REGULATION OF LIPID METABOLISM AND OXIDATIVE STRESS IN BIOF1B HAMSTERS: A COMPARISON OF FISH OIL AND SEAL OIL RICH DIETS

PRATIBHA DUBEY

Regulation of Lipid Metabolism and Oxidative Stress in BioF1B Hamsters: a Comparison of Fish Oil and Seal Oil Rich Diets

Pratibha Dubey

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Department of Biochemistry

Memorial University of Newfoundland

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St. John's **Newfoundland and Labrador** **Canada**

ABSTRACT

Dietary supplementation of fish oil has been associated with reduced risk of cardiovascular disease. However, previous studies from our lab showed severe hyperlipidemia and elevated oxidative stress levels in BioF1B hamsters fed 20% (w/w) fish oil supplemented diet. BioF1B hamster, an inbred strain from Bio87.2 and Bio1.5 parent strains, is an animal model for diet induced hyperlipidemia and atherosclerosis. BioF1B hamsters have significantly lower post-heparin lipoprotein lipase (LPL) activity as compared to Golden Syrian hamsters, which is further reduced in response to 20% (w/w) fish oil diet. Reduced LPL activity in BioF1B hamsters is believed to interfere with the clearance of chylomicron-like particles and thus responsible for fish oil induced hyperlipidemia. In the present study we compared the effects of two ω-3 PUFA-rich sources, fish oil and seal oil, on regulation of lipid metabolism and oxidative stress in BioF1B hamsters. The two marine sources of ω-3 PUFA differ in the intramolecular distribution of ω-3 PUFA on their triglyceride (TG) molecules. While EPA and DHA are primarily located in *sn*-2 position in fish oil TG, these are distributed in *sn*-1 and *sn*-3 positions in seal oil TG. Fish oil and seal oil further differ in their fatty acid composition with significantly higher levels of DPA and MUFA in seal oil as compared to fish oil. We hypothesized that BioF1B hamsters will be able to tolerate seal oil better than fish oil due to differences in positional distribution of ω-3 PUFA in TG molecule as well as differences in the fatty acid composition. Moreover, increased resistance to oxidation has been reported with seal oil. Significantly lower plasma and liver lipid levels were observed with 20% (w/w) seal oil fed BioF1B hamsters as compared to 20% (w/w) fish

oil fed BioF1B hamsters. RT-PCR analysis showed significantly reduced SREBP-1c mRNA expression levels in seal oil fed hamsters which can partially explain the suppression of lipogenesis in response to dietary seal oil compared to fish oil. Seal oil fed BioF1B hamsters also showed significantly lower plasma and liver TBARS levels, thus suggesting reduced oxidative stress relative to fish oil fed hamsters. Since fish oil fed hamsters showed elevated levels of oxidative stress, we wanted to investigate possible beneficial effects of antioxidant supplementation in hamsters fed high fat diets. Berry extract rich in anthocyanins has gained prominence as a potent antioxidant in recent years. Study of the role of anthocyanin enriched (25% w/w) elderberry extract supplementation on plasma lipid levels in marine oil fed BioF1B hamsters also revealed significant reduction in all plasma lipid parameters upon addition of elderberry extract to respective marine oil fed BioF1B hamsters. While cosupplementation with elderberry extract resulted in significantly lower hepatic total cholesterol and cholesterol ester concentrations in both fish oil and seal oil fed BioF1B hamsters, reductions in hepatic TG and free cholesterol levels was seen in fish oil fed group alone. Moreover, both plasma and hepatic TBARS levels showed significant reductions upon elderberry extract supplementation in fish oil fed BioF1B hamsters. Thus, current findings suggest that seal oil may confer greater benefits compared to fish oil in lowering lipid and oxidative stress levels under certain genetic conditions. Furthermore, co-supplementation of fish oil with anthocyanin enriched elderberry extract may be beneficial under these conditions than fish oil alone.

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ABBREVIATIONS

ACC	acetyl CoA carboxylase
ACAT-2	acyl CoA: cholesterol acyltransferase 2
ApoB	apolipoprotein B
bp	base pair
BHT	butylated hydroxytoluene
CVD	cardiovascular disease
CETP	cholesterol ester transfer protein
Cyp7a	cholesterol 7α hydroxylase
CEH	cholesteryl ester hydrolase
CM	chylomicrons
cDNA	complementary DNA
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
FAT	fatty acid transporters
FAS	fatty acyl synthase
FO	fish oil
FFA	free fatty acids
GLC	gas liquid chromatography
HDL	high density lipoprotein
HCl	hydrochloric acid

LCAT	lecithin:cholesterol acyl transferase
LPL	lipoprotein lipase
LXRs	liver X receptors
LDL	low density lipoprotein
MUFA	monounsaturated fatty acids
ω-3 PUFA	omega-3 polyunsaturated fatty acids
PPAR	peroxisome proliferator activated receptor
RT-PCR	reverse transcriptase PCR
SO	seal oil
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SCD1	stearoyl CoA desaturase-1
SREBP	sterol regulatory element binding protein
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TG	triglyceride
TMP	1,1,3,3 tetramethoxy propane
VLDL	very low density lipoprotein
w/w	weight/weight

Chapter 1: Introduction

1.1 Fish oil in cardiovascular disease

Dietary fish oil consumption is widely believed to confer beneficial effects in cardiovascular disease (CVD). Fish oil first gained attention when Bang and Dyerberg (1976) and Kromann and Green (1980) reported low incidence of heart disease in Eskimos and Greenland Inuits consuming large doses of fish and marine mammals. Since these preliminary observations, epidemiological and clinical studies and research initiatives have substantiated a beneficial role of fish oil consumption in heart disease (Hirai *et al.*, 1980; Kromhout *et al.*, 1995; Harris, 1997; Hu *et al.*, 2002). Fish and marine mammals are a rich source of essential omega-3 polyunsaturated fatty acids (ω-3 PUFA), especially eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). In 1999, GISSI Prevenzione study, a randomized controlled trial to study the effects of dietary ω-3 PUFA supplementation, demonstrated significantly reduced cardiovascular mortality in patients who had previously experienced myocardial infarction. Dietary ω-3 PUFA has a beneficial role in the prevention of arrythmias and sudden cardiac death (Jones and Lau, 2002; Lemaitre *et al.*, 2003). The anti-atherogenic effects of fish oil intake have been associated with the hypolipidemic, anti-thrombotic and anti-atheromatous effects of ω-3 PUFA (Simopoulos, 1999).

1.2 Beneficial effects of fish oil on lipid and lipoprotein profile

Hypertriglyceridemia was accepted as an independent risk factor for CVD when a meta-analysis of 17 population-based studies revealed a strong correlation between elevated triglyceride (TG) concentrations and the risk of CVD (Hokanson and Austin,

1996). Alterations in lipid and lipoprotein metabolism, which affect the synthesis, secretion and clearance of TG-rich lipoproteins, viz. chylomicrons (CM), very-low density- lipoprotein (VLDL) and low density-lipoprotein (LDL) are the major cause of hypertriglyceridemia. Fish oil feeding studies in normolipidemic and hypertriglyceridemic subjects have consistently demonstrated dramatic reductions in plasma TG and the rate of VLDL secretion (Connor *et al.*, 1993; Rambjor *et al.*, 1996; Putadechakum *et al.*, 2005; Harris 1997; Menuet *et al.*, 2005). In addition to lowering fasting TG levels, dietary fish oil supplementation also effectively reduces postprandial plasma TG concentrations (Sanders *et al.*, 1997; Roche and Gibney, 1996). Moreover, significant reduction in postprandial chylomicronemia reported in ω-3 PUFA fed rats has been attributed to accelerated chylomicron clearance and not to reduced chylomicron production/ secretion (Harris *et al.*, 1997a). The net reduced postprandial lipemic response following ω-3 PUFA consumption is either due to reduced production/secretion or enhanced clearance/uptake of these TG-rich lipoproteins. Thus, fish oil consumption is gaining immense popularity in light of its beneficial hypotriglyceridemic effects.

1.2.1 Mechanisms for the regulation of lipid and lipoprotein metabolism by fish oil

There are two important postprandial pathways for the metabolism of TG-rich lipoproteins. The exogenous pathway involves CM secreted by the intestine, which transport dietary TG between the gut and blood and are finally taken up by the liver. The second pathway involves the synthesis of hepatic VLDL that transports endogenous TG. Circulating VLDL is then converted to low density lipoprotein (LDL), which delivers

cholesteryl esters to peripheral tissues and liver (Chan *et al.*, 2004). Thus, the net lipoprotein levels in circulation depend on their rates of secretion and removal from the plasma. Dietary ω-3 PUFA regulate TG concentrations at various stages of CM and VLDL metabolism i.e. assembly, secretion and clearance from circulation.

Apolipoprotein B (ApoB) is an essential surface apolipoprotein involved in the synthesis of both CM and VLDL. While the liver secretes ApoB100 exclusively, the intestine secretes ApoB48, a truncated form of ApoB100. ω-3 PUFA supplementation in normolipidemic subjects has revealed reduced absolute concentrations of ApoB48 and VLDL ApoB100 (Tinker *et al.*, 1999; Bordin *et al.*, 1998; Park and Harris 2003). These reduced levels may be due to suppression at the level of ApoB synthesis or secretion (Tinker *et al.*, 1999). Reduced VLDL-ApoB100 pool size observed in normolipidemic males fed fish oil was not accompanied by any alteration in ApoB100 synthesis, but showed a significant decrease in VLDL assembly (Bordin *et al.*, 1998; Wilkinson *et al.*, 1998). DHA and EPA have been shown to reduce ApoB secretion by fifty percent in cell culture studies using newborn swine enterocytes (Wang *et al.*, 2001). Significantly decreased hepatic secretion of ApoB has been reported in men with visceral obesity and hyperlipidemic patients upon fish oil supplementation (Chan *et al.*, 2003; Shidfar *et al.*, 2003). Several fish oil feeding studies have confirmed that ω-3 PUFA reduce ApoB secretion by targeting ApoB for post-translational degradation within the rough endoplasmic reticulum (Kendrick and Higgins 1999; Fisher *et al.*, 2001). However, other studies by Fisher *et al.* (1998) show that ApoB production was not responsive to fish oil

supplementation in hypertriglyceridemic patients with diabetes. Thus, fish oil can have variable effects on CM/ VLDL assembly and secretion by influencing ApoB levels.

