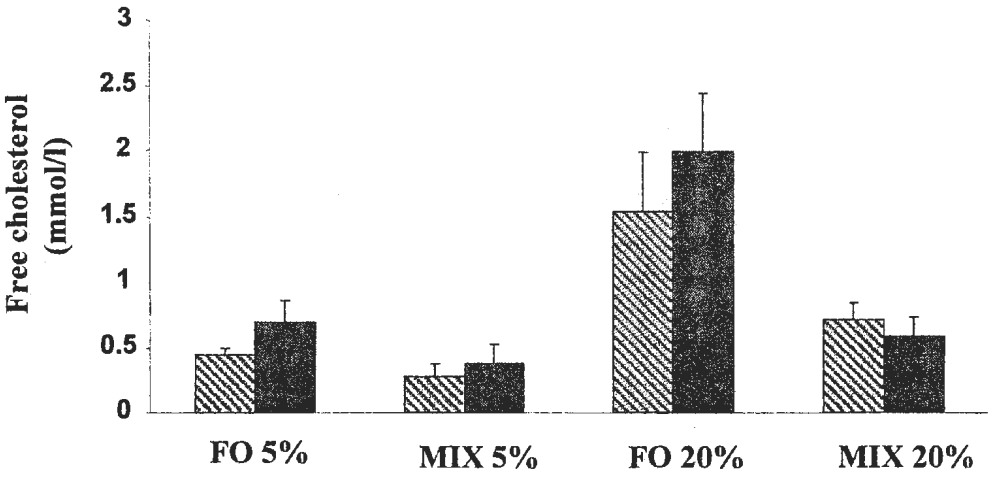


Figure 3.13: Changes in LDL free cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Free cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.13: 3-way ANOVA results for LDL free cholesterol concentrations.

Source	F-Value	P-Value
Diet type	2.742	0.1087
Diet level	82.731	0.0001
Cholesterol	2.124	0.1551
Diet type * diet level	0.029	0.8665
Diet type * cholesterol	1.472	0.2342
Diet level * cholesterol	0.034	0.8546
Diet type * diet level * cholesterol	0.407	0.5284

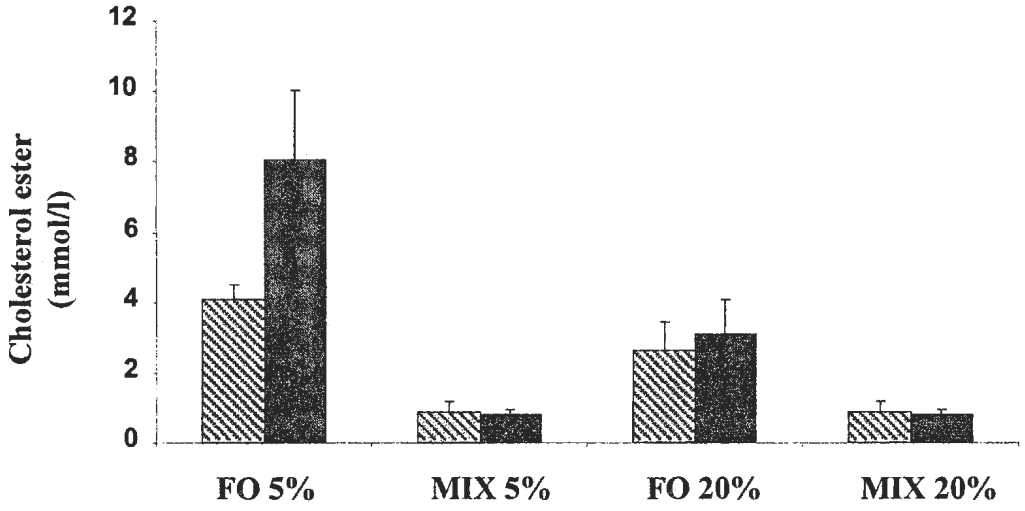


Figure 3.14: LDL cholesterol ester concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Cholesterol ester concentration was calculated as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.14: 3-way ANOVA results for LDL cholesterol ester concentrations.

Source	F-Value	P-Value
Diet type	19.273	0.0001
Diet level	46.008	0.0001
Cholesterol	20.807	0.0001
Diet type * diet level	5.340	0.0279
Diet type * cholesterol	2.800	0.1046
Diet level * cholesterol	15.556	0.0004
Diet type * diet level * cholesterol	1.042	0.3154

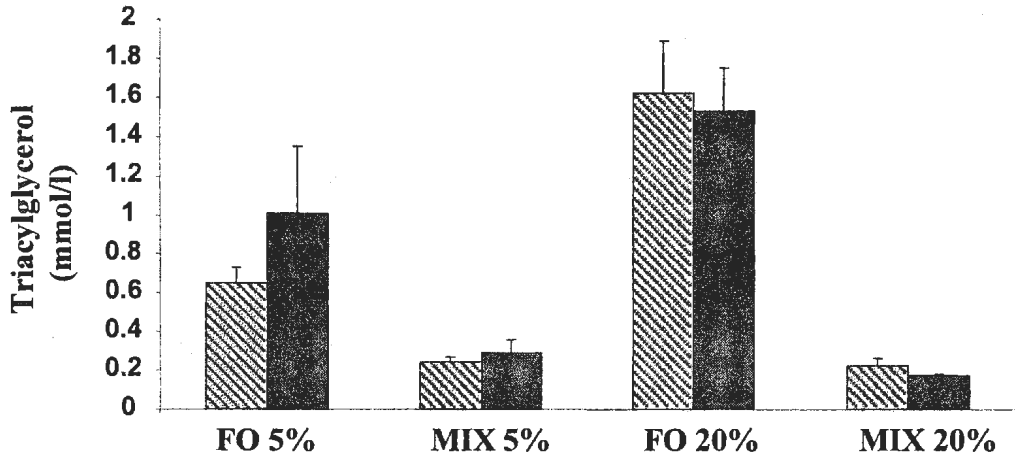


Figure 3.15: LDL triacylglycerol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Triacylglycerol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.15: 3-way ANOVA results for LDL triacylglycerol concentrations.

Source	F-Value	P-Value
Diet type	358.713	0.0001
Diet level	44.572	0.0001
Cholesterol	1.652	0.2086
Diet type * diet level	62.868	0.0001
Diet type * cholesterol	1.708	0.2011
Diet level * cholesterol	7.442	0.0105
Diet type * diet level * cholesterol	2.799	0.1047

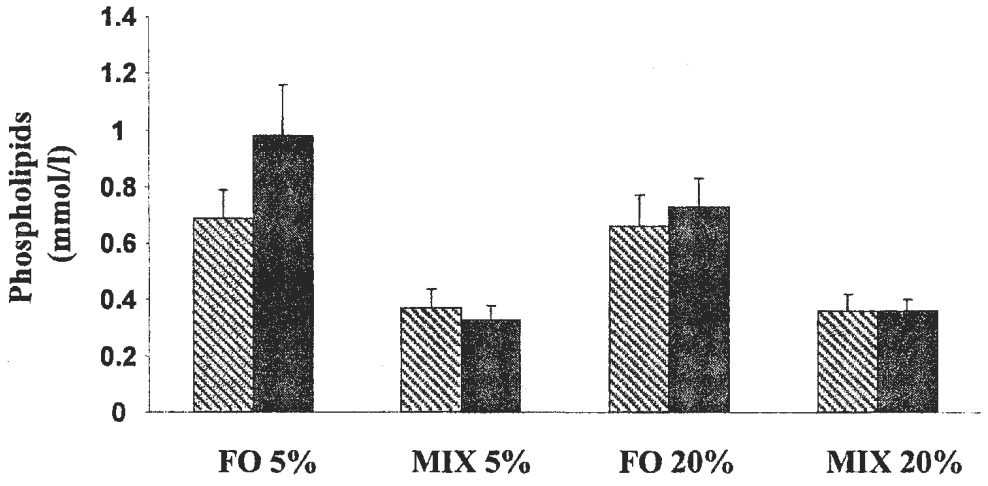


Figure 3.16: LDL phospholipid concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Phospholipid concentration was assayed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.16: 3-way ANOVA results for LDL phospholipid concentrations.

Source	F-Value	P-Value
Diet type	135.799	0.0001
Diet level	3.431	0.0754
Cholesterol	4.957	0.0349
Diet type * diet level	4.999	0.0342
Diet type * cholesterol	7.980	0.0090
Diet level * cholesterol	1.707	0.2029
Diet type * diet level * cholesterol	3.628	0.0679

3.4.3.2 Effect of dietary fat level on HDL lipids

Increasing the dietary fat level from low fat to high fat resulted in a decrease in HDL total cholesterol, cholesterol ester and phospholipid concentrations in fish oil diet fed hamsters (DT X DL interaction, $p= 0.0005$) and an increase in these parameters in MIX diet fed hamsters (Figure 3.17-3.21). Furthermore, there was dietary fat level dependent increase in HDL triacylglycerol concentration in fish oil diet fed hamsters (DT X DL interaction, $p< 0.04$).

3.4.3.3 Influence of addition of cholesterol to the diets on HDL lipids

Cholesterol supplementation to diets resulted in an increase in HDL total cholesterol, cholesterol ester and phospholipid concentrations irrespective of diet type (Figure 3.17-3.21). However, there was no consistent effect of cholesterol supplementation on HDL triacylglycerol concentrations.

3.5 Effect of dietary fats on hepatic lipid composition

3.5.1 Effect of diet type on hepatic lipids

Changes in hepatic lipid composition are shown in Figure 3.22-3.25. Hamsters fed a low fat fish oil diet show lower hepatic total cholesterol, cholesterol ester and triacylglycerol levels compared to hamsters fed a low fat MIX diet. In contrast, high fat fish oil diet fed hamsters show significantly higher levels of total cholesterol, cholesterol esters and triacylglycerols compared to high fat MIX diet fed hamsters (DT X DL interaction, $p< 0.0001$). There was no significant difference in free cholesterol levels between two

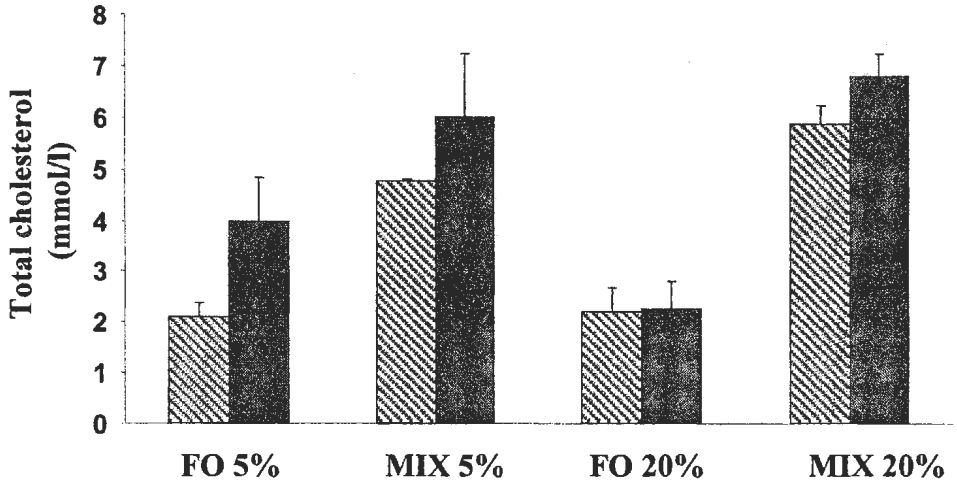


Figure 3.17: HDL total cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Total cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.17: 3-way ANOVA results for HDL total cholesterol concentrations.

Source	F-Value	P-Value
Diet type	150.815	0.0001
Diet level	0.393	0.5385
Cholesterol	11.917	0.0018
Diet type * diet level	20.343	0.0001
Diet type * cholesterol	1.062	0.3116
Diet level * cholesterol	2.147	0.1540
Diet type * diet level * cholesterol	0.602	0.4445

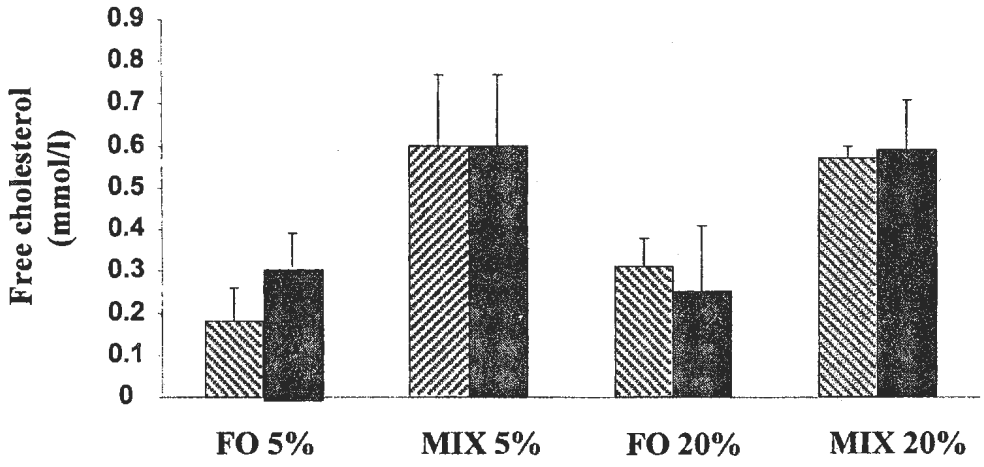


Figure 3.18: Changes in HDL free cholesterol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Free cholesterol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.18: 3-way ANOVA results for HDL free cholesterol concentrations.

Source	F-Value	P-Value
Diet type	66.224	0.0001
Diet level	0.102	0.7517
Cholesterol	0.409	0.5278
Diet type * diet level	0.395	0.5350
Diet type * cholesterol	0.075	0.7859
Diet level * cholesterol	1.160	0.2911
Diet type * diet level * cholesterol	1.700	0.2033

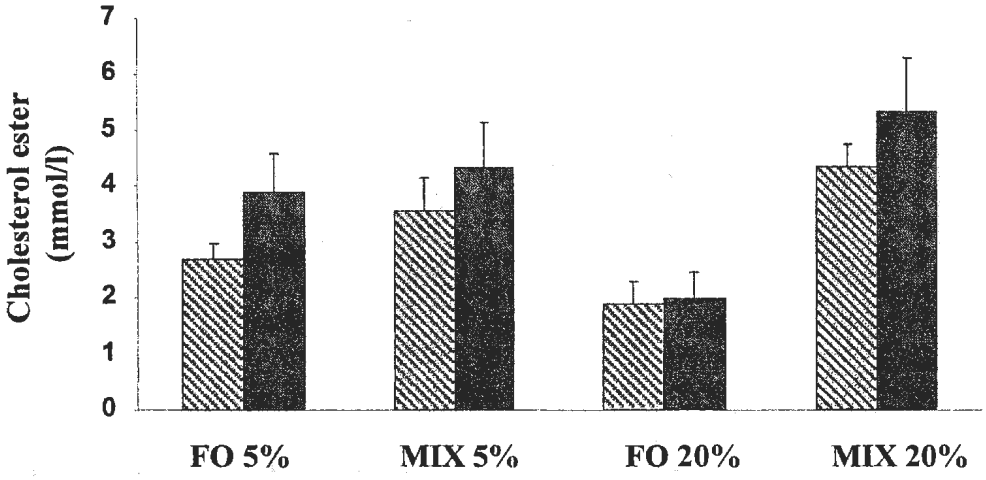


Figure 3.19: Effect of various diets on HDL cholesterol ester Concentrations. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Cholesterol ester concentration was calculated as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.19: 3-way ANOVA results for HDL cholesterol ester concentrations.

Source	F-Value	P-Value
Diet type	56.626	0.0001
Diet level	2.587	0.1173
Cholesterol	13.300	0.0009
Diet type * diet level	25.002	0.0001
Diet type * cholesterol	0.224	0.6390
Diet level * cholesterol	1.106	0.3005
Diet type * diet level * cholesterol	2.689	0.1105

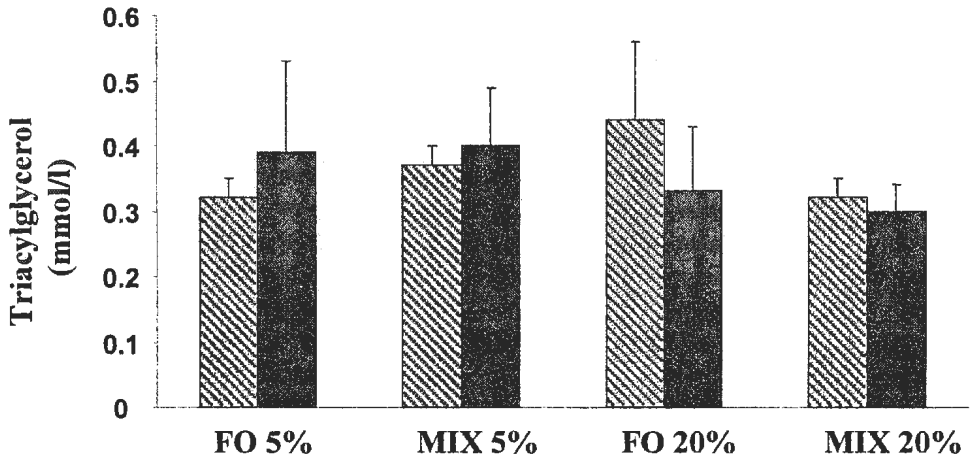


Figure 3.20: HDL triacylglycerol concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Triacylglycerol concentration was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.20: 3-way ANOVA results for HDL triacylglycerol concentrations.

Source	F-Value	P-Value
Diet type	1.201	0.2810
Diet level	0.939	0.3395
Cholesterol	0.194	0.6624
Diet type * diet level	4.825	0.0352
Diet type * cholesterol	0.064	0.8017
Diet level * cholesterol	4.643	0.0386
Diet type * diet level * cholesterol	0.666	0.4204

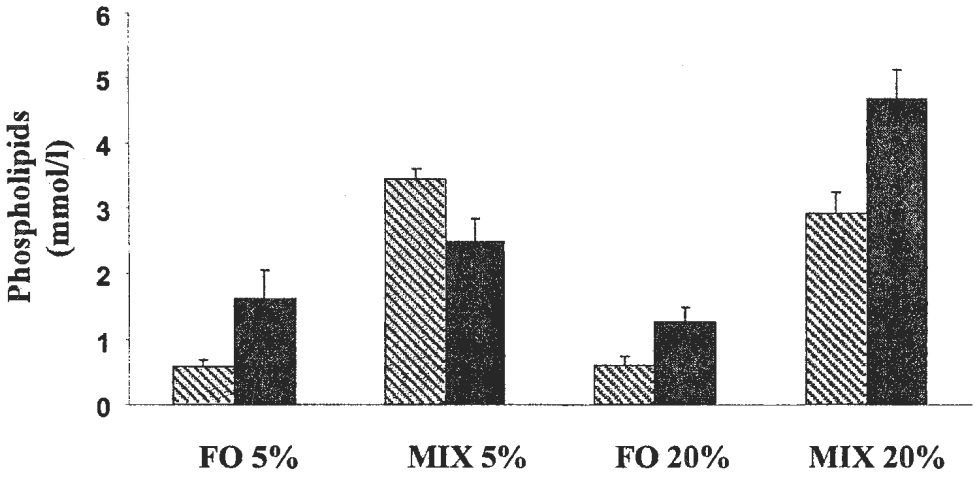


Figure 3.21: HDL phospholipid concentrations of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Phospholipid concentration was assayed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.21: 3-way ANOVA results for HDL phospholipid concentrations.

Source	F-Value	P-Value
Diet type	314.690	0.0001
Diet level	7.924	0.0101
Cholesterol	18.854	0.0003
Diet type * diet level	16.319	0.0005
Diet type * cholesterol	4.280	0.0505
Diet level * cholesterol	15.549	0.0007
Diet type * diet level * cholesterol	28.213	0.0001

diet types (Figure 3.23).

3.5.2 Effect of amount of fat on hepatic lipids

Increasing the dietary fat level from low fat (5% w/w) to high fat (20% w/w) in fish oil diet fed hamsters resulted in an increase in hepatic total cholesterol, cholesterol ester and triacylglycerol concentrations (DT X DL interaction, $p< 0.0001$) while there was no effect on free cholesterol concentrations (Figure 3.22-3.25). However, increasing the fat level in MIX diet fed hamsters did not have any effect on hepatic levels of total cholesterol, cholesterol ester and triacylglycerol.

3.5.3 Effect of cholesterol supplementation on hepatic lipids

Addition of cholesterol to the diets increased hepatic total cholesterol and cholesterol ester concentrations (Figure 3.22 and 3.24). However, this effect was prominent in fish oil diet fed hamsters compared to MIX diet fed hamsters (DT X DL interaction, $p< 0.05$). Further more, only fish oil diet fed hamsters showed a dietary cholesterol-induced elevation of hepatic triacylglycerol levels (DT X CHOL interaction, $p< 0.03$). In addition, the effect of dietary cholesterol on hepatic cholesterol ester levels was greater in low fat diet fed hamsters (DL X CHOL interaction, $p< 0.03$) compared to high fat diet fed hamsters.