In addition to the effects of fish oil feeding on CM/VLDL assembly and secretion, hypolipidemia can also result owing to enhanced clearance of CM/VLDL from the circulation. The clearance of circulating CM/VLDL involves lipoprotein lipase (LPL) which hydrolyses the TG molecules of CM and VLDL to free fatty acids (FFA) and glycerol. Fish oil supplementation has yielded conflicting results for the regulation of LPL activity. While some studies in normolipidemic and hypertriglyceridemic subjects show that there is no effect of ω-3 PUFA from fish oil on LPL activity (Bordin *et al.*, 1998; Nozaki *et al.,* 1991), others showed an increase in pre-heparin LPL activity during the fed state with no effect on post-heparin LPL. The latter study also demonstrated reduced CM-TG half lives and decreased CM particle sizes, thus suggesting a role for ω-3 PUFA supplementation in accelerating CM-TG clearance through increased LPL activity (Park and Harris 2003). Insulin resistant rats fed fish oil demonstrated a two-fold higher LPL activity in adipose tissue but not in muscle tissue (Peyron-Caso *et al.*, 2003). These findings indicated that fish oil might exert its effects on the regulation of LPL activity in a tissue specific manner.

Upon the action of LPL, CM or VLDL are converted into CM or VLDL remnants and are taken up by specific receptors present on the liver. Tissues such as liver, intestine, heart and adipose tissue take up FFAs via membrane associated fatty acid transporters (FAT), e. g. fatty acid translocase CD36 and fatty acid binding protein. Fish oil treatment can influence plasma FFA levels by altering the activity of membrane associated FAT.

Exposure of skeletal muscle cells to EPA increased the activity of FAT CD36 compared with control cells (Aas *et al.*, 2006). Thus, fish oil potentially exerts its hypolipidemic effects by regulating various metabolic pathways involved in lipid transport and storage.

1.2.2 Fish oil mediated regulation of gene expression

Besides regulating metabolic pathways at the enzymatic level, fish oil supplementation appears to regulate the expression of various genes involved in lipid metabolism. ω-3 PUFA has been shown to alter plasma TG levels by regulating the expression of genes involved in lipid synthesis and oxidation. Downregulation of hepatic genes involved in lipogenesis e.g. Fatty acyl synthase (FAS) and Acetyl CoA carboxylase (ACC) has been reported in rodents fed ω-3 PUFA (Clarke *et al.*, 1990; Salati and Clarke 1986). In addition to the negative regulation of lipogenic genes, ω-3 PUFA upregulates several hepatic genes involved in fatty acid oxidation and storage, such as fatty acyl CoA synthetase (Martin *et al.*, 1997) and carnitine palmitoyl transferase-1 (Chatelain *et al.*, 1996). DHA-fed pigs demonstrated significantly increased liver and muscle acyl-CoA oxidase mRNA expression, suggesting that DHA treatment may increase peroxisomal fatty acid oxidation in these tissues (Hsu *et al.*, 2004). Thus, ω-3 PUFA exerts a dual action on the lipid metabolism by decreasing the expression of lipogenic enzymes and activating the genes involved in lipid oxidation and storage. This results in a net negative fat balance, attesting to the beneficial role of ω-3 PUFA in the management of hyperlipidemia (Sampath and Ntambi 2005).

Besides regulating the expression of genes involved in lipid synthesis and oxidation, ω-3 PUFA have also been shown to alter the expression of genes involved in the clearance of lipids. There is evidence for the tissue specific regulation of LPL gene expression by ω-3 PUFA. Rats fed fish oil showed significantly higher LPL mRNA expression in the epididymal adipose tissue compared with maize oil fed rats, while LPL mRNA was higher in perirenal adipose tissue in the maize oil fed rats compared with the fish oil fed rats (Murphy *et al.*, 1993). On the other hand, human studies examining the effects of ω-3 PUFA on LPL gene expression in adipose tissue demonstrated no significant effect on LPL gene expression (Murphy *et al.*, 1999a). However, ω-3 PUFA have been shown to increase LPL gene expression in the adipose tissue of subjects with atherogenic lipoprotein phenotype (Khan *et al.*, 2002). These studies suggest tissue-specific regulation of LPL gene expression by fish oil.

ω-3 PUFA also regulates the gene expression of specific FAT, which determines fatty acid uptake, oxidation and storage. FAT/CD36 mRNA expression was found to increase after EPA treatment in human skeletal muscle cells (Aas *et al.*, 2006). Similarly, spontaneously hypertensive rats fed ω-3 PUFA showed increased adipose tissue CD36 mRNA levels as compared to the control Kyoto-Wistar rats (Aguilera *et al.*, 2006). Thus, ω-3 PUFA appears to regulate plasma TG concentrations by altering lipid transport and storage pathways at the molecular level.

Recently, cDNA microarrays have been used to study the effects of ω-3 PUFA on the transcription of hepatic genes involved in lipid metabolism in mice models. These microarray experiments have further confirmed the role of ω-3 PUFA in the regulation of

genes involved in fatty acid synthesis, desaturation, transport and oxidation (Berger *et al.*, 2002; Lapillonne *et al.*, 2004). Evidently, ω-3 PUFA play a major role in the regulation of gene expression in lipid metabolism.

1.2.3 Regulation of transcription factors and nuclear receptors by fish oil

ω-3 PUFA mediated changes in gene expression were previously attributed to alterations in signaling by eicosanoid metabolites. However, the rapid and sustained changes in gene expression by ω-3 PUFA were more consistent with a ligand mediated event, such as a PUFA binding transcription factor (Clarke 2000). The key transcription factors and nuclear receptors studied in response to ω-3 PUFA mediated regulation are: 1) Sterol-Regulatory Element Binding Proteins, 2) Peroxisome Proliferator-Activated Receptors and 3) Liver X-Receptors

1.2.3.1 Sterol-Regulatory Element Binding Proteins (SREBP): SREBPs are helix-loop-helix transcription factors which regulate lipid levels by binding to the sterol regulatory elements in promoters of genes involved in lipogenesis and cholesterol metabolism (Sampath and Ntambi 2005). There are three known isoforms of SREBP i.e. SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1c preferentially activates lipogenesis, SREBP-2 preferentially enhances the transcription of genes involved in cholesterogenesis (Le Jossic-Corcos *et al.*, 2005). SREBPs play an important role in the ω-3 PUFA induced suppression of lipogenic enzymes e.g. FAS, stearoyl CoA desaturase-1 (SCD1) and S14 (Jump *et al.*, 1994; Xu *et al.*, 1999b; Kim *et al.*, 1999; Yahagi *et al.*, 1999). DHA has

been shown to regulate nuclear SREBP-1 abundance in rat hepatocytes (Botolin *et al.*, 2006). Regulation of SREBP-1 proteolytic degradation by ω-3 PUFA is considered to be the main mechanism controlling SREBP nuclear abundance. PUFA have also been shown to lower intracellular levels of SREBP-1c mRNA [Xu *et al.*, 1999b; Kim *et al.*, 1999; Yahagi *et al.*, 1999]. This inhibition is dependent on chain length and degree of unsaturation, with EPA and DHA being more potent inhibitors than linoleic or oleic acids (Sampath and Ntambi 2005). While PUFA mediated regulation of SREBP-1c mRNA levels involves an enhanced rate of SREBP-1c mRNA turnover rather than inhibition of gene transcription in primary hepatocytes, several reports have demonstrated inhibition at the transcriptional level (Jump *et al.*, 2002; Ou *et al.*, 2001; Hannah *et al.*, 2001; Sealls *et al.*, 2008).

1.2.3.2 Peroxisome Proliferator-Activated Receptors (PPARs): Three isoforms of PPAR, PPAR -α, -β/δ, - γ have been identified. The isoform PPARα is predominant in the liver and regulates genes involved in lipid transport and oxidation. On the other hand, PPARγ is expressed in muscle and adipose tissue and aids in adipocyte differentiation. All PPAR isoforms bind EPA with K_d ranging from 1-4 μM (Jump 2002). EPA is reported to bind to PPAR in two conformations. In the first conformation, EPA gets completely buried in the binding pocket and alters the conformation to stabilize the activator function 2 helix. This stabilization allows for coactivator recruitment and subsequent PPAR mediated gene expression. Second conformation reveals that fatty acid hydrophobic tail stays exposed to the solvent. Since fatty acids <14 carbons or >20 carbons cannot fit in the docking site

and stay exposed to solvent, thereby hindering helix stabilization, 18 carbon (oleic acid) and 20 carbon (EPA) fatty acids appear to be optimal for PPAR activation (Xu *et al.*, 1999a). Next, though PPARα binds EPA and oleic acid with similar affinity, EPA but not oleic acid activates PPARα in primary rat hepatocytes (Xu *et al.*, 1999a; Ren *et al.*, 1997). This physiological discrimination is explained by the fact that compared to EPA, oleic acid is the preferred substrate for diacylglycerol acyl transferase in triglyceride synthesis. Reduced metabolism of EPA results in elevated cellular levels of EPA needed for PPAR activation. EPA, but not DHA, has been shown to induce mRNA expression of PPARγ (Chambrier *et al.*, 2002). Structural analysis showed that EPA is an endogenous ligand for PPARs and DHA needs to be converted to EPA to activate PPARs (Xu *et al.*, 1999a; Sprecher 2000). Moreover, eicosanoid metabolites of PUFA are more potent activators of PPARs than their fatty acid precursors (Sampath and Ntambi 2005). Genetic polymorphisms in PPARγ2 have been associated with the inter-individual variability in serum TG response to ω-3 PUFA (Lindi *et al.*, 2003). Some recent reports have shown agonist-driven PPARα activation to induce hepatic FAT/CD36 expression (Bonen *et al.*, 2004) and hepatic LPL mRNA as well as LPL activity (Auwerx *et al.*, 1996). These findings emphasize a need for a better understanding of the regulation of PPARs by ω-3 fatty acids *in vivo*.

1.2.3.3 Liver X Receptors (LXRs): LXRα and LXRβ regulate the expression of several genes involved in lipid metabolism, e.g. cholesterol 7α-hydroxylase (Cyp7a), LPL, FAS, ACC and SREBP-1c (Sampath and Ntambi 2005). While fish oil enrichment of diet

resulted in the induction of PPARs and repression of SREBP, dietary fish oil did not reveal any effect on classical LXR target genes such as Cyp7a and ABCG5 in HEK293 cells (Pawar *et al.*, 2003). Previously, SREBP mediated repression of lipogenesis by PUFA was considered to be LXR dependent (Yoshikawa *et al.*, 2002b), however recent evidence suggests that the repression of SREBP-1c mRNA levels by PUFA is independent of LXR. It was observed that treatment of HEK293 cells with EPA resulted in SREBP-1c mRNA inhibition both in the presence and absence of an LXR agonist (Pawar *et al.*, 2003). However, to date there is no *in vivo* evidence for the regulation of LXR by ω-3 PUFA.