3.6 Influence of dietary fat on hepatic MTTP activity

Hepatic MTTP activity of hamsters fed various diets is given in Figure 3.26. There was no significant difference in MTTP activity between low fat fish oil and MIX

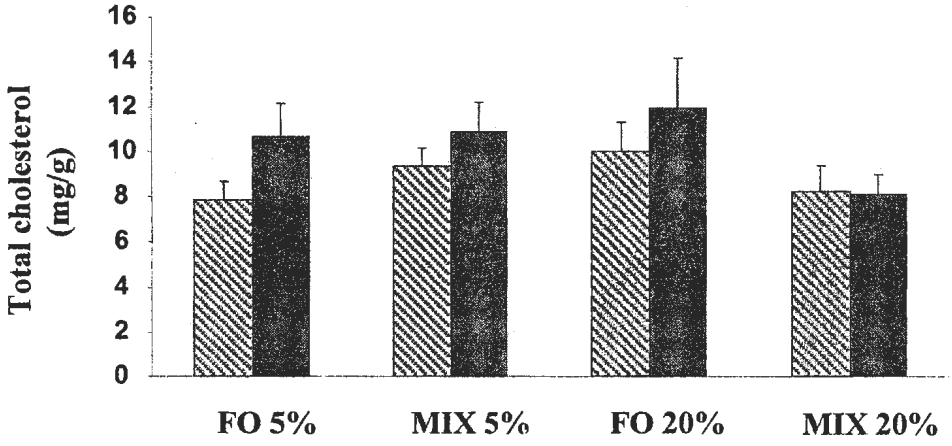


Figure 3.22: Hepatic total cholesterol concentration of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Lipids were extracted from liver samples and total cholesterol level was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.22: 3-way ANOVA results for hepatic total cholesterol concentrations.

Source	F-Value	P-Value
Diet type	6.518	0.0148
Diet level	0.060	0.8072
Cholesterol	15.217	0.0004
Diet type * diet level	21.339	0.0001
Diet type * cholesterol	4.098	0.0500
Diet level * cholesterol	2.846	0.0998
Diet type * diet level * cholesterol	0.275	0.6028

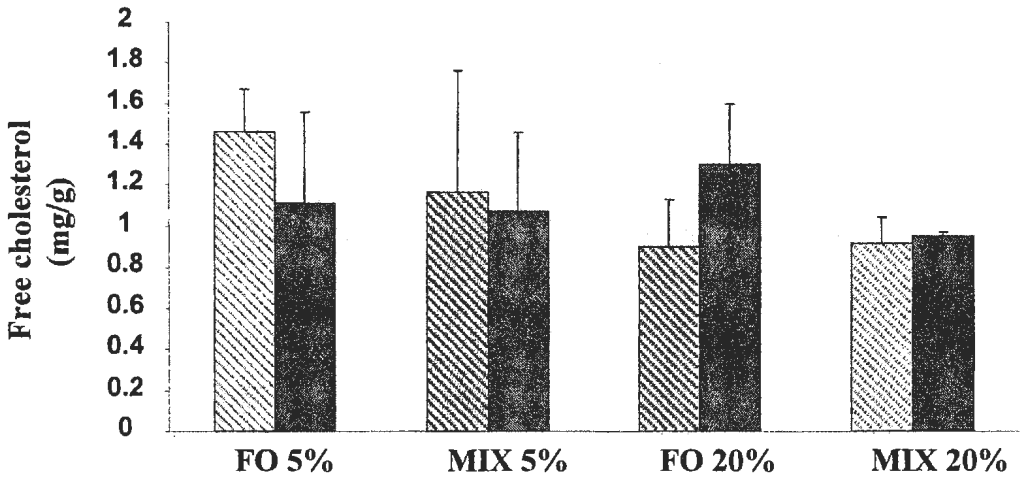


Figure 3.23: Hepatic free cholesterol concentration of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Lipids were extracted from liver samples and free cholesterol level was analyzed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 23: 3-way ANOVA results for hepatic free cholesterol concentrations.

Source	F-Value	P-Value
Diet type	2.914	0.0960
Diet level	2.804	0.1022
Cholesterol	0.020	0.8879
Diet type * diet level	0.010	0.9198
Diet type * cholesterol	0.030	0.8625
Diet level * cholesterol	3.996	0.0528
Diet type * diet level * cholesterol	2.119	0.1537

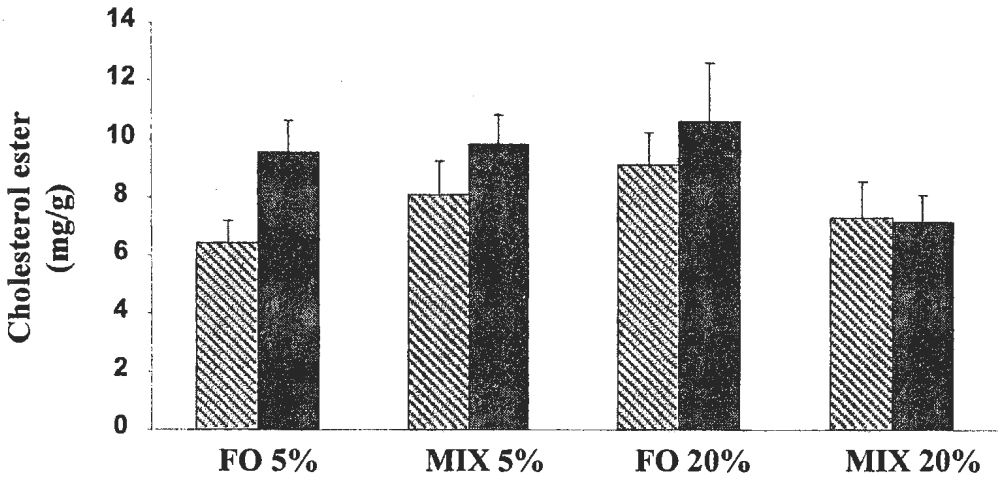


Figure 3.24: Effect of various diets on hepatic cholesterol ester concentration. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Lipids were extracted from liver samples and cholesterol ester level was calculated as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.24: 3-way ANOVA results for hepatic cholesterol ester concentrations

Source	F-Value	P-Value
Diet type	4.884	0.0332
Diet level	0.031	0.8609
Cholesterol	17.855	0.0001
Diet type * diet level	24.251	0.0001
Diet type * cholesterol	4.284	0.0453
Diet level * cholesterol	5.749	0.0215
Diet type * diet level * cholesterol	0.037	0.8485

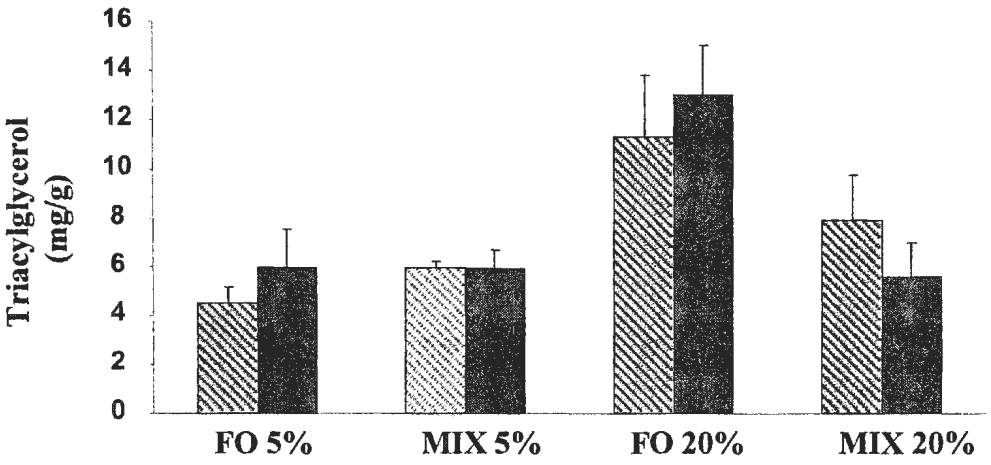


Figure 3.25: Hepatic triacylglycerol concentration of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Lipids were extracted from liver samples and triacylglycerol level was analyzed as outlined in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.25: 3-way ANOVA results for hepatic triacylglycerol concentrations.

Source	F-Value	P-Value
Diet type	15.893	0.0003
Diet level	55.271	0.0001
Cholesterol	1.046	0.3135
Diet type * diet level	26.569	0.0001
Diet type * cholesterol	5.562	0.0241
Diet level * cholesterol	0.009	0.9248
Diet type * diet level * cholesterol	0.672	0.4181

diet fed hamsters. However, hamsters fed a high fat fish oil diet show a significant increase in MTTP activity compared to high fat MIX diet fed hamsters (DT X DL interaction, $p< 0.05$). Dietary fat level had no effect on MTTP activity. Addition of cholesterol to the low fat fish oil diet resulted in an increase in MTTP activity.

3.7 Effect of dietary fats on adipose tissue LPL activity

The adipose tissue LPL activity of hamsters fed various diets is given in figure 3.28. There was a trend of increase in LPL activity in fish oil diet fed hamsters compared to MIX diet fed hamsters. In general, dietary supplementation of cholesterol had no effect on LPL activity, but hamsters fed low fat fish oil diet showed dietary cholesterol dependent decrease in LPL activity (DT X CHOL and DL X CHOL interactions, $p< 0.0004$).

3.8 Effect of dietary fat on plasma CETP activity and CETP mass

Changes in CETP activity and CETP mass are shown in Figure 3.28 and 3.29 respectively. The CETP activity and the CETP mass were significantly lower in hamsters fed the fish oil diet compared to MIX diet fed hamsters. Increasing the dietary fat level from low fat to high fat caused a decrease in CETP mass in fish oil diet fed hamsters whereas, an increase in MIX diet fed hamsters (DT X DL interaction, $p< 0.002$). However, there was no effect of dietary fat level on plasma CETP activity in both fish oil and MIX diet fed hamsters.

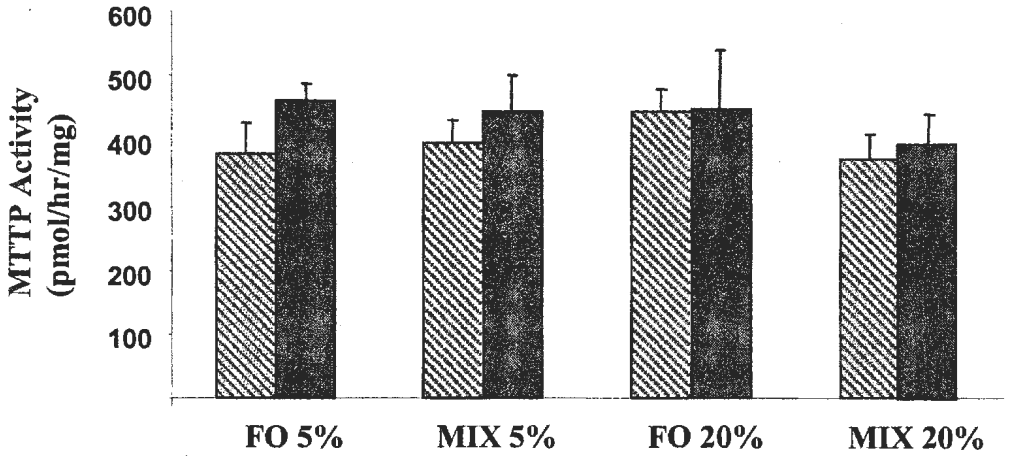


Figure 3.26: Effect of various diets on hepatic MTTP activity. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. MTTP activity of liver homogenate was assayed as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.26: 3-way ANOVA results for MTTP activity.