1.3 Controversies associated with the beneficial health effects of fish oil

A large systematic study on the effects of ω-3 PUFA on total mortality due to CVD suggested that the beneficial effects of ω-3 PUFA were not conclusive (Hooper *et al.*, 2006). Recently, a meta-analysis of randomized controlled trials in patients at risk of ventricular arrhythmia showed enormous heterogeneity in patient response to fish oil supplementation (Jenkins *et al.*, 2008). Moreover, while the hypotriglyceridemic effects of fish oil are well established, the effects of fish oil on plasma total cholesterol and LDL- cholesterol are still controversial. While some studies show LDL-cholesterol reducing effects of fish oil feeding in normolipidemic patients, other studies report elevated plasma total- and LDL-cholesterol levels in response to fish oil in normolipidemic and hyperlipidemic individuals (Illingworth *et al.*, 1984; Nestel *et al.*, 1984; Sullivan *et al.*, 1986; Hsu *et al.*, 2000; Harris, 1997; Rivellese *et al.*, 2003). Pre-

existing dyslipidemia, e.g. hypertriglyceridemia and Type II diabetes, are believed to cause greater elevations in plasma total- and LDL-cholesterol concentrations upon fish oil consumption (Farmer *et al.*, 2001).

Dietary fish oil has also been reported to induce hyperlipidemia, which was more evident in animals fed fish oil and cholesterol (Kubow *et al.*, 1996; Lu *et al.*, 1996; Lin *et al.*, 1995). Addition of cholesterol (0.5% w/w) to fish oil diet primarily resulted in an increase in total plasma, VLDL- and LDL-cholesterol levels in Golden Syrian hamsters (Lin *et al.*, 1995). Interestingly, however, TG levels were reduced even further upon supplementation of fish oil diet with 0.5% w/w cholesterol. Dose dependent hypercholesterolemic effect of dietary cholesterol given with the fish oil diet was shown in the hamster model. Fish oil in Golden Syrian hamsters has been reported to increase the ApoB/ApoA1 ratio, which has been linked to an increased risk of atherosclerosis (Hayes *et al.*, 1990).

Previous studies from our laboratory have shown that BioF1B hamsters are highly susceptible to fish-oil induced hyperlipidemia at high fat levels (de Silva *et al.*, 2004). Another study from our laboratory compared the effects of fish oil feeding in Golden Syrian hamsters and BioF1B hamsters (Cheema and Cornish, 2007). High levels of fish oil feeding in BioF1B hamsters demonstrated dramatic hyperlipidemic response as compared to Golden Syrian hamsters. This study showed for the first time that alterations in LPL activity and mRNA expression levels play an important role in the varied response of these hamsters to dietary fats (Cheema and Cornish, 2007). These findings

highlight the significance of genetic background in the regulation of lipid and lipoprotein metabolism in response to fish oil.

1.3.1 Dietary fish oil and oxidative stress

Fish oil supplementation has been commonly associated with increased *in vivo* lipid peroxidation and compromised anti-oxidant status in organs, blood and urine of experimental animals and humans (Kaasgaard *et al.*, 1992, Cho *et al.*, 1995; Ando *et al.*, 1998; Ando *et al.*, 2000). High fish oil diet (19% (w/w) menhaden oil) markedly increases the oxidative potential in the mammary gland of spontaneously hypertensive rats (Mehta *et al.*, 1994). High dietary fish oil (20% (w/w) menhaden oil) has also been reported to increase the hepatic and faecal levels of oxidized lipids in male F344 rats (Dommels *et al.*, 2003). Yuan and Kitts (2003) reported the effect of high menhaden oil diet on lipid oxidation and anti-oxidant enzyme status in spontaneously hypertensive rats and Wistar Kyoto rats. Significantly elevated levels of lipid peroxidation in the hearts and livers of menhaden oil fed animals were attributed to alterations in anti-oxidant enzyme activities. Interestingly, addition of cholesterol (5g/kg diet) significantly lowered the hepatic reduced glutathione levels and exerted a protective effect against enhanced oxidative stress in these rats. It was concluded that high doses of ω-3 PUFA enhance the tissue susceptibility to oxidation, which can be modulated by supplementing dietary cholesterol in spontaneously hypertensive rats and Wistar Kyoto rats. Atalay *et al.* (2000) demonstrated increased activity of catalase, glutathione peroxidase and glutathione –S-transferase in rats in response to fish oil and exhaustive exercise. Increased aortic lesions

and elevated plasma lipid peroxidation levels have been reported for rabbits fed fish oil (Thiery and Seidel, 1987). Additionally, fish oil supplementation induced oxidative stress resulted in an increased requirement for vitamin E (Atalay *et al.*, 2000).

ω-3 PUFA in dietary fish oil can efficiently be incorporated into tissue membrane phospholipids by displacing the ω-6 PUFA. The increased unsaturation of membrane phospholipids due to incorporation of EPA and DHA from fish oil is thought to be the major cause of elevated *in vivo* lipid peroxidation since these membranes are likely to be more susceptible to oxidative stress (Leibovitz *et al.*, 1990). Fish oil induced lipid peroxidation has further been related to fish oil induced hyperlipidemia (Kubow, 1998).

On the contrary, some investigators have reported protective effects of dietary fish oil against oxidative stress. Erdogan *et al.*, (2004) assessed the effect of fish oil supplementation on plasma thiobarbituric acid-reactive substances (TBARS), nitric oxide, xanthine oxidase, superoxide dismutase and glutathione peroxidase in rats. These authors concluded that ω-3 PUFA enhanced resistance to free radical attack and lowered lipid peroxidation. ApoE knockout mice fed fish oil showed increased anti-oxidant enzyme activities in macrophages and reduced atherosclerotic lesions compared to corn oil fed animals (Wang *et al.*, 2004). These findings further support the anti-oxidant effects of ω-3 PUFA in spontaneously hypertensive rats (Frenoux *et al.*, 2001). Thus, fish oil supplementation may help restore the balance between anti-oxidant status and oxidative stress in the cell. Increased anti-oxidant enzyme activity can lead to increased free radical scavenging, thereby conferring enhanced resistance against free radical damage and delay the progression of atherosclerosis.

Interestingly, inverse correlation has been reported between the extent of lipid peroxidation and DNA damage in response to dietary fish oil. Kikugawa *et al.* (2003) suggested that dietary fish oil co-administered with vitamin E induced lipid peroxidation via increased levels of hydroperoxides and TBARS but lowered DNA damage in rat liver *in vivo.* DNA damaging potencies of the peroxidation products is proposed to be lower compared to that seen with ROS mediated oxidative stress. This explains how an increase in the extent of lipid peroxidation results in the attenuation of oxidative stress induced DNA damage, pointing to the protective effects of fish oil feeding. Consuming ω-3 PUFA is also believed to confer resistance to CM remnants against free radical attack and thus attenuates their potential atherogenic properties (Napolitano *et al.*, 2004). Fish oil supplementation has also been associated with reduced oxidative stress in hyperinsulinemic rats (Nyby *et al.*, 2005). These contradictory results of fish oil feeding emphasize the need to study the regulation of fish oil induced oxidative stress under hyperlipidemic conditions.

1.3.2. Combination of fish oil and vitamin E in the prevention of CVD

ω-3 PUFA such as DHA has been reported to decrease vitamin E levels in plasma and tissues of several experimental animals (Farwer *et al.*, 1994; Kubo *et al.*, 1997; Surai and Sparks, 2000). Thus, fish oil induced lipid peroxidation can be correlated with reduced vitamin E levels. Elevated lipid peroxidation and low tocopherol concentrations have further been associated with increased tissue cholesterol concentrations (Chupukcharoen *et al.*, 1985). Hence, an increase in oxidative stress may be a causative

factor in diet induced hyperlipidemia. Cho and Choi (1994) proposed that increasing vitamin E supplementation could be an effective strategy for preventing tissue peroxidation in ω-3 PUFA supplemented subjects. Vitamin E has been shown to protect erythrocyte and liver microsome lipids rich in ω-3 PUFA against lipid peroxidation during postnatal development of rats (Suarez' *et al.*, 1999). Vitamin E is believed to efficiently inhibit fatty acid peroxidation via its anti-oxidant effects. This further helps to enhance the incorporation of ω-3 PUFA in different tissues, thereby lowering ω-6/ω-3 ratio and improving the efficiency of dietary fish oil. High supplemental dosage of vitamin E has been shown to significantly enhance the hypotriglyceridemic effects of dietary fish oil in normolipidemic humans (Haglund *et al.*, 1991).

Kubow *et al.* (1996) reported that supplementing dietary fish oil with vitamin E significantly reduced the extent of tissue lipid peroxidation and hyperlipidemia in Golden Syrian hamsters. Cosupplementation of vitamin E with fish oil markedly decreased fish oil induced antioxidant enzyme activities in the livers of rats after exhaustive exercise (Atalay *et al.*, 2000). These studies suggest that vitamin E supplementation can potentially confer protection against CVD either by regulating oxidative stress or lipid metabolism. However, the beneficial effects of combination of vitamin E and ω-3 PUFA on CVD mortality in the randomized controlled GISSI trial were attributed to ω-3 PUFA alone with no significant effects of vitamin E supplementation (Shekelle *et al.*, 2004). Other independent randomized controlled trials to evaluate the effects of vitamin E on cardiovascular events/cardiovascular mortality have also revealed that anti-oxidant

vitamin E does not affect CVD either positively or negatively (Vivekananthan *et al.*, 2003; Morris and Carson, 2003).

O'Malley (2004) offered possible explanations for the failure of potential anti-oxidant therapy in clinical outcomes. Firstly, anti-oxidant therapy might be more effective in retarding/inhibiting the progression of atherosclerosis if implemented at early stages of the disease. Anti-oxidant therapy in most randomized controlled trials is either aimed at secondary prevention in patients who had experienced myocardial infarction or primary prevention in older high-risk patients. Secondly, optimal anti-oxidant activity necessary to affect complex atherogenic processes might not be achieved in the form of supplemental pills due to limited absorption and bioavailability. These observations emphasize the need to investigate more biologically active antioxidants in future.

1.4 Anthocyanins as biologically active anti-oxidants

In an attempt to discover biologically active anti-oxidants, research has now focused on anthocyanins, due to their high anti-oxidant potential and abundance in fruits such as berries. Anthocyanins are water soluble, glycosylated and non-acetylated polyphenolic compounds which belong to the flavanoid class of compounds and impart red, blue and purple colour to various fruits and vegetables (Bell and Gochenaur, 2006; Clifford, 2000; Galvano *et al.*, 2004). While approximately 400 individual anthocyanins have been identified, six are most commonly found in colored fruits especially berries. These are classified on the basis of number and position of hydroxyl and methoxyl moieties on the flavan nucleus (Mazza, 2007). Cyanidin is believed to be the most

abundant anthocyanidin in nature. Free radical scavenging capacity of anthocyanins is reduced upon glycosylation due to reduced ability of the anthocyanin radical to delocalize electrons (Mazza, 2007). Thus, a comparison between the anti-oxidant potential of different anthocyanidin molecules revealed direct proportionality to the number of hydroxyl groups and inverse proportionality to the number of glycosyl groups (Fukomoto and Mazza, 2000). Anthocyanins possess a wide spectrum of therapeutic properties such as anti-oxidant, hypolipidemic, anti-inflammatory and anti-thrombotic effects (Wang *et al.*, 1997; Fukumoto and Mazza, 2000; Mazza *et al.,* 2002; Wang and Mazza, 2002; Morazzoni and Magistretti, 1990; Satue-Gracia *et al.,* 1997).

1.4.1 Anti-oxidant potential of anthocyanins

Anthocyanins are potent anti-oxidants and reactive oxygen species scavengers and thus can be beneficial in reducing oxidative stress associated with CVD. Dietary flavanoids have been demonstrated to significantly reduce the amounts of dienes produced during 12 hours of oxidation in PUFA rich diet fed rats (Fre'mont L *et al.*, 1998) and lengthened the lag time in rats fed monounsaturated fatty acids (MUFA). These flavanoids can potentially protect circulating and membrane lipids by sparing vitamin E and endogenous antioxidants. Proanthocyanidin administered rats showed increased resistance against copper-ion induced oxidation of blood plasma (Koga *et al.*, 1999). Proanthocyanidin rich extracts can trap reactive oxygen species in plasma and interstitial fluid of the arterial wall, thereby inhibiting LDL oxidation and potentially preventing the progression of atherosclerosis. Dietary supplementation with grape seed

proanthocyanidins lowered the postprandial oxidative stress by decreasing the oxidant and increasing the anti-oxidant levels in the plasma of healthy human volunteers (Natella *et al.,* 2002). Proanthocyanidin rich grape seed extract also resulted in improved resistance to oxidative modification of LDL by reducing plasma lipid hydroperoxides and the oxidant/ antioxidant status. Grape seed proanthocyanidins significantly inhibited the progression of atherosclerosis in the aorta of cholesterol fed rabbits (Yamakoshi *et al.,* 1999).

Analysis of anthocyanins to detect radical scavenging activity present in different berries showed potent antiradical activities in all berry extracts (Nakajima *et al.*, 2004). Interestingly, anthocyanins have significantly higher anti-oxidant potential compared to classical anti-oxidants such as butylated hydroxyanisole, butylated hydroxytoluene and vitamin E and vitamin C (Wang *et al.*, 1997; Fukumoto and Mazza, 2000). *In vitro* inhibition of enzymatic and non-enzymatic PUFA-mediated peroxidation occurred in a dose dependent manner by purified anthocyanins (Narayan *et al.*, 1999). Many studies report inhibition of LDL oxidation *in vitro*, further attesting to the anti-oxidant activity of anthocyanins (Teissedre *et al.*, 1996; Aviram and Fuhrman, 2002). Cyanidin-3-O-beta-glucopyranoside, an anthocyanin, is known to significantly inhibit malondialdehyde generation in a dose dependent manner, with the extent of inhibition being significantly higher than those obtained with similar concentrations of resveratrol and ascorbic acid (Amorini *et al.*, 2001). Fruits and vegetables rich in anthocyanins showed concentration dependent anti-oxidant activities in the inhibition of copper-induced liposome

peroxidation as well as in inhibiting co-oxidation of linoleic acid and beta-carotene (Hassimotto *et al.*, 2005).

Ramirez-Tortosa *et al.* (2001) reported that dietary anthocyanins significantly improved plasma anti-oxidant capacity and lowered the vitamin E deficiency-enhanced hydroperoxides and 8-oxo-deoxyguanosine concentrations in rat livers. Anthocyanins from chokeberry significantly reduced the levels of TBARS and thiol protein groups and improved the overall anti-oxidant status in Wistar rats (Kowalczyk *et al.*, 2002). A study on male subjects consuming freeze dried blueberries reported that the serum anthocyanins concentration correlated positively with the serum anti-oxidant capacity (Mazza *et al.*, 2002). Elderberry anthocyanins are efficiently incorporated into the plasma membrane and cytosol of vascular endothelial cells (Youdim *et al.*, 2000). This enrichment of endothelial cells is proposed to confer significant protection against oxidative insult. Bagchi *et al.* (2004) evaluated several combinations of berry extracts and developed a synergistic formula OptiBerry IH141, which exhibited higher anti-oxidant and anti-angiogenic potential compared to other combinations studied.

Anthocyanin doses of 40 mg/kg improved the total anti-oxidant capacity with increased superoxide dismutase activity and reduced serum malondialdehyde levels in models of Freund's adjuvant induced arthritis (He *et al.*, 2006). Han *et al.* (2006) reported antioxidant capacities of pigmented fractions from purple potato flakes *in vitro* due to increased radical scavenging activity and inhibition of linoleic acid oxidation. Authors further studied the anti-oxidant potential of anthocyanin-rich purple potato flake diet in rats. In comparison to the rats fed cornstarch rich diet, rats on purple potato flake diet had

significantly lower hepatic lipid peroxidation and elevated hepatic Cu/ Zn- and Mn-superoxide dismutase and glutathione peroxidase mRNA expression and thus, improved anti-oxidant potential. Thus, anthocyanins can potentially exert anti-oxidant effects by regulating the genes involved in oxidative stress. These interesting findings emphasize the need to elucidate the efficacy of anthocyanins against oxidative stress *in vivo* and to establish whether anthocyanin supplementation could be a better alternative to vitamin E supplementation.

1.4.2 Anti-atherogenic potential of anthocyanins

Recently, some studies have reported that anthocyanins appear to play a major role in regulating various pathways involved in the development or progression of heart disease. Valcheva *et al.* (2007a) reported significant hypoglycemic and hypolipidemic effects of anthocyanin-rich aronia melanocarpa fruit juice (10 and 20mL/kg body weight) in streptozotocin-induced diabetic rats. Fruit juice rich in anthocyanins significantly reduced plasma glucose and TG to levels comparable to normal rats and also counteracted the influence of streptozotocin on total cholesterol, LDL-cholesterol and high-density lipoprotein- (HDL) cholesterol. These authors also showed antihyperlipidemic effects of anthocyanin-rich aronia melanocarpa fruit juice in rats with dietary induced hyperlipidemia (Valcheva *et al.,* 2007b). Anthocyanin-rich juice significantly hindered elevations in plasma total cholesterol, LDL-cholesterol and TG levels seen in rats fed high cholesterol in the diet. Lyophilized grape powder supplementation, a rich source of flavans such as anthocyanins, significantly reduced

plasma TG, LDL-cholesterol, ApoB, ApoE levels as well as cholesterol ester transfer protein (CETP) activity in both pre-and post-menopausal women (Zern *et al.,* 2005). While LDL oxidation was not affected upon supplementation, whole-body oxidative stress assessed by urinary-F2-isoprostanes was significantly reduced in these subjects. In another study, rats fed a combination of high fat diet and black soybean anthocyanins had significantly lower serum TG and cholesterol levels and markedly elevated HDL-cholesterol concentrations as compared to animals fed high fat diet alone (Kwon *et al.,* 2007). A randomized, placebo controlled trial studied the effects of elderberry juice (containing 10% anthocyanins) on serum lipids and anti-oxidant status in healthy volunteers (Murkovic *et al.*, 2004). While low dose treatment (equivalent to 5mL juice/day for 2 weeks) did not reveal any significant differences in serum lipid levels and anti-oxidant status, higher, but nutritionally relevant doses (single dose equivalent of 50 mL juice) significantly lowered postprandial serum lipids. Thus this dosage was used as a reference standard for the current thesis investigation (details in methodology section).

Furthermore, soybean anthocyanins have been proposed to possess anti-obesity effects which can potentially reverse the effects of high fat diets on body weight, adipose tissue weight and serum lipid profile (Kwon *et al.,* 2007). Other studies have also shown that anthocyanin supplementation can effectively suppress increases in body weight gain observed in response to high fat diet and may be beneficial in prevention and control of obesity (Tsuda *et al.,* 2003; Jayaprakasam *et al.,* 2006). Polyphenol compounds might have anti-obesity effects in female Zucker fatty rats through inhibition of fat metabolizing enzymes (pancreatic lipase, adipose-tissue derived LPL and glycerphosphate

dehydrogenase activities) and enhanced lipolysis (Yoshikawa *et al.*, 2002a). Anthocyanins can also exert anti-atherogenic effects by modulating several inflammatory pathways. Berry extract anthocyanins have been shown to play an immuno-modulatory role by attenuating various parameters of inflammation such as nitric oxide synthesis, adhesion molecules and prostaglandins (Pergola *et al.*, 2006; Rossi *et al.*, 2003).

Moreover, atherosclerotic plaque stability was shown to improve remarkably in ApoE –deficient mice upon dietary supplementation with an anthocyanins-rich extract from black rice (Xia *et al.*, 2006); extracts rich in anthocyanins dramatically improved serum lipid profile by lowering TG, total-cholesterol and non-HDL cholesterol levels. Lipid-lowering and anti-inflammatory properties of anthocyanins are thought to be primarily responsible for the beneficial effects of anthocyanins in ApoE deficient mice. On the contrary however, adverse effects of anthocyanin supplementation on plasma cholesterol and LDL- cholesterol levels were observed in Watanabe Heritable Hyperlipidemic rabbits fed purified anthocyanins (Finné-Nielsen IL *et al.*, 2005). Thus, therapeutics involving anthocyanins must be viewed with caution, particularly in patients with Familial Hypercholesterolemia (Frolov and Hui, 2007). More studies need to be done to evaluate the safety and efficacy of anthocyanins with regard to variability in genetic background of experimental models.