Source	F-Value	P-Value
Diet type	5.125	0.0305
Diet level	0.289	0.5946
Cholesterol	7.139	0.0118
Diet type * diet level	4.207	0.0485
Diet type * cholesterol	0.018	0.8933
Diet level * cholesterol	2.145	0.1528
Diet type * diet level * cholesterol	1.147	0.2921

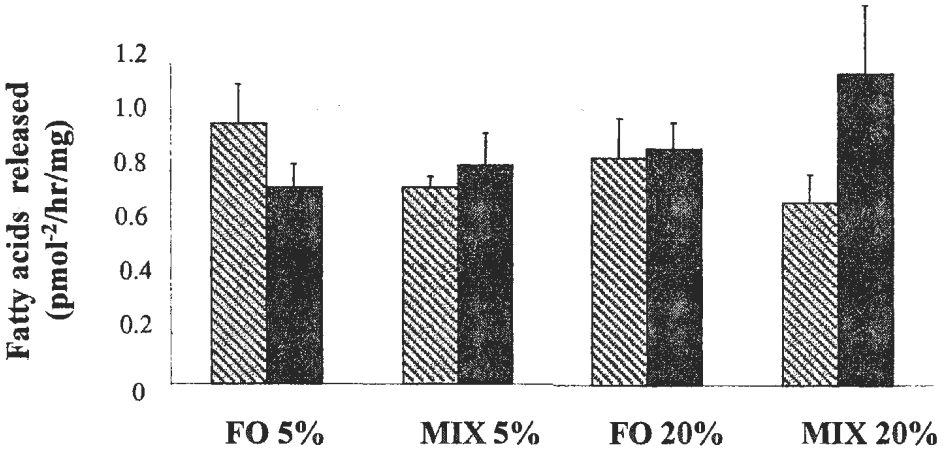


Figure 3.27: Adipose tissue LPL activity in various diet fed hamsters.

Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Adipose tissue was collected and LPL activity was assayed as described in the method section. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.27: 3-way ANOVA results for adipose tissue LPL activity.

Source	F-Value	P-Value
Diet type	2.593	0.1269
Diet level	7.923	0.0125
Cholesterol	1.095	0.3109
Diet type * diet level	1.844	0.1934
Diet type * cholesterol	22.926	0.0002
Diet level * cholesterol	19.982	0.0004
Diet type * diet level * cholesterol	1.028	0.3258

Supplementation of cholesterol to diets showed an increase in CETP activity and CETP mass in both fish oil and MIX diet fed hamsters. Furthermore, the effect of dietary cholesterol on plasma CETP activity was prominent in low fat diet fed hamsters (DL X CHOL interaction, $p< 0.0001$).

3.9 Effect of dietary fat on hepatic LDLr mRNA expression

The LDLr mRNA expression is shown in Figure 3.30. There was a significant reduction in hepatic LDLr mRNA expression in fish oil diet fed hamsters compared to MIX diet fed hamsters. Increasing the dietary fat level show a significant reduction in LDLr mRNA expression in fish oil diet fed hamsters ($p< 0.001$).

Dietary cholesterol supplementation had no effect on LDLr mRNA expression in fish oil diet fed hamsters, whereas supplementation of cholesterol to the MIX diet reduced the LDLr mRNA expression (DT X CHOL interaction, $p< 0.0001$)

3.10 Influence of dietary fat on lipoprotein particle size

3.10.1 LDL particle size

The average particle size of LDL from hamsters fed the fish oil diet was larger compared to hamsters fed the MIX diet (Figure 3.32). The average particle size is approximately 7nm in the MIX diet groups and approximately 15nm in the fish oil fed groups ($n= 3$ for each diet group). The amount of fat in the diet had no significant effect on LDL particle size (data not shown). The ratio of LDL surface lipids (free cholesterol and phospholipids) to core lipids (cholesterol esters and triacylglycerols) was lower

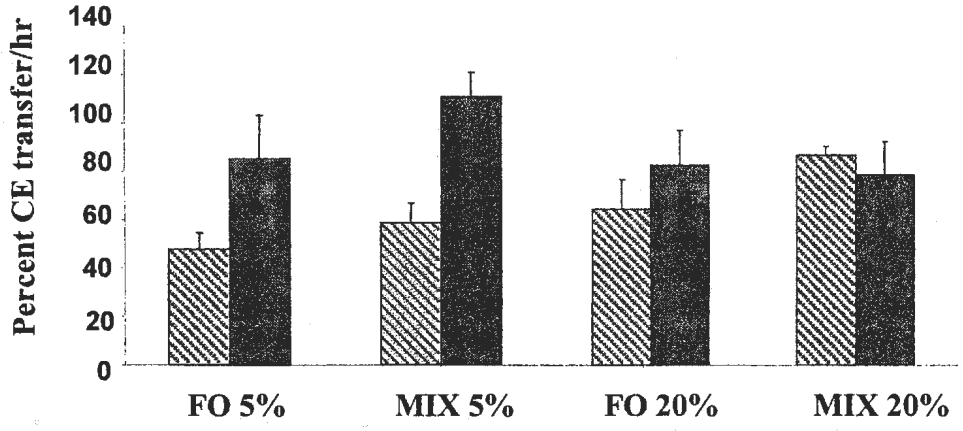


Figure 3.28: Plasma CETP activity of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Plasma was collected and assayed for CETP activity using the radioisotope method as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.28: 3-way ANOVA results for plasma CETP activity.

Source	F-Value	P-Value
Diet type	12.538	0.0015
Diet level	0.110	0.7429
Cholesterol	43.341	0.0001
Diet type * diet level	1.210	0.2815
Diet type * cholesterol	0.587	0.4505
Diet level * cholesterol	27.181	0.0001
Diet type * diet level * cholesterol	6.974	0.0138

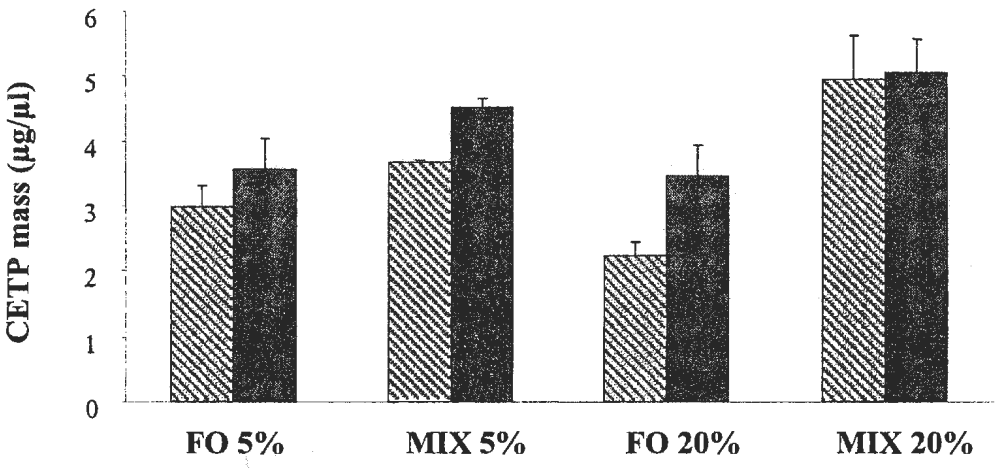


Figure 3.29: Plasma CETP mass of hamsters fed various diets. Hamsters were fed a fish oil diet (FO) or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Plasma was collected and assayed for CETP mass using ELISA as described in the methods. Differences between groups were evaluated using 3-way ANOVA. Values are means for 4 animals with SD shown by vertical bars.

Table 3.29: 3-way ANOVA results for plasma CETP mass.

Source	F-Value	P-Value
Diet type	81.932	0.0001
Diet level	1.339	0.2642
Cholesterol	22.183	0.0002
Diet type * diet level	15.148	0.0013
Diet type * cholesterol	0.388	0.5419
Diet level * cholesterol	0.079	0.7820
Diet type * diet level * cholesterol	3.188	0.0931

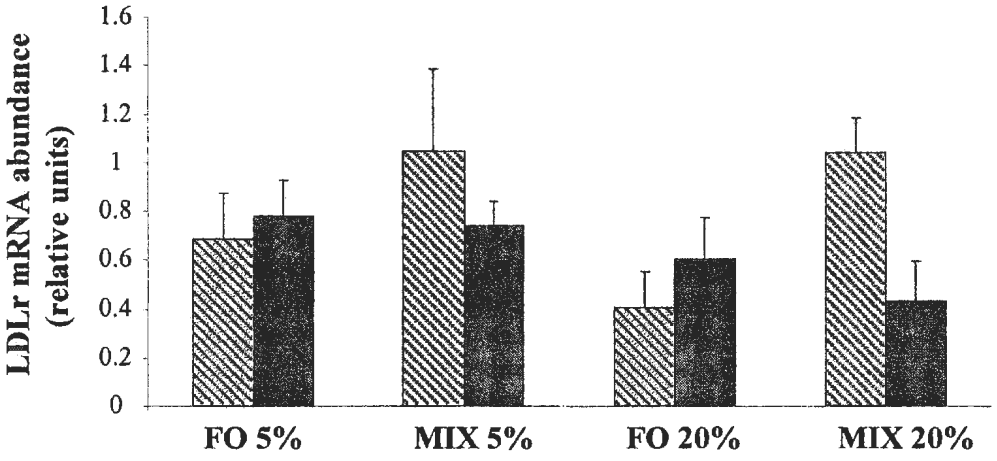


Figure 3.30: LDL-receptor gene expression of hamsters fed various diets..

Hamsters were fed a fish oil diet or a MIX diet, at 5% w/w or 20% w/w fat level, in the absence (hatched bars) or presence (solid bars) of 0.25% w/w cholesterol. Total liver RNA was extracted, reverse transcribed and the cDNA template for hamster LDL-receptor and hamster β-actin was amplified as described in the method section. The amounts of amplified templates were quantified and the abundance of LDLr mRNA is expressed relative to β-actin mRNA content. Differences between groups were evaluated using 3-way ANOVA. Values are means for 12 animals with SD shown by vertical bars.

Table 3.30: ANOVA results for LDLr mRNA abundance.

Source	F-Value	P-Value
Diet type	11.587	0.0036
Diet level	15.949	0.0010
Cholesterol	11.77	0.0034
Diet type * diet level	0.162	0.6930
Diet type * cholesterol	25.934	0.0001
Diet level * cholesterol	0.011	0.9160
Diet type * diet level * cholesterol	2.095	0.1671

in fish oil fed hamsters (0.36 at low fat level and 0.46 at high fat level) compared to hamsters fed the MIX diet (0.85 at low fat level and 1.16 at high fat level) (Table 3.32).