1.5 Other marine source of ω-3 PUFA: Seal oil

To date studies reporting health benefits of ω-3 PUFA have primarily focused on increasing the consumption of fish oil. These studies, however, have failed to consistently

achieve low levels of CVD upon fish oil supplementation as that observed in the Eskimo and Greenland Inuit populations. The inconsistencies could partly be due to the fact that the Greenland Eskimos also consumed other marine mammals such as seal and whale besides fish. Seal oil supplementation in healthy volunteers was found to significantly reduce plasma TG and ω-6/ω-3 ratio of plasma and erythrocytes without any significant effects on cholesterol levels (Bonefeld-Jorgensen *et al.*, 2001). Another study by Conquer *et al.* (1999) demonstrated that seal oil supplementation resulted in lower ω-6/ω-3 ratio and higher EPA, DHA and docosapentaenoic acid (DPA; 22:5) levels in healthy, normocholesterolemic subjects. They further showed that seal oil increased the ratio of EPA/arachidonic acid and DHA/arachidonic acid in the serum phospholipids and FFAs. However, no significant differences were observed on glucose, plasma TG and cholesterol levels in response to seal oil consumption.

There are some differences in the ω-3 PUFA composition of seal oil and fish oil. While EPA and DHA content of seal oil is slightly lower as compared to fish oil, DPA levels are approximately 3 fold higher in seal oil (Brox *et al.*, 2001). The two marine sources of ω-3 PUFA further differ in the intramolecular distribution of ω-3 PUFA on their TG molecules. While EPA and DHA are primarily distributed in the *sn*-1 and *sn*-3 positions of seal oil TGs, they are located in the *sn*-2 position in case of fish oil TGs (Christensen *et al.*, 1994). Seal oil and fish oil fed guinea pigs showed significantly higher concentration of ω-3 PUFA in different organs and plasma as compared to corn oil fed animals (Murphy *et al.*, 1999b). Differences in the pattern of incorporation of ω-3 PUFA were also noted for the two marine oils. While fish oil feeding resulted in higher

levels of EPA in plasma TG, dietary seal oil led to maximal incorporation of EPA in heart polar lipids. DHA levels were significantly higher in heart TG from fish oil fed guinea pigs compared to seal oil fed guinea pigs. In contrast, heart polar lipids had significantly higher levels of DPA in response to dietary seal oil compared to fish oil.

Yoshida *et al.* (1999) reported the effects of dietary seal and fish oils on TG metabolism in rats. Their findings showed that seal oil was more effective compared to fish oil in lowering serum and liver TG concentrations. Significantly lower activities of fatty acid synthase, glucose-6–phosphate dehydrogenase and hepatic TG lipase were observed in seal oil fed rats compared to those fed linoleic acid. In a similar study on hamsters, these authors also showed that dietary seal oil reduced arachidonic acid content in liver phosphatidylcholine and phosphatidylethanolamine, and serum phosphatidylcholine more effectively as compared to fish oil (Yoshida *et al.*, 2001).

Different intramolecular distribution of ω-3 PUFA in dietary fats is believed to regulate lipid metabolism differently in experimental animals. These structural differences can potentially influence the uptake and bioavailability of ω-3 PUFA due to the stereospecificity of pancreatic lipase and gastrointestinal lipase, both of which hydrolyze primary ester bonds of TG molecule. While ω-3 PUFA are mostly absorbed as *sn*-2 monoacylglycerols upon fish oil consumption, these are absorbed as FFAs after consuming seal oil. This can potentially alter the digestion and absorption efficiency of different ω-3 PUFA (Christensen *et al.*, 1995). A comparison of the rate of CM clearance following either seal oil or fish oil injection showed a faster clearance of seal oil CM

compared to fish oil CM. This has been attributed to structural differences in intramolecular distribution of ω-3 PUFA in TG of dietary fats (Christensen *et al.*, 1995).

Another potential advantage of seal oil supplementation is enhanced resistance to oxidation compared to fish oil (Nakhla, 1997). While ω-3 PUFA from fish oil are highly susceptible to oxidation resulting in the formation of lipid hydroperoxides, seal oil ω-3 PUFA are relatively resistant to oxidation. In light of these findings, there is a need to further investigate the role of seal oil in comparison with fish oil in the regulation of lipid metabolism and oxidative stress.

1.6 Choice of hamster as an animal model

The hamster is an animal model of choice to study lipoprotein metabolism and atherosclerosis because the lipoprotein profile of hamsters closely resembles that of humans (Nistor *et al.*, 1987; Spady and Dietschy, 1988). Hamsters carry a significant proportion of their circulating lipoprotein in LDL fraction, which is similar to humans (Ohtani *et al.*, 1990). Moreover, as opposed to rodents, hamsters possess plasma CETP activity similar to that in humans (Ahn *et al.*, 1994; Ha and Barter, 1982). Hamsters also resemble humans in the secretion of TG-rich lipoproteins in that the origin of ApoB48 and ApoB100 is intestinal and hepatic respectively, however in case of rats and mice both the ApoB isoforms are secreted from liver. In the context of cholesterol metabolism, hamsters show hepatic cholesterol synthesis similar to that in humans (Spady and Dietschy 1988; Woollett *et al.*, 1989) and partial regulation of cholesterol metabolism by LDL receptors (Chen *et al.*, 1996; Remillard *et al.*, 2001). Dietary cholesterol and

saturated fat have been shown to result in greater serum LDL-cholesterol concentrations similar to that observed in humans (Sullivan *et al.*, 1993). Moreover, genetic variability in hamster strains has been associated with differences in response to dietary fats (Dorfman *et al.*, 2003).

BioF1B hamster, an inbred strain from Bio 87.2 and Bio 1.5 parent strains, is an established model for diet-induced hyperlipidemia and atherosclerosis. Kowala *et al.* (1991) reported that the BioF1B strain of hamsters showed atherosclerotic lesions even at low concentrations of dietary cholesterol. Increased concentrations of TG-rich lipoproteins upon cholesterol supplementation have been associated with the increased susceptibility of BioF1B hamsters to hyperlipidemia and atherosclerosis (McAteer *et al.*, 2003). Dyslipidemic individuals (hypertriglyceridemic, diabetic) have been reported to show greater elevations in plasma total and LDL cholesterol levels (Hsu et al., 2000, Farmer et al., 2001), compared to normolipidemic subjects in response to dietary fish oil. Similar to hyperlipidemic humans, BioF1B hamsters fed an atherogenic diet showed increased total cholesterol/HDL levels and increased susceptibility to atherosclerosis (Trautwein et al., 1993). Our lab has previously demonstrated that BioF1B hamsters are susceptible to fish oil induced hyperlipidemia at high fish oil levels, in contrast to GS hamsters fed similar levels of fish oil. These lipid profile changes further suggest that genetic variability might play an important role in determining the final outcome of ω-3 PUFA supplementation. More studies are needed to ascertain whether BioF1B hamsters are representative of differential effects (if any) of high fish oil diet in hyperlipidemic humans. However, the relevance of genetic variability in the regulation of lipid

metabolism upon fish oil feeding emphasizes the need for investigating lipid regulation in fish oil fed hyperlipidemic animal models and subsequently hyperlipidemic human populations.

1.7 Justification of the study

Existing data suggests that differential responses to dietary treatments might arise due to heterogeneity in genetic background and gene-nutrient interactions in different experimental models (Katan *et al.*, 1986; Overturf *et al.*, 1990). Previous studies from our laboratory to investigate the effects of high fish oil feeding i.e. 20% (w/w) vs 5% (w/w), on the regulation of lipoprotein metabolism have reported the occurrence of diet induced hyperlipidemia in genetically susceptible BioF1B hamsters (de Silva *et al.*, 2004). Interestingly, BioF1B hamsters fed the high fish oil diet showed the presence of milky plasma rich in CM like particles after an overnight fast. High fish oil supplementation also resulted in elevated plasma TG and plasma-, VLDL- and LDL- cholesterol concentrations in BioF1B hamsters as compared to BioF1B hamsters fed a mixture of lard and safflower oil (1.5:1). Dietary fish oil supplemented BioF1B hamsters further showed reduced hepatic LDL-receptor mRNA expression and significantly lowered CETP activity (de Silva *et al.*, 2005). Following these preliminary observations from our laboratory using BioF1B hamsters, another study was undertaken to compare the effects of different types of high fat diets (fish oil, MUFA and ω-6/ ω-3 ratio of 5:1) in Golden Syrian hamsters and BioF1B hamsters (Cheema and Cornish 2007). Contrary to the observations with fish oil fed BioF1B hamsters, fish oil fed Golden Syrian hamsters did

not reveal any milky plasma after an overnight fast. BioF1B hamsters fed high fat fish oil diet showed significantly elevated lipid levels as compared to fish oil fed Golden Syrian hamsters, highlighting the importance of genetic heterogeneity in response to dietary fat. Moreover, among the three different dietary treatments in BioF1B hamsters, diet induced hyperlipidemia was specific only to high fat fish oil diet. Molecular analysis revealed that fish oil fed BioF1B hamsters had higher levels of ApoB48 and ApoB100 as compared to other dietary treatments, suggesting a reduced clearance of TG-rich lipoproteins (Cheema and Cornish, 2007). BioF1B hamsters revealed significantly lower post-heparin LPL activity as compared to Golden Syrian hamsters. Fish oil supplementation further inhibited the LPL activity in BioF1B hamsters compared to MUFA and ω-6/ ω-3 fed BioF1B hamsters (Cheema and Cornish, 2007).

In an attempt to investigate the extent of oxidative stress in Golden Syrian and BioF1B hamsters in response to high fat diets, we measured the levels of lipid peroxidation and anti-oxidant enzymes in the liver. We observed significantly higher lipid peroxidation in BioF1B hamsters as compared to Golden Syrian hamsters. Fish oil feeding in BioF1B hamsters resulted in a further increase in the extent of lipid peroxidation compared to MUFA and ω-6/ω-3 fed BioF1B hamsters (Dubey and Cheema, unpublished data). Fish oil fed BioF1B hamsters also showed compromised anti-oxidant enzyme status suggesting an oxidative potential of high dietary fish oil in genetically susceptible BioF1B hamsters.