3.10.2 VLDL particle size

The average particle size of VLDL from hamsters fed fish oil diet was smaller compared to hamsters fed the MIX diet (Figure 3.33). The particle size of VLDL from fish oil fed hamsters was 33 nm, whereas the VLDL particle size from MIX diet fed hamsters was 60 nm (n= 3 for each group). The amount of fat in the diet had no significant effect on VLDL particle size (data not shown

Figure 3.31: The effect of dietary fish oil on LDL particle size. Purified LDL fractions (d<1.065) were negatively stained as explained in methods and electron microscopy was performed at 80 KV. Panel **A** shows the LDL particles of fish oil fed hamsters and panel **B** shows the particles of MIX diet fed hamsters. Magnification is x 180. Bar=100 nm.

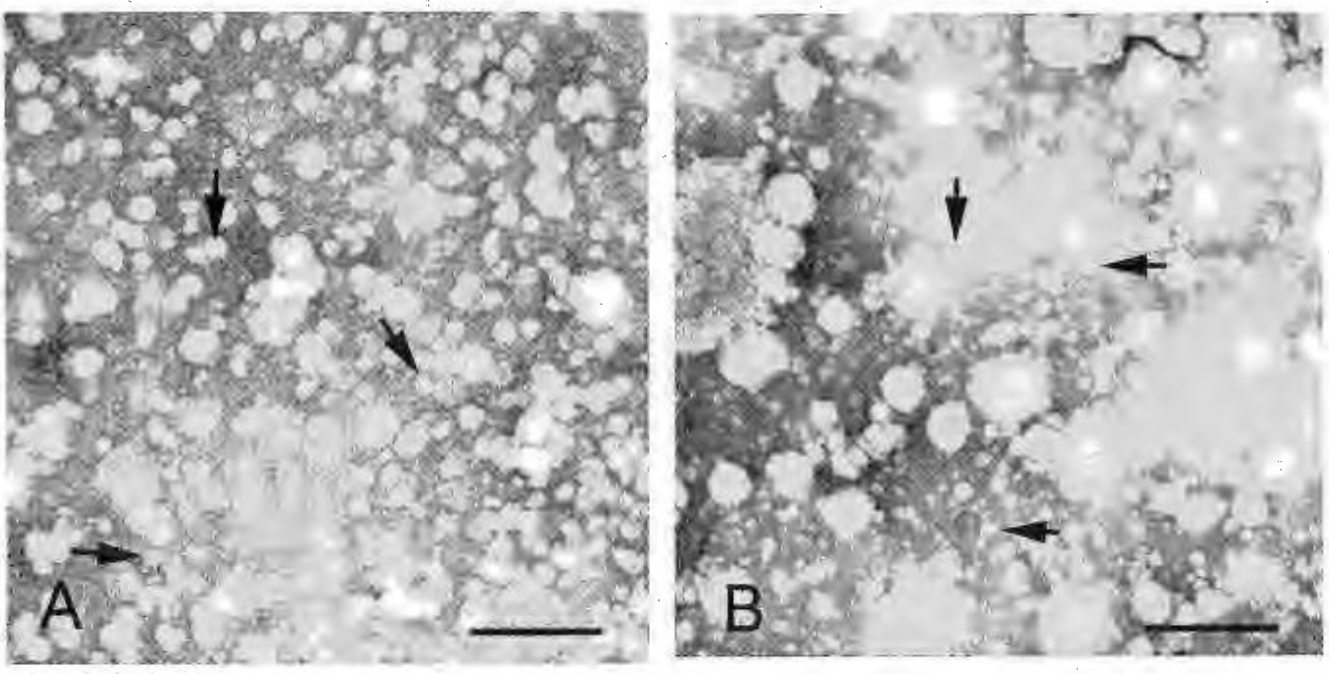
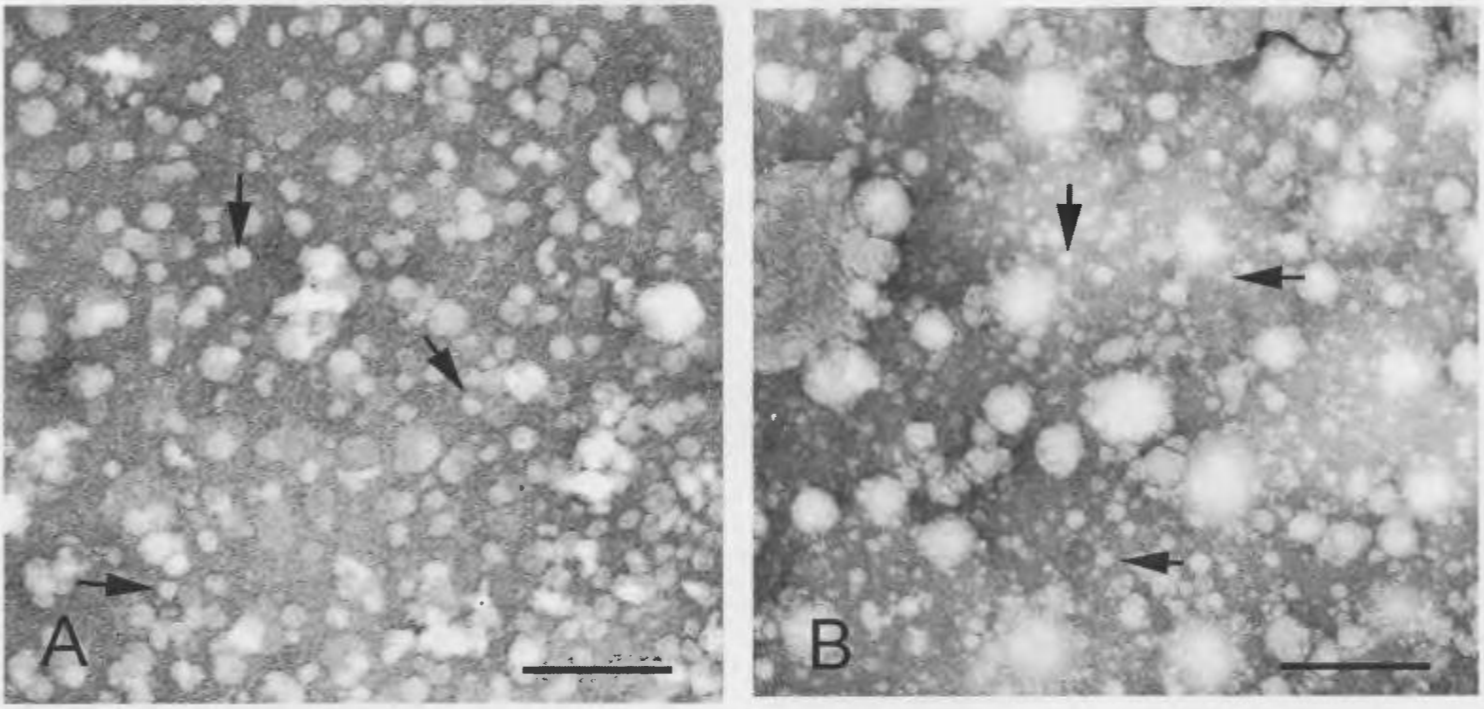

A
B

Table 3.32: Surface lipid to Core lipid ratio of LDL particles from hamsters fed various diets. Surface lipids are composed of free cholesterol and phospholipids. Core lipids are composed of cholesterol esters and triacylglycerols. The surface lipid to core lipid ratio of LDL-particles was calculated by dividing the surface lipid concentration by the core lipid concentration of LDL. Differences between groups were evaluated using Student's *t* test. Values shown are mean ± SD, n= 12 for each diet group. [a, b]Mean values with unlike superscript letters are significantly different.

Fat level	Fish oil				MIX			
	- Cholesterol		+ Cholesterol		- Cholesterol		+ Cholesterol	
	(Surface lipid: core lipid)				(Surface lipid: core lipid)			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5%	0.43[a]	0.12	0.31[a]	0.05	0.81[b]	0.32	0.82[b]	0.10
20%	0.45[a]	0.08	0.47[a]	0.18	1.04[b]	0.35	0.99[b]	0.35

Figure 3.32: The effect of dietary fish oil on VLDL particle size. VLDL fractions (d<1.065) were negatively stained as explained in methods and electron microscopy was performed at 80 KV. Panel **A** shows the VLDL particles of fish oil fed hamsters and panel **B** shows the particles of MIX diet fed hamsters. Magnification is x 180. Bar=200 nm.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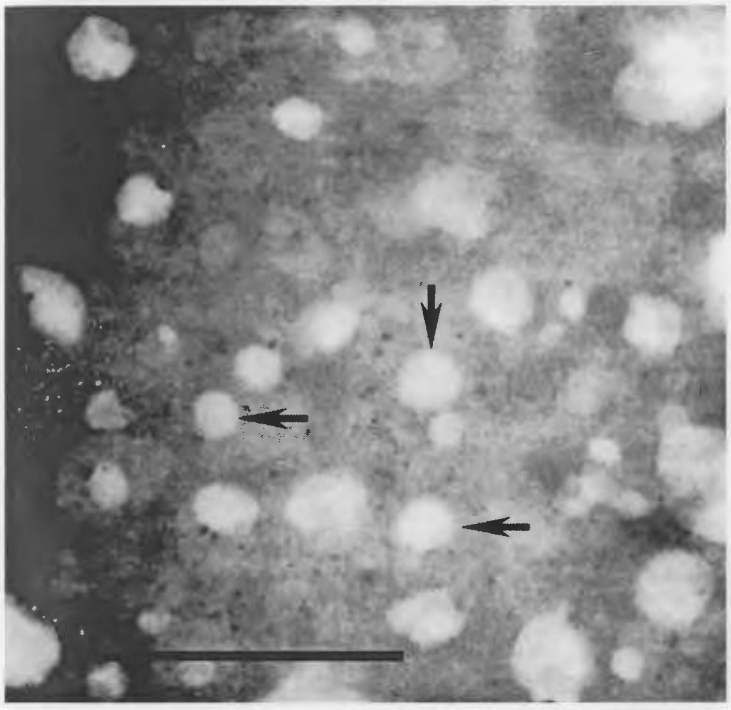

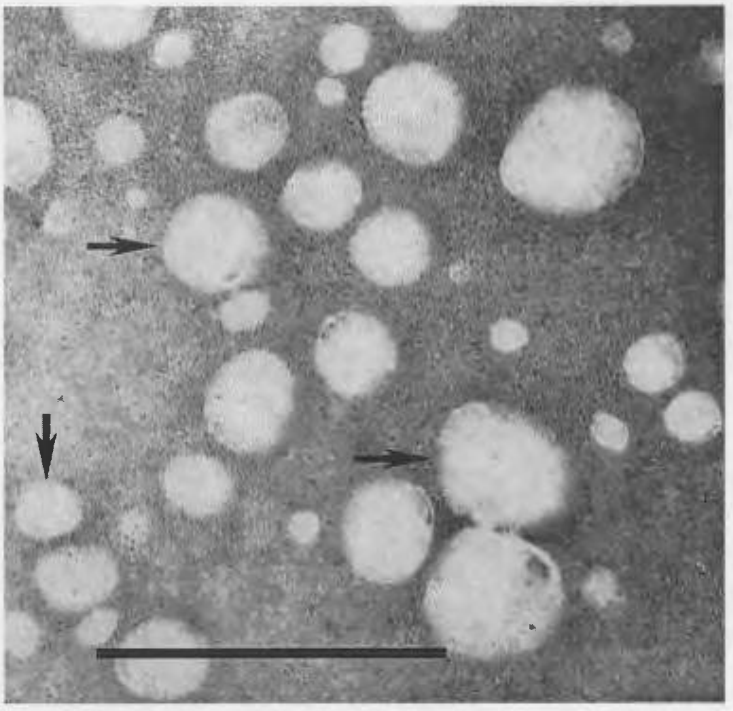

Chapter 4: Discussion

4.1 Effect of fish oil on plasma and lipoprotein lipids

Many studies have shown that fish oil suppresses plasma triacylglycerol concentrations (Wong *et al.,* 1984, Surette *et al.*, 1992b). Furthermore it has been observed that this hypotriglyceridemic effect of fish oil is dose dependent (Sanders *et al.*, 1983, Blonk *et al.*, 1990). However, the effect of fish oil on plasma cholesterol concentration is inconsistent. Human subjects with type-2 diabetes mellitus or dyslipidemia show a significant increase in LDL-cholesterol concentration following a high fat fish oil diet. Our study was designed to determine the effect of fish oil on plasma and lipoprotein lipid composition under different dietary fat levels and the regulation of metabolic pathways involved in lipoprotein lipid metabolism. We examined these effects of fish oil at low (5% w/w) and high fat (20% w/w) levels with or without added cholesterol (0.25% w/w) using the F_1B hamster as the animal model because of the similarity of its lipid profile to humans and their high susceptibility to diet-induced hyperlipidemia (Kowala *et al.*, 1991, Trautwein *et al.*, 1993). The proportions of PUFA, MUFA and saturated fatty acids in the control diet (MIX diet) was kept similar to the fish oil diet by using a mixture of lard and safflower oil (1.5: 1), thus the main difference in the diets is the amount of n-6 or n-3 fatty acids. Since menhaden oil contains low levels of cholesterol, cholesterol was added to the MIX diets to maintain the cholesterol levels consistent in both MIX and fish oil diets.

4.1.1 Effect of fish oil on plasma lipids

Plasma cholesterol and triacylglycerol concentrations were higher in fish oil diet fed hamsters compared to MIX diet fed hamsters, which was dependent on the dietary fat

level (Figure 3.1-3.4). In addition, dietary cholesterol level dependent hypercholesterolemic and hypertriglyceridemic effects were observed in fish oil diet fed hamsters. Interestingly, these effects were attenuated in MIX diet fed hamsters. A hypercholesterolemic effect of fish oil has been observed in previous studies especially when diets are fed with moderate to high levels of cholesterol in hamsters (Surette *et al.*, 1992a, Lin *at al.*, 1995, Lu *et al.*, 1996, Kubow., 1996) and this effect was dependent on dietary cholesterol level and fat level (Surette *et al.*, 1992a). Furthermore, this hypercholesterolemic effect was mainly attributed to changes in plasma VLDL and LDL cholesterol concentrations. In addition, these studies have postulated that the hypercholesterolemic effect of fish oil might be due to decreased catabolism of VLDL (Lin *at a.*, 1995) and suppressed LDLr mediated removal of LDL (Surette *et al.*, 1992). However, none of the above studies observed a hypertriglyceridemic effect of fish oil.

4.1.2 Effect of fish oil on VLDL lipid composition

Many studies have shown beneficial effects of fish oil in lowering the plasma VLDL levels in humans (Nestel *et al.*, 1984, Wong *et al.*, 1987). However, studies in the hamster model indicate elevation of VLDL cholesterol by fish oil feeding (Surette *et al.*, 1992a, Lu *et al.*, 1996, Kubow *et al.*, 1996 Lin *at al.*, 1995) with no change in VLDL triacylglycerol concentrations. We found that feeding fish oil for two weeks to F1B hamsters, a strain susceptible to diet induced hyperlipidemia, increases plasma VLDL and LDL lipids compared to hamsters fed a MIX diet rich in n-6 PUFA. Furthermore, this hyperlipidemic effect was dependent on the dietary fat level and cholesterol level.

Surette *at al.* (1992a) observed that an incremental increase in dietary n-3 PUFA from fish oil increases plasma VLDL cholesterol concentrations in Syrian golden hamsters when the diet is supplemented with 0.1% (w/w) cholesterol. However, with low dietary cholesterol (0.015% w/w), this dietary fat dependent hypercholesterolemic effect of fish oil was not seen. In our study, both the low fat fish oil and MIX diets contained 0.025% (w/w) cholesterol but the hamsters fed the low fat fish oil diet showed higher levels of VLDL- total cholesterol, triacylglycerols and phospholipid concentrations compared to hamsters fed the MIX diet (Figure 3.7-3.11). This suggests the possibility of an interaction of dietary cholesterol with fish oil in rendering the hyperlipidemic effect.

Addition of cholesterol to the fish oil diet to a final concentration of 0.25% (w/w) increased all the VLDL lipids (Figure 3.7-3.11). Although, this dietary cholesterol dependent increase in VLDL lipids was also observed in MIX diet fed hamsters, the effect on VLDL cholesterol was lower (Figure 3.7 and 3.9) compared to fish oil diet fed hamsters. This indicates that the hyperlipidemic effect of fish oil is due to a combination of n-3 PUFA and the amount of dietary cholesterol, and that unlike n-3 PUFA, the n-6 PUFA prevent cholesterol induced hyperlipidemia except at very high cholesterol concentrations. This suggestion is supported by the observations of previous studies that cholesterol supplementation at a level of 0.5% (w/w) to both n-3 PUFA and n-6 PUFA, induce hypercholesterolemia in hamsters, and this effect was more prominent in n-3 PUFA fed hamsters (Lin *et al.*, 1995, Lu *et al.*, 1996).

The most interesting observation made in this study was that the plasma from hamsters fed high fat fish oil diets was packed with chylomicron like particles and very high levels of VLDL that was rich in cholesterol and triacylglycerols. This observation

clearly delineates that the increase in VLDL and chylomicron by dietary fish oil is dependent on the dietary fat level. Since the high fat fish oil diet contains 0.1% (w/w) cholesterol compared to the low fat fish oil diet that contains 0.025% (w/w) cholesterol, the hyperlipidemic effect may not only be attributed to increased dietary fat level, but might be a result of an interaction between moderately high dietary cholesterol and high n-3 PUFA intake.

4.1.2.1 Possible mechanisms for increased VLDL concentrations in fish oil fed hamsters

4.1.2.1.1 Role of VLDL synthesis and secretion

There is a possibility that higher VLDL cholesterol and triacylglycerol concentrations in fish oil fed hamsters is a result of increased hepatic secretion of VLDL. The rate of VLDL secretion is affected by the rate of endogenous production and exogenous supply of its contents and their precursors such as cholesterol, cholesterol ester, fatty acid, triacylglycerol, phospholipids, apoB and the activity of microsomal triglyceride transfer protein (MTTP) which plays the major role in the process of assembly of VLDL particles in hepatocytes. We measured the changes in MTTP activity in hamsters fed various diets to understand whether increased MTTP activity contributes for increased VLDL levels (Figure 3.26). We observed that, at low dietary fat level there was no difference in MTTP activity between fish oil fed hamsters and MIX diet fed hamsters. The MTTP activity of high fat fish oil fed hamsters increased significantly (1.2 fold) compared to MIX diet fed hamsters. However, the increase in VLDL

triacylglycerol levels is 7 fold. Thus, increased MTTP activity does not account for the massive increase in plasma VLDL concentrations in fish oil fed hamsters. Botham *et al.* (2003) has recently shown that the MTTP mRNA expression in hepatocytes incubated with n-3 PUFA-enriched chylomicron remnants is not significantly different from hepatocytes incubated with chylomicron remnants rich in n-6 PUFA. These findings also imply that fish oil has no significant effect on MTTP activity compared to an n-6 PUFA rich diet, indicating that the increase in plasma VLDL levels is not due to induction of MTTP activity by fish oil.

4.1.2.1.1.1 Fish oil decreases VLDL particle size

Hamsters fed the fish oil diet had smaller VLDL particles compared to hamsters fed the MIX diet (Figure 3.33). This is consistent with the data showing that VLDL particles from hypertriglyceridemic patients fed fish oil are smaller than normal (Sullivan *et al.*, 1986). This reduction in particle size is reported to be due to hepatic secretion of smaller VLDL particles as a result of inhibition of hepatic triacylglycerol synthesis by fish oil (Harris, 1989). Thus, decreased particle size of VLDL from fish oil diet fed hamsters indicates a decrease in hepatic triacylglycerol synthesis, there by leading to smaller VLDL particles.

4.1.2.1.2 Role of triacylglycerol clearance

Another possible explanation for the increased VLDL concentration in fish oil fed hamsters is decreased removal of VLDL triacylglycerol rich in n-3 PUFA. Increased levels of chylomicron like particles in high fat fish oil fed hamsters also raise the

possibility of reduced triacylglycerol clearance from plasma. Recent studies indicate that triacylglycerol, which are esterified with EPA and DHA are resistant to lipoprotein lipase mediated lipolysis (Botham *et al.*, 1997, Levy *et al.*, 1999, Oliveira *et al.* 1997)

Lipoprotein lipase, located on the luminal surface of the blood vessels is responsible for hydrolysis of VLDL and chylomicron triglycerides into glycerol and fatty acids, so that peripheral cells can consume the hydrolyzed products. Although this enzyme functions on the luminal surface of the blood vessels, it is synthesized mainly in the adipose and muscle parenchymal cells. The total adipose tissue lipoprotein lipase activity might indicate alterations in overall lipoprotein lipase activity in the plasma. However, it is not an accurate reflection of lipoprotein lipase activity as both lipoprotein lipase and hormone sensitive lipase activities are measured. We measured the lipoprotein lipase activity of white adipose tissue (Figure 3.27). There was a trend of increase in lipoprotein lipase activity in fish oil diet fed hamsters compared to MIX diet fed hamsters. High levels of lipoprotein lipase activity in adipose tissue were observed by Anil *et al.* (1992), Yoshiba *et al.* (1999) and Benhizia *et al.* (1994) in n-3 PUFA fed rats compared to n-6 PUFA fed rats, but this response occurred along with lower plasma triacylglycerol concentrations. As opposed to above observations, a decrease in post heparin lipoprotein lipase activity in fish oil fed swine (Groot *et al.*, 1989) and rats (Haug *et al.*, 1987) has been reported with decreased plasma triacylglycerol concentrations. With the help of the latter findings, our results suggest that increased lipoprotein lipase activity observed in fish oil fed hamsters might be an adaptive response to clear elevated triacylglycerol from plasma. Since triacylglycerols, esterified with n-3 PUFA are known to be resistant to lipoprotein lipase mediated hydrolysis (Botham *et al.*, 1997, Levy *et al.*,

1999, Oliveira *et al.* 1997), increased adipose tissue lipoprotein lipase activity in fish oil fed hamsters might be an adaptive response to maintain adequate supply of fatty acids and glycerol to peripheral cells. Furthermore, McAteer *et al.* (2003) demonstrated an association of increased accumulation of chylomicrons with decreased post-heparin lipoprotein lipase activity in F_1B hamsters fed a cholesterol supplemented diet. On the other hand, Harris *et al.* (1997) observed an increase in endogenous (nonheparin-stimulated) lipoprotein lipase activity and decreased plasma triacylglycerol concentrations in healthy human subjects treated with fish oil concentrate. These studies show the critical role of intravascular lipase activity in regulating plasma triacylglycerol concentrations. We only measured the adipose tissue LPL activity, which is increased in fish oil fed hamsters. Thus, increased VLDL in fish oil fed hamsters cannot be explained based on adipose tissue LPL activity.