The ω-3 PUFA, such as EPA and DHA are primarily located in *sn*-2 position in fish oil TGs, while these are distributed in *sn*-1 and *sn*-3 positions on seal oil TGs. The

structural differences in location of ω-3 PUFA in the fish oil and seal oil TGs are considered to be important in producing better clearance and uptake of lipids from seal oil as compared to fish oil. Since fish oil-induced hyperlipidemia in BioF1B hamsters was attributed to hindered clearance of TG-rich lipoproteins and lower post heparin LPL activity, the current study was designed to investigate whether seal oil, another rich source of ω-3 PUFA, with different distribution of ω-3 PUFA in TG molecule as compared to fish oil, will differentially regulate lipid metabolism in BioF1B hamsters. We hypothesized that BioF1B hamsters will be able to metabolize dietary seal oil better as compared to fish oil due to positional distribution of ω-3 PUFA. Moreover, seal oil is believed to be relatively resistant to oxidation as compared to fish oil. It was further hypothesized that the extent of oxidative stress will be lower in seal oil fed BioF1B hamsters as compared to fish oil fed hamsters. Anthocyanins are potent anti-oxidants with higher anti-oxidant potential as compared to classical anti-oxidants such as Vitamin E and Vitamin C. It was further hypothesized that a combination of anthocyanins and fish oil diet will suppress diet-induced hyperlipidemia and oxidative stress as compared to fish oil diet alone. We treated BioF1B hamsters with high fat diets (20% fat w/w) containing either fish oil or seal oil alone, or a combination of high fat diets (fish oil and seal oil) containing anthocyanin-rich elderberry extract (25% w/w anthocyanin).

Thus, the present study was undertaken with two key objectives:

1. To evaluate the effects of dietary seal oil, an alternative source of ω-3 PUFA, on the regulation of lipoprotein metabolism and oxidative stress in BioF1B hamsters.

2. To investigate the effects of combination of anthocyanin-rich elderberry extract and ω-3 PUFA (fish oil and seal oil) on lipid profile and oxidative stress in BioF1B hamsters.

The findings will help us to understand whether seal oil differentially regulates lipid and lipoprotein metabolism in BioF1B hamsters, and whether combinational therapy with anthocyanin rich elderberry extract and marine oils will have a greater effect on lowering plasma lipids and oxidative stress as compared to marine oils alone.

Chapter 2: Methodology

2.1 Animals and diets

Bio F1B hamsters (male, 7 weeks old) were obtained from Bio Breeders Inc (Water Town, MA). During the initial equilibration period, hamsters were kept on a chow diet for 1 week. Hamsters were then divided into 4 groups (n = 6) and kept on specified diets for 4 weeks. The specified diets consisted of custom-made fat-free semipurified diet (ICN Biomedical Inc., OH, USA) that was either supplemented with 20% (w/w) fish oil (FO) (Menhaden oil, Sigma- Aldrich, Ontario, Canada) or 20% (w/w) seal oil (SO) (Ocean Choice Ltd, NL, Canada). Within each high fat diet group, hamsters were either fed 20% (w/w) fat alone or were given a combination of 20% (w/w) fat and elderberry extract (40 g/kg diet) (Murkovic *et al.*, 2004). Elderberry (25% w/w anthocyanin), spray-dried powder (Nutrican Nutritionals Ltd. ON, Canada) was mixed with fat and added to the diets. All the diets were stored at -20°C. The diet composition is given in Table 2.1. Experimental design for different dietary treatments is given in Figure 2.1.

The animals were housed in individual cages in a single room. The room conditions were maintained at 12-12hr light-dark cycles (room was lit from 07:00 to 19:00 hrs), temperature kept at 23 ± 1°C and humidity at 35 ± 5%. Fresh diets were given to the animals daily, and experimental diets and water were given ad libitum for 4 weeks. Food intake was measured daily and body weight was measured once a week. All experiments were approved by Memorial University's Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. At the end of 4 week feeding period, animals from each diet group were euthanized after

Table 2.1: Composition (g/kg) of fish oil and seal oil diet.

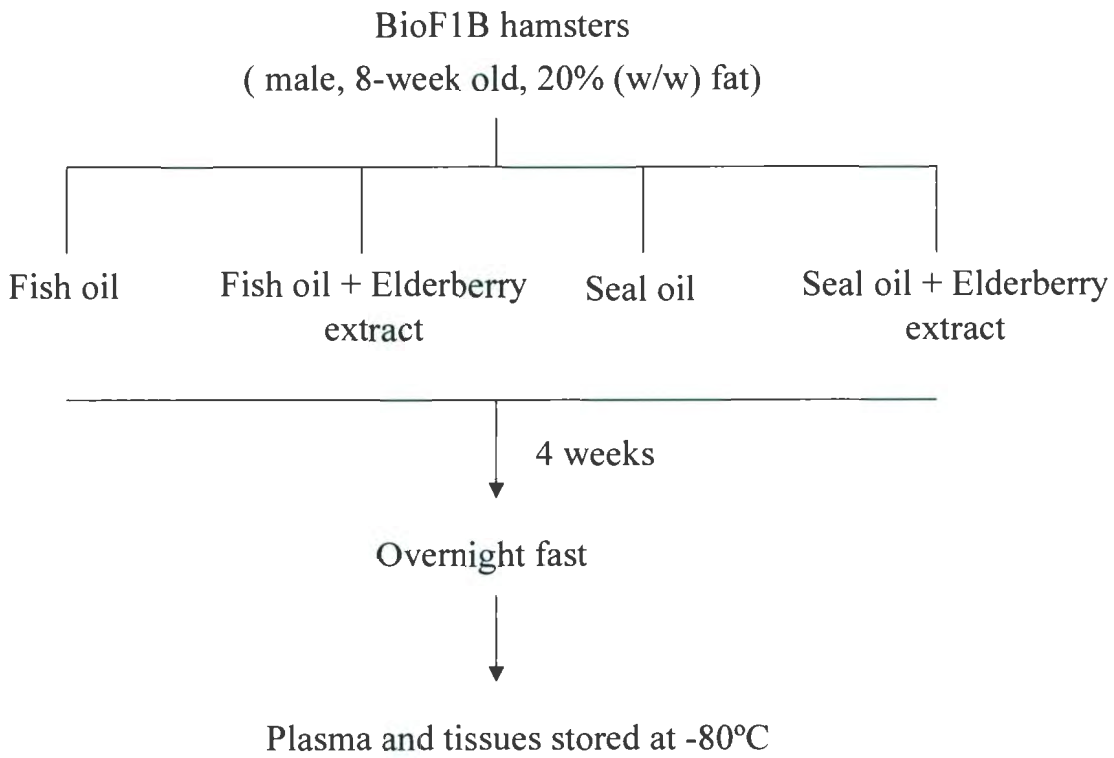
Components (g/kg)	Fish Oil Diet*	Seal Oil Diet*
Casein	200	200
DL-Methionine	3	3
Sucrose	305	305
Corn Starch	190	190
Vitamin Mix $^{\alpha}$	11	11
AIN Mineral Mix $^{\alpha}$	40	40
Fibre $^{\beta}$	50	50
Fat	200	200

* Semi-purified diets were designed to obtain 200g/kg fat

$^{\alpha}$ Formulated to meet the national requirements (National Research Council, 1995)

$^{\beta}$ Supplied as Alphacel non-nutritive bulk (ICN Biomedicals Inc., OH, USA)

Figure 2.1: Diagrammatic representation of the experimental design for different dietary treatments.



an overnight fast. Blood samples were obtained by cardiac puncture and collected in tubes containing EDTA, centrifuged immediately at 3000 rpm, 4°C for 15 min to separate plasma. Plasma samples were stored on ice at 4°C. Liver tissues were snap frozen in liquid nitrogen and stored at -80°C until further use.

2.2 Gas liquid chromatography

The fatty acid composition of the diets was analyzed by Gas Liquid chromatography (GLC) (Table 2.2). Lipids were extracted from diets with 2:1 chloroform: methanol using the method of Yokode *et al.* (1990). Trans-methylation of lipids was done by adding 2 mL of transmethylation reagent (94% methanol, 6% HCl with few crystals of hydroxyquinone) followed by incubation at 65°C for 2 hrs. Fatty-acid methyl esters were then extracted into the organic, hexane layer by adding hexane (1 mL) and deionised water (1 mL). The upper hexane layer was evaporated under nitrogen and the fatty acid methyl esters were dissolved in 20 µL of carbon disulfide. The fatty acid methyl esters were run on an Omegawax x 320 (30 mm*0.32 mm) column from Supelco (Sigma-Aldrich, ON, Canada) using a flame ionization detector (FID) for 1 hr. The GLC parameters were set as follows: oven at 196°C, injector at 240°C and detector at 250°C. Fatty acids were identified by comparison of retention times with those of known standards (Sigma-Aldrich, ON, Canada).

Table 2.2: Fatty acid composition of fish oil and seal oil diets using Gas Liquid Chromatography

Fatty acids*	Fish oil	Seal oil
14:0	11	6.5
16:0	18	10.1
16:1 ω-7	12.2	16.8
18:0	3.1	1.4
18:1 ω-9	8.9	22.9
18:2 ω-6	1.9	1.8
18:3 ω-3	1.5	0.6
18:4 ω-3	3.5	ND
20:1 ω-9	1.1	10.9
20:4 ω-6	0.8	0.5
20:5 ω-3	12.6	9
22:5 ω-3	2.2	5.2
22:6 ω-3	12.1	11.7
∑ SFA	32.1	18
∑ MUFA	22.2	50.6
∑ PUFA	34.6	28.8
∑ ω-3PUFA	31.9	26.5
∑ ω-6PUFA	2.7	2.3
ω-6/ ω-3 ratio	0.085	0.087

* Fatty acids are shown as % of the total fatty acids and represent the average of duplicate values.
ND- Not detected

2.3 Plasma lipoprotein fractionation and lipid analysis

Plasma samples were allowed to sit overnight on ice at 4°C and the top layer containing the CM fraction was removed after centrifugation at 10000 rpm for 15 min. Density gradient ultracentrifugation was done to isolate VLDL, LDL (LDL+IDL) and HDL fractions corresponding to specific densities of <1.006, 1.006-1.060 and >1.060 g/mL respectively (de Silva *et al.*, 2004). Sequential separation of different fractions was obtained by using solutions containing increasing amounts of NaCl (VLDL and LDL fraction), or NaBr (HDL fraction) to adjust the density of plasma.