In general, amount of dietary cholesterol had no effect on lipoprotein lipase activity in fish oil diet fed hamsters (Figure 3.27). However, there was decrease in lipoprotein lipase activity with addition of cholesterol to low fat fish oil diet. A dietary cholesterol dependent decrease in lipoprotein lipase activity has also been observed by McAteer *et al.* (2003) in F_1B hamsters fed a cholesterol rich coconut oil diet. This suggests that the effect of dietary cholesterol on lipoprotein lipase activity can be modified by the type and amount of dietary fat.

4.1.2.1.4 Do changes in chylomicron level modify VLDL clearance?

It has been suggested that changes in plasma chylomicron levels can affect VLDL clearance due to alteration of the competition for lipoprotein lipase mediated catabolism

of VLDL (Harris, 1989). We observed a marked increase in chylomicron like particles in high fat fish oil diet fed hamsters. Thus, there is a possibility that the very high levels of VLDL found in high fat fish oil fed hamsters might partly be due to increased competition for lipoprotein lipase mediated clearance.

4.1.3 Effect of fish oil on LDL lipid composition

The findings of previous studies on the influence of n-3 PUFA on plasma LDL concentrations are inconsistent, especially in type-2 diabetes mellitus, where fish oil therapy is questioned. We found that LDL cholesterol, triacylglycerol and phospholipid concentrations were significantly higher in hamsters fed the fish oil diet compared to hamsters fed the MIX diet (Figure 3.12-3.17).

In the present study, increased LDL cholesterol concentrations in fish oil diet fed hamsters are not associated with increase in dietary fat level. Interestingly, with the increase of dietary fat level, there was a decrease in total cholesterol and cholesterol ester concentrations in fish oil diet fed hamsters. On the other hand, LDL triacylglycerol concentrations showed a dietary fat level dependent increase in hamsters fed a fish oil diet. This dietary fat level dependent alteration in LDL composition might be attributed to fish oil-induced changes in cholesterol ester transfer between HDL and LDL.

We observed an increase in LDL cholesterol and triacylglycerol levels with supplementation of cholesterol to fish oil diet, although the effect was prominent only at low dietary fat level (Figure 3.12-3.15). Unlike the fish oil diet, the MIX diet attenuated the cholesterol-induced elevation of LDL cholesterol concentrations. It has been observed in Syrian golden hamsters that dietary cholesterol supplementation at a level of

0.5% w/w to a diet rich in n-3 PUFA or n-6 PUFA increase LDL cholesterol levels (Lin et al., 1995, Lu *et al.*, 1996). However, the effect of addition of cholesterol was more prominent in n-3 PUFA rich diet fed hamsters (Lu *et al.*, 1996). Together with the results of the previous studies, our observations suggest that the fish oil-induced increase in LDL cholesterol concentration is dependent on the amount of dietary cholesterol. The mechanisms involved in regulating plasma LDL levels are discussed below.

Increased LDL lipids in fish oil fed hamsters might be due to increased LDL production from VLDL, decreased clearance of LDL from the plasma or increased transfer of cholesterol esters from HDL to LDL by reverse cholesterol transport pathway.

4.1.3.1 Effect of fish oil on LDL production

There is a possibility for increased production of LDL from VLDL in fish oil diet fed hamsters, as the levels of VLDL are significantly raised under these conditions (Figure 3.7). In addition, we observed a trend of increased adipose tissue lipoprotein lipase activity in fish oil fed hamsters (Figure 3.27). Increased plasma lipoprotein lipase activity and the availability of high levels of VLDL might result in an increase in LDL levels. However, as discussed in section 4.1.2.1.2 there is a possibility of decreased intravascular lipase activity in fish oil fed hamsters rather than increased activity, although the lipoprotein lipase activity in adipose tissue is high. On the other hand, increased VLDL levels in fish oil diet fed hamsters are possibly due to decreased lipoprotein lipase mediated lipolysis rather than increased secretion of VLDL. We have no evidence for fish oil induced increased VLDL secretion due to changes in MTTP activity. However, there is still a possibility of increased secretion of VLDL and/or

increased breakdown of VLDL to LDL. We did not measure post-heparin lipoprotein lipase activity or VLDL secretion, which might give a better understanding of fish oil mediated regulation of VLDL and LDL synthesis.

4.1.3.2 Effect of fish oil on LDL clearance

The main way of LDL clearance from plasma is via hepatic LDL-receptor (LDLr) mediated LDL uptake, which can be modified by the levels of LDLr mRNA expression or affinity of LDL particles to LDL-receptors. We measured LDLr mRNA expression in the liver to investigate whether the increase in LDL levels might be due to down regulation of LDLr mRNA levels.

We observed a decrease in LDLr mRNA expression in fish oil fed hamsters at both low and high fat levels compared to MIX diet fed hamsters (Figure 3.30). Fish oil induced suppression of LDLr mRNA expression has also been observed previously in cell culture studies (Lindsey *et al.*, 1992) and animal studies (Wilkinson *et al.*, 1998). Thus, increased LDL levels in fish oil fed hamsters might be due to the suppression of LDLr mRNA expression.

Furthermore, hamsters fed the high fat fish oil diet showed significantly lower levels of LDLr mRNA abundance compared to low fat fish oil diet fed hamsters. This dietary fat level dependent suppression of LDLr mRNA expression was associated with a dietary fat level dependent increase in LDL triacylglycerol concentrations (figure 3.15). However, hamsters fed the high fat fish oil diet showed decreased LDL cholesterol concentrations compared to hamsters fed low fat fish oil diet, which cannot be explained by modification of LDLr mRNA expression.

We observed a decrease in LDLr mRNA expression in cholesterol supplemented MIX diet fed hamsters compared to hamsters fed MIX diet alone. Previous studies also have shown that dietary cholesterol down-regulates the expression of LDLr mRNA in animals (Spady *et al.*, 1995) and healthy human subjects (Boucher *et al.*, 1998). Although with suppression of LDLr mRNA expression, elevation of plasma LDL cholesterol and triacylglycerol concentrations is expected in cholesterol supplemented MIX diet fed hamsters, plasma lipids remained similar to that of hamsters fed MIX diet alone.

Dietary cholesterol supplementation had no effect on LDLr mRNA expression in fish oil diet fed hamsters. Furthermore, the increase in plasma LDL cholesterol concentrations with cholesterol supplementation to low fat fish oil diet cannot be explained by the changes of LDLr mRNA abundance. These observations suggest that the influence of dietary cholesterol on plasma LDL levels in fish oil and MIX diet fed hamsters may not be attributed to LDLr mediated LDL uptake alone.

4.1.3.2.1 Fish oil increases LDL particle size

LDL particle size is known to be associated with LDLr affinity and hence LDLr mediated LDL uptake (Nigon *et al.*, 1991). There was an increase in LDL particle size in fish oil diet fed hamsters compared to MIX diet fed hamsters at both low and high dietary fat levels (Figure 3.31). The difference in particle size measured by electron microscopy was consistent with the LDL surface lipid: core lipid ratio (the lower the ratio, bigger the particle size), which was lower in fish oil diet fed hamsters compared to MIX diet fed hamsters (Table 3.31). An increase in LDL particle size has also been observed in

hypertensive human subjects (Suzukawa *et el.*, 1995) and in normal human subjects (Sanchez-muniz *et al.*, 1999) following dietary supplementation of fish oil. According to these observations, it can be suggested that increased LDL levels in fish oil diet fed hamsters might partly be due to decreased affinity of LDL particles to LDL-receptors because of increase in the LDL particle size, hence decreased hepatic LDL uptake. This hypothesis is further supported by the findings that larger or smaller LDL particles have less affinity for LDL-receptor compared to intermediate size particles (Nigon *et al.*, 1991).

4.1.3.3 Fish oil suppresses cholesteryl ester transfer protein activity

Cholesteryl ester transfer protein (CETP) plays the major role in transferring cholesterol ester from HDL to VLDL and LDL, in exchange for triacylglycerols (Quinet *et al.*, 1991), thus determines plasma LDL and HDL composition. We measured the plasma CETP activity and CETP mass in hamsters fed fish oil and MIX diets to understand whether the increase in LDL and decrease in HDL levels in fish oil diet fed hamsters is due to increased CETP activity. There was a significant decrease in both CETP activity (Figure 3.28) and CETP mass (Figure 3.29) in fish oil diet fed hamsters irrespective of dietary fat level. Findings of studies done on hypercholesterolemic human subjects (Abbey *et al.*, 1990), normolipidemic patients with insulin dependent diabetes mellitus (Bagdade *et al.*, 1996) and cell culture studies (Hirano *et al.*, 2001) also suggest decreased reverse cholesterol transport from HDL to LDL following dietary supplementation of n-3 PUFA. The CETP gene is activated by sterol regulatory element-binding protein-1 (SREBP1) (Chouinard *et al.*, 1998) and fish oil is reported to suppress

SREBP1 by dietary fish oil has been reported (Kim *et al.*, 1999). Thus, the fish oil induced decrease in plasma CETP activity and CETP mass is possibly due to suppression of CETP gene expression. Although, CETP activity was lower in fish oil diet fed hamsters compared to MIX diet fed hamsters, the LDL cholesterol concentrations were higher and HDL cholesterol concentrations were lower. Thus, these fish oil-induced changes in LDL and HDL levels might not be attributed to fish oil-induced changes in plasma reverse cholesterol ester transfer.

We observed a reduction in CETP mass with an increase of dietary fat level from 5% to 20% w/w in fish oil fed hamsters. This might explain the high levels of triacylglycerol and low levels of cholesterol ester detected in LDL from high fat fish oil diet fed hamsters compared to that of low fat fish oil diet fed hamster. However, in contrast to CETP mass, CETP activity did not change with the increase of dietary fat level. Since the plasma used for CETP activity assay in high fat fish oil fed hamsters contained very high levels of chylomicrons, VLDL and LDL, which are potential acceptors of cholesterol esters from radiolabeled exogenous HDL, CETP activity of high fat fish oil fed hamsters might be an exaggerated reflection of CETP mass.

Dietary cholesterol is known to suppress plasma CETP activity (Tall, 1995). However, the interactive effect of dietary cholesterol and n-3 PUFA on CETP activity, CETP mass and CETP gene expression is not yet known. Considering both CETP activity and CETP mass results, cholesterol supplementation caused an increase in plasma CETP activity in both fish oil and MIX diets fed hamsters. Fusegawa *et al.* (2001) observed an increase in CETP activity in St. Kitts vervet monkeys with addition of cholesterol to diets rich in n-6 PUFA. These findings suggest that supplementation of

cholesterol to n-3 or n-6 PUFA enriched diet causes increase in CETP activity. The cholesterol-mediated increase in CETP activity is firmly associated with up regulation of the CETP gene and, interaction of SREBP1a, -2 and Yin Yang 1 (YY1) transcription factors with cholesterol (Gauthier et al., 1999).

4.1.4 Effect of fish oil on HDL lipids

HDL cholesterol and phospholipid concentrations were significantly lower in fish oil diet fed hamsters compared to MIX diet fed hamsters, while there was no difference in HDL triacylglycerol concentrations (Figure 3.17-3.21). Findings of previous studies done on hamsters (Kubow *et al.*, 1996, Surette *et al.*, 1992a) and non-human primates (Ward *et al.*, 1985) have shown decreased HDL cholesterol concentrations following fish oil feeding, which supports our observation. Plasma HDL cholesterol, triacylglycerol and phospholipid concentrations are mainly regulated by the reverse cholesterol transport pathway and cellular cholesterol and phospholipid efflux (Dietschy 1997, Bruce *et al.*, 1998). HDL acquires triacylglycerol from VLDL and LDL in exchange for cholesterol esters via the reverse cholesterol transport pathway and acquires cholesterol and phospholipids from peripheral cells. Reduction of HDL cholesterol and phospholipid concentrations, with no change in triacylglycerol concentrations suggests that the fish oil induced HDL cholesterol lowering effect might be attributed to decreased efflux of cholesterol and phospholipids from peripheral cells rather than increased reverse cholesterol transport. Syeda *et al.* (2003) demonstrated that, cellular cholesterol efflux is positively correlated with HDL phospholipid concentrations and the efflux is not dependent on the plasma CETP activity. These findings are consistent with our results

that decreased HDL phospholipid concentrations in fish oil diet fed hamsters are associated with decreased HDL cholesterol concentrations, which reflects decreased cellular cholesterol efflux.

Increasing the dietary fat level from 5% w/w to 20% w/w did not affect the HDL free cholesterol levels, but HDL cholesterol ester levels decreased significantly. This might be due to poor esterification of HDL free cholesterol to cholesterol esters by lecithin cholesterol acyl transferase (LCAT) as n-3 PUFA are known to be less utilized by LCAT for the formation of cholesterol esters (Parks et al., 1997, Parks *et al.*, 1998).

In the present study, there were significant increases in HDL cholesterol and phospholipid concentrations with addition of cholesterol to the fish oil diet. Similarly, Kubow *et al.* (1996) observed high levels of HDL cholesterol in hamsters fed a fish oil diet supplemented with high cholesterol (0.88% w/w) compared to hamsters fed a fish oil diet supplemented with low cholesterol (0.285 w/w). Since HDL phospholipid concentrations are known to be positively correlated with cellular cholesterol efflux (Syeda *et al.*, 2003), cholesterol-induced increase in HDL cholesterol concentrations in low fat fish oil fed hamsters might be due to increased cholesterol efflux from peripheral cells.

4.2 Hepatic lipids in fish oil and MIX diet fed hamsters

We observed lower hepatic total cholesterol, cholesterol ester and triacylglycerol levels in low fat fish oil diet fed hamsters compared to MIX diet fed hamsters (Figure 3.22, 3.24 and 3.25). Similar results have been observed by Field *et al.* (1987) in rabbits fed a fish oil diet (10% w/w) compared with those fed a saturated fat diet. Furthermore,

Syrian golden hamsters fed a cholesterol supplemented fish oil diet showed a significant decrease in hepatic total cholesterol and cholesterol ester concentrations (Lin *et al.*, 1995). Both field *et al.* (1987) and Lin *et al.* (1995) observed that reduction in hepatic cholesterol concentrations was associated with high levels of microsomal acyl-coenzymeA:cholesterol acyl transferase (ACAT) activity. Thus, lower hepatic cholesterol ester levels in low fat fish oil fed hamsters might be due to less availability of cholesterol for esterification resulting from suppressed LDLr mediated hepatic LDL uptake. Decreased hepatic triacylglycerol levels in fish oil diet fed hamsters might be due to fish oil-induced inhibition hepatic triacylglycerol synthesis that is established by several studies (Wong *et al.*, 1984)

In contrast to low fat fish oil diet fed hamsters, high fat fish oil diet fed hamsters had high levels of hepatic cholesterol ester and triacylglycerol levels. These findings are supported by the observation made by Gaiva *et al.* (2003) in rats that showed higher levels of hepatic lipids, lipogenesis and increase in liver weight following high fat fish oil diet (15% w/w) feeding compared to rats fed a soybean oil diet. However, they observed a concomitant decrease in plasma total lipid levels. Thus, increased levels of hepatic lipids in high fat fish oil diet fed hamsters might be due to increased accumulation and deposition of lipids resulted from fish oil-induced inhibition of VLDL secretion and/or increased hepatic lipogenesis.

There was increased hepatic cholesterol ester and triacylglycerol levels in high fat fish oil diet fed hamsters compared to low fat fish oil diet fed hamsters. This dietary fat level dependent increase in hepatic lipid content might be due to increased hepatic lipogenesis rather than increased hepatic influx of dietary fats, as formation of

chylomicron remnants is possibly suppressed in high fat fish oil diet fed hamsters, that is discussed in section 4.1.2.1.2. Although, the hepatic lipid content seems to be correlated with dietary fat level in fish oil diet fed hamsters, the effect of dietary fat level was attenuated in MIX diet fed hamsters (Figure 3.22-3.25). Similar results have been reported by Fungwe *et al.* (1993) in rats that were fed a cholesterol supplemented corn oil diet (rich in n-6 PUFA) at 5% w/w and 20% w/w dietary fat levels; although both diets increased the hepatic lipid content compared to those fed a cholesterol free diet, there was no effect of dietary fat level. These findings suggest that the fish oil-induced increase in hepatic lipid content is dietary fat level dependent and a diet rich in n-6 PUFA attenuates the dietary fat level dependent increase in hepatic lipid content.

Cholesterol supplemented diets fed hamsters had increased levels of hepatic total cholesterol and cholesterol ester compared to hamsters fed low fat diet alone. This effect was observed in both fish oil and MIX diets fed hamsters. Rats fed a low fat (5% w/w) corn oil diet with incremental increase in cholesterol content showed elevation of hepatic free cholesterol and cholesterol ester levels (Fungwe *et al.*, 1992). These findings support our results that cholesterol supplemented diets fed hamsters had increased levels of hepatic cholesterol ester compared to hamsters fed diets alone irrespective of the diet type. Since, dietary cholesterol is known to suppress hepatic cholesterol synthesis (Fungwe *et al.*, 1992), the dietary cholesterol-induced increase in hepatic cholesterol content might be due to increased hepatic accumulation of cholesterol caused by increased hepatic uptake of cholesterol from plasma. On the other hand, the effect of dietary cholesterol on hepatic lipids was lesser in MIX diet fed hamsters compared to fish

oil diet fed hamsters, that implies the n-6 PUFA-mediated attenuation of cholesterol-induced increase in hepatic cholesterol content.

4.3 Role of oxidative stress in hyperlipidemic effect of fish oil

Increased oxidative stress is known to be associated with increased tissue cholesterol (Chupukcharoan *et al.*, 1985) and increased LDL cholesterol (Katayama *et al.*, 1991). Animal studies have shown a fish oil-induced reduction in anti-oxidant capacity of the body due to enhanced *in vivo* lipid peroxidation of n-3 PUFA (Sanders, 1989). Moreover, administration of vitamin E could reverse a hyperlipidemic effect of fish oil, that was associated with lowered tissue lipid peroxidation in Syrian golden hamsters (Kubow *et al.*, 1996). Kubow at al. (1996) further observed a highly significant correlation between serum levels of lipid peroxides and various lipoprotein fractions in fish oil fed hamsters. Thus, there is possibility that the dietary fat level dependent hyperlipidemic effect of fish oil in F1B hamsters might be due to increased oxidative stress rendered by increased peroxidation of n-3 PUFA in tissue membranes.

4.4 Conclusion and Future works

Fish oil, in general is considered to be beneficial by lowering plasma triacylglycerol concentrations. However, the effect of fish oil on plasma LDL and HDL cholesterol concentrations is inconsistent. Our study showed that fish oil increases the cholesterol and triacylglycerol concentrations of VLDL and LDL lipoprotein fractions while decreasing HDL cholesterol concentrations in F_1B hamsters. The increase in plasma triacylglycerol concentrations was mainly attributed to increased VLDL and

chylomicron like particles. The increase in VLDL cholesterol and triacylglycerol concentrations was dependent on dietary fat level and dietary cholesterol level. Whether increased secretion or decreased removal caused increased VLDL levels could not be determined from our results. Future investigations need to be carried out to understand how fish oil modifies hepatic lipogenesis, fatty acid oxidation and the rates of VLDL and apoB secretion. In addition, the effect of plasma and tissue antioxidant capacity on the fish oil-induced hyperlipidemic effect has to be investigated, since vitamin E supplementation is known to protect hamsters against this hyperlipidemic effect. Plasma post-heparin lipoprotein lipase activity, apoE and apoCII levels would give a better reflection of plasma VLDL and chylomicron catabolism. Increased LDL cholesterol and triacylglycerol concentrations in fish oil diet fed hamsters is probably due to decreased LDLr mediated LDL uptake, caused by suppression of LDLr and/or increased LDL particle size. The decrease in HDL cholesterol concentrations in fish oil fed hamsters might not be dependent on plasma CETP activity. However, the mechanism behind fish oil-induced decrease in HDL cholesterol concentrations has to be investigated by future studies.

This study shows that the changes in plasma lipoproteins with n-3 PUFA feeding in F_1B hamsters are not consistent with the hypolipidemic and anti atherogenic properties of fish oil observed in other animal models and human trials. Fish oil is known to increase LDL-cholesterol concentration in human subjects with type-2 diabetes mellitus, although the mechanism of this increase is not known. Understanding the regulation of lipoprotein metabolism by fish oil in F_1B hamsters, a model susceptible to high fat-

induced type-2 diabetes mellitus will shed new lights into the cause of fish oil-induced increase in LDL-cholesterol concentration and to design therapeutic strategies.

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