For VLDL separation, 0.5 mL of plasma was mixed with 0.5 mL NaCl (density: 1.0063 g/mL). Samples were then centrifuged at 100,000 rpm at 16°C for 2.5 hrs with an acceleration of 5 and a deceleration of 7 using TL 100 fixed angle rotor (Beckman instruments Inc., CA). The top 0.5 mL (density <1.006) was then transferred carefully to a tube labeled VLDL and kept on ice at 4°C. To isolate LDL, the remaining 0.5 mL bottom layer was mixed with 0.5 mL NaCl (density: 1.12 g/mL) and centrifuged under conditions similar to VLDL isolation. After centrifugation, 0.5 mL of upper layer (LDL fraction) was again removed carefully and kept on ice at 4°C. Finally, to separate HDL, 0.5 mL of NaBr (density: 1.36 g/mL) was mixed with the remaining sample and centrifuged for 3.5 hrs under similar conditions as above. After centrifugation, 0.5 mL of upper layer was removed and kept on ice at 4°C. Isolated lipoprotein fractions i.e. VLDL, LDL and HDL were stored on ice at 4°C for lipid analysis.

Whole plasma and the individual lipoprotein fractions were assayed immediately for total TG (Kit# 236-60, Diagnostic Chemicals Ltd, PE, Canada), total cholesterol (Kit#

225-S7, Diagnostics Chemicals Ltd, PE, Canada) and free cholesterol (Kit# 274-47109E, Free cholesterol Enzymatic Kit, Wako Chemicals, VA, USA). Cholesterol ester concentrations were determined by subtracting free cholesterol concentrations from total cholesterol concentrations.

2.4 Liver lipid analysis

Lipids were extracted from liver tissue by homogenizing liver in chloroform/methanol (2:1) (Folch *et al.*, 1957). The lower organic phase was dried under nitrogen, and resuspended in 100 μL of isopropanol. Hepatic lipids were analyzed for total cholesterol (Kit# 225-S7, Diagnostic Chemicals Ltd, PE, Canada), total TG (Kit# 236-60, Diagnostic Chemicals Ltd, PE, Canada) and free cholesterol (Kit# 274-47109E, Free cholesterol Enzymatic Kit, Wako Chemicals, VA, USA). Cholesterol ester concentrations were determined by subtracting free cholesterol concentrations from total cholesterol concentrations. Standards were also suspended in isopropanol for consistency.

2.5 Plasma thiobarbituric acid reactive substances (TBARS) assay

Plasma TBARS were assayed using the method of Ohkawa *et al.* (1979). The standards were prepared with 0.1mM 1,1,3,3 tetramethoxy propane (TMP) solution in absolute ethanol and 29 mmol/L thiobarbituric acid (TBA) solution was prepared in 8.75M acetic acid. The following were added to 75 μL of plasma or TMP standards: 100 μL of 0.9% sodium chloride, 100 μL of 15% trichloroacetic acid (TCA), 100 μL of 2.5mM butylated hydroxytoluene (BHT), 150 μL of TBA and 60 μL of 8.1% sodium

dodecyl sulfate (SDS). Tubes were then vortexed for 15 sec and kept in a water bath at 95°C for 60 min. The samples were cooled on ice for 10 min and 750 µL of n-butanol was added to all the tubes. This was followed by centrifugation at 4400 rpm (4°C) for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS concentration in the blood plasma was expressed as nmol MDA/µL plasma from the standard curve.

2.6 Liver TBARS assay

The protocol for liver TBARS was similar to plasma TBARS with minor modifications (Williamson *et al.*, 2003). TMP standards were prepared as described earlier for plasma TBARS assay. Approximately 100 mg of liver tissue was homogenized in 1 mL PBS and 300 µL of the sample or standard was transferred to eppendorf tubes. This was followed by adding 150 µL of 15% TCA, 150 µL of 0.25 N Hydrochloric acid (HCl), 150 µL of 2.5mM BHT, 150 µL of 0.375% TBA and 60 µL of 8.1% SDS to each tube. Tubes were then vortexed for 15 sec and kept in a water bath at 95°C for 60 min. All samples were cooled on ice and centrifuged at 4400 rpm (4°C) for 20 min. The supernatant was filtered through 0.45 µm syringe filters and the absorbance of the filtrate was measured at 532 nm. TBARS concentration in the tissue samples was expressed as nmol MDA/mg tissue from the standard curve.

2.7 RNA isolation and reverse transcriptase PCR (RT-PCR) analysis

Total RNA from hamster livers was extracted using TRIZOL reagent (Invitrogen Life Technologies Inc., Gaithersburg, MD, USA) and Fast Prep (Bio101, ThermoSavant)

instrument. The yield of RNA samples was assessed by measuring the absorbance at 260 nm and 280 nm. The integrity of RNA samples was verified by running the samples on 1.2% agarose gels at 50V. Isolated RNA samples were stored at –20°C until further analysis.

RT-PCR was done to analyze the hepatic mRNA expression of various transcription factors of BioF1B hamsters fed different diets. Isolated RNA samples were treated with RNase free DNase I (Promega) to remove any genomic DNA. Briefly, 4 μg RNA sample was treated with 4 μL DNase (1 μg/μL) and 1 μL DNase buffer (10X). The final volume was made up to 10 μL with DEPC water. The samples were incubated at 37°C for 30 min and reaction was terminated by adding 2 μL DNase stop solution. The samples were then incubated at 70°C for 10 min to inactivate DNase.

Complementary DNA (cDNA) was synthesized by reverse transcription from DNase treated total RNA using AMV reverse transcriptase (Roche, QC, Canada) and used as template for PCR amplification. Briefly, DNase treated RNA (5 μL), 1X random primers (1 μL) and 5 μL DEPC water were mixed and incubated at 70°C for 10 min. The samples were cooled on ice and centrifuged briefly. This was followed by addition of 8 μL reaction mixture (4 μL of 5X RT buffer, 1 μL RNase inhibitor, 2 μL of 10 mM dNTPs and 1 μL DEPC water). Finally 1 μL of reverse transcriptase was added to each tube (except negative control) and incubated at 42°C for 1 hr. The cDNA template was stored at -20°C until PCR analysis.

PCR reactions were performed in the Genius PCR machine (Roche, QC, Canada) using the cDNA templates. PCR conditions were as follows: initial denaturation at 95°C for 10 min and then amplification cycles (denaturation at 95°C for 1 min, annealing for 2 min and elongation at 72°C for 2 min). This was followed by a final elongation step of 10 min at 72°C. The total number of cycles was chosen to remain within the exponential phase of the reaction. The specific primers used for SREBP-1c (Shimomura *et al.*, 1997), SREBP-2 (Field *et al.*, 2003), PPAR-α (Valeille *et al.*, 2005) and LXR-α (Valeille *et al.*, 2005) and their specific annealing temperatures are given in Table 2.3. Different genes were amplified simultaneously with hamster β-actin primers (sense 5`-CATCGTACTCCTGCTTGCTG-3' and antisense 5'-GCTACAGCTTCACCACCACA-3') in a multiplex PCR. Figure 2.2 – Figure 2.4 depict the PCR amplification of SREBP-1c, PPAR-α and LXR-α genes respectively. The mRNA expression of these genes was normalized to β-actin mRNA content and expressed as relative units.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software (GraphPad Software Inc., CA, USA). Differences between high fat diets were assessed using unpaired t-test and the effects of fat type and elderberry extract supplementation were analyzed using 2-way ANOVA. Newman-Keuls post-hoc test was used to test significant differences obtained by ANOVA analysis. Results are expressed as group means (n = 6) and ± SEM (standard error of mean). Differences were considered statistically significant at p-value ≤ 0.05.

Table 2.3: Primer sequences and annealing temperatures for different genes studied.

Gene	Primer Sequence	PCR (bp)	Temp (°C)
PPAR-α	Sense: 5'-GAGAAAGCAAAACTGAAAGCAGAGA-3' Antisense: 5'-GAAGGGCGGGTTATTGCTG-3'	179	62
LXR-α	Sense: 5'-GCAACTCAATGATGCCGAGTT-3' Antisense: 5'-CGTGGGAACATCAGTCGGTC-3'	171	62
SREBP-1c	Sense: 5'-GCGGACGCAGTCTGGG-3' Antisense: 5'-ATGAGCTGGAGCATGTCTTCAAA-3'	95	60
SREBP-2	Sense: 5'-AGCTGGCAAATCAGAAAAACAAG-3' Antisense: 5'-GATTAAAGTCTTCAATCTTCAAGTCCAC-3'	93	56

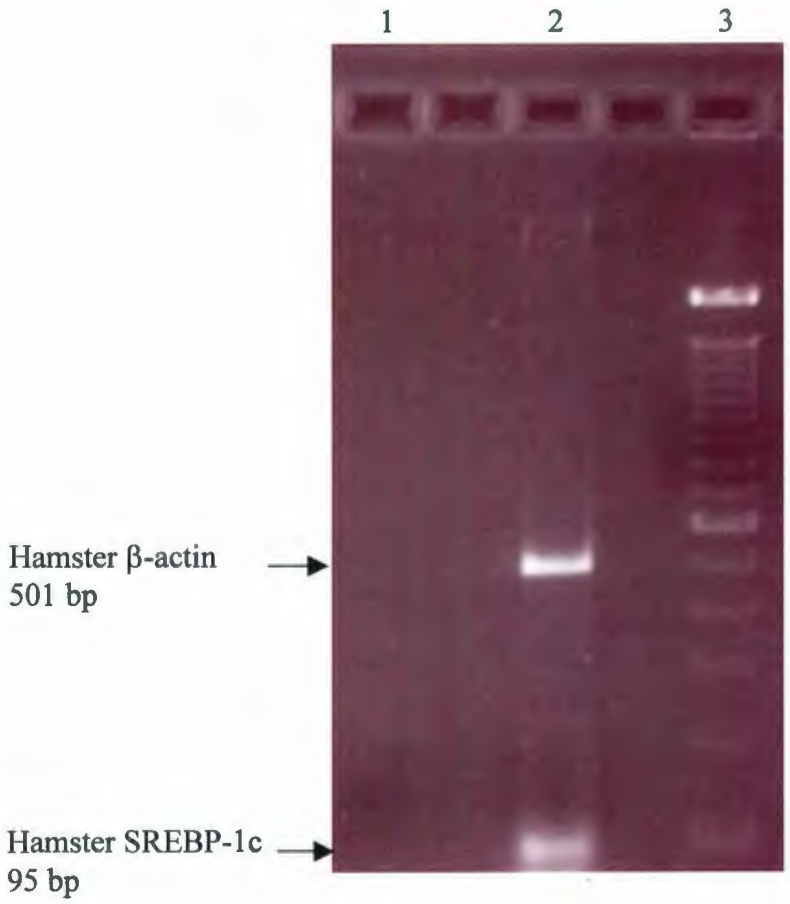


Figure 2.2: RT-PCR of SREBP-1c and β-actin. Hepatic mRNA from high fat fed BioF1B hamsters was reverse transcribed and multiplex PCR was performed to simultaneously amplify SREBP-1c and β-actin (described under the methods section). Lane1 depicts amplification in the absence of reverse transcriptase, lane2 shows the amplification of SREBP-1c and β-actin in the presence of reverse transcriptase and lane3 shows the 100bp ladder (molecular marker).

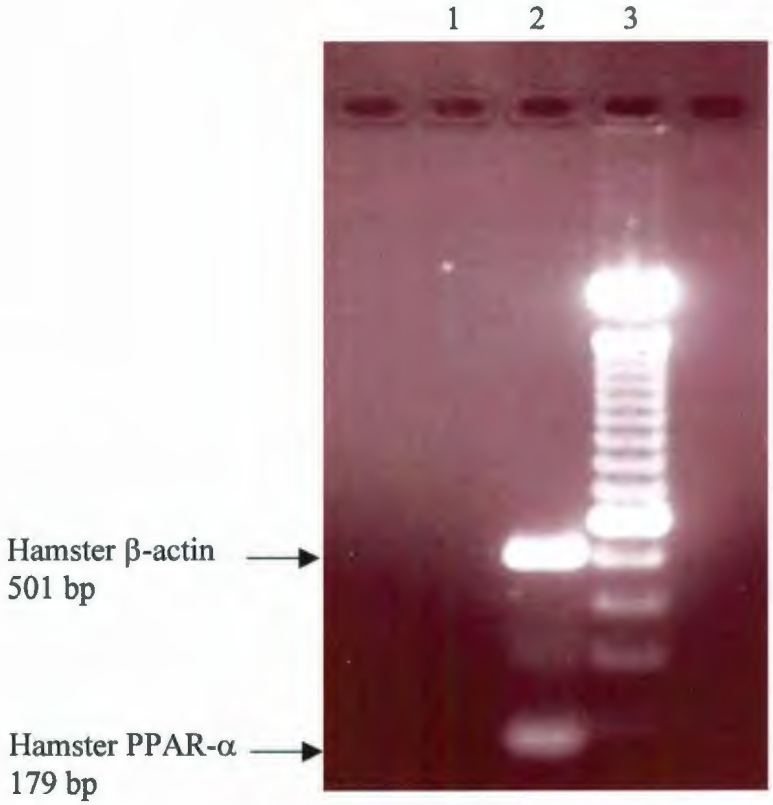


Figure 2.3: RT-PCR of PPAR-α and β-actin. Hepatic mRNA from high fat fed BioF1B hamsters was reverse transcribed and multiplex PCR was performed to simultaneously amplify PPAR-α and β-actin (described under the methods section). Lane1 depicts amplification in the absence of reverse transcriptase, lane 2 shows the amplification of PPAR-α and β-actin in the presence of reverse transcriptase and lane 3 shows the 100bp ladder (molecular marker).

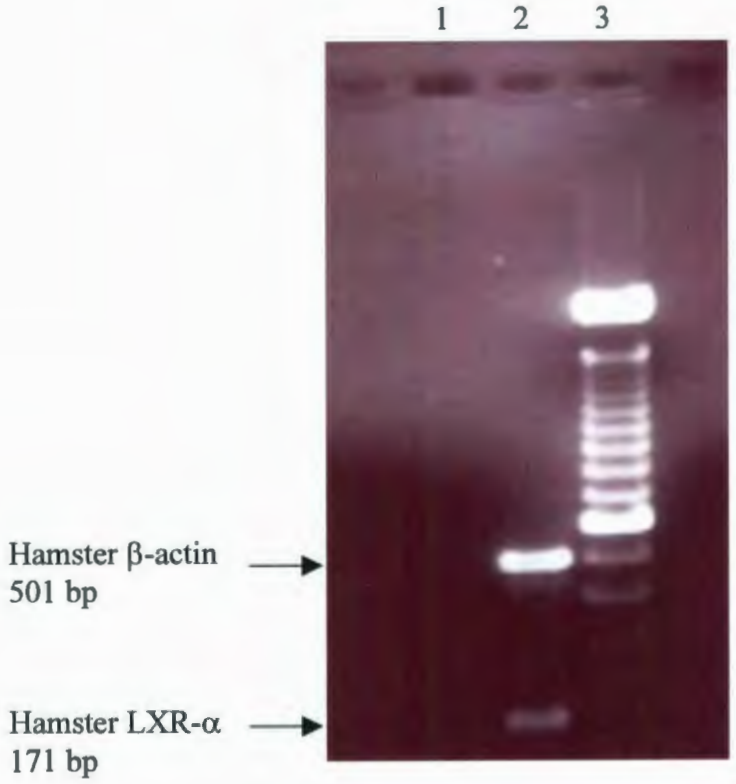


Figure 2.4: RT-PCR of LXR-α and β-actin. Hepatic mRNA from high fat fed BioF1B hamsters was reverse transcribed and multiplex PCR was performed to simultaneously amplify LXR-α and β-actin (described under the methods section). Lane1 depicts amplification in the absence of reverse transcriptase, lane 2 shows the amplification of LXR-α and β-actin in the presence of reverse transcriptase and lane 3 shows the 100bp ladder (molecular marker).

Chapter-3: Results

3.1 Effect of fish oil and seal oil

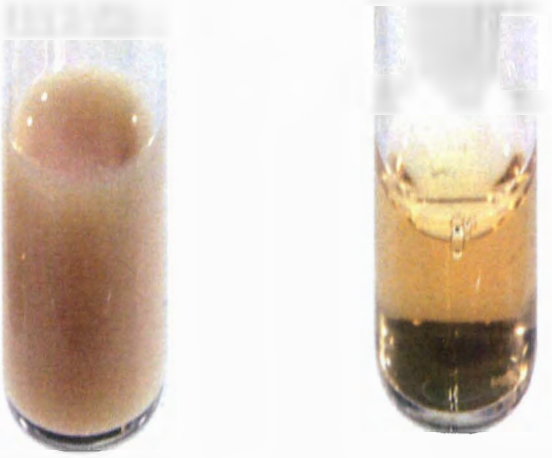
3.1.1 Body weight and diet consumption

At the end of 4-week dietary treatment, body weight gain (g) for BioF1B hamsters fed either fish oil (19.18±3.58) or seal oil (17.07±3.68) did not reveal significant differences. The average daily food consumption (g/day) of fish oil fed hamsters was also not significantly different as compared to hamsters fed seal oil (5.41 ± 0.21 and 6.05 ± 0.56 respectively).

3.1.2 Plasma and lipoprotein lipid profile

Plasma samples from BioF1B hamsters fed fish oil showed milkiness due to the presence of CM like particles. Milky plasma was however, not seen in seal oil fed BioF1B hamsters (Figure 3.1).

TG concentrations in whole plasma and different lipoprotein fractions in fish oil and seal oil fed BioF1B hamsters are given in Figure 3.2 and Figure 3.3 respectively. Whole plasma TG concentrations in fish oil fed hamsters were significantly higher compared to seal oil fed hamsters ($p < 0.0001$). Significantly higher levels of TG were also noted in different lipoprotein fractions from fish oil fed hamsters compared to seal oil fed hamsters ($p < 0.0001$ for VLDL and LDL; $p = 0.002$ for HDL fractions). BioF1B hamsters fed fish oil also showed significantly higher whole plasma total cholesterol levels ($p < 0.0001$) as compared to the seal oil fed animals (Figure 3.4). Analysis of different lipoprotein fractions for total cholesterol concentration showed a similar pattern where fish oil fed BioF1B hamsters revealed significantly higher concentrations of total



Fish Oil Seal Oil

Figure 3.1: Fish oil fed BioF1B hamsters showed milky plasma containing chylomicron like particles. BioF1B hamsters were fed 20% (w/w) fish oil or 20% (w/w) seal oil diets for 4 weeks as described under the methods section. Blood was collected after 14 hours of fasting and plasma was separated.

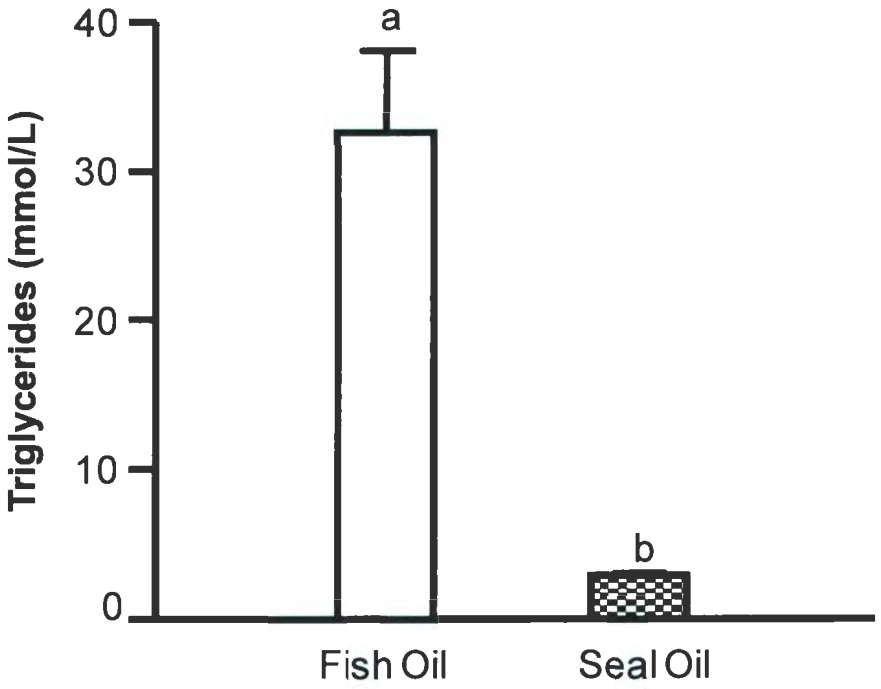


Figure 3.2 Whole plasma triglyceride concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were assayed for triglycerides as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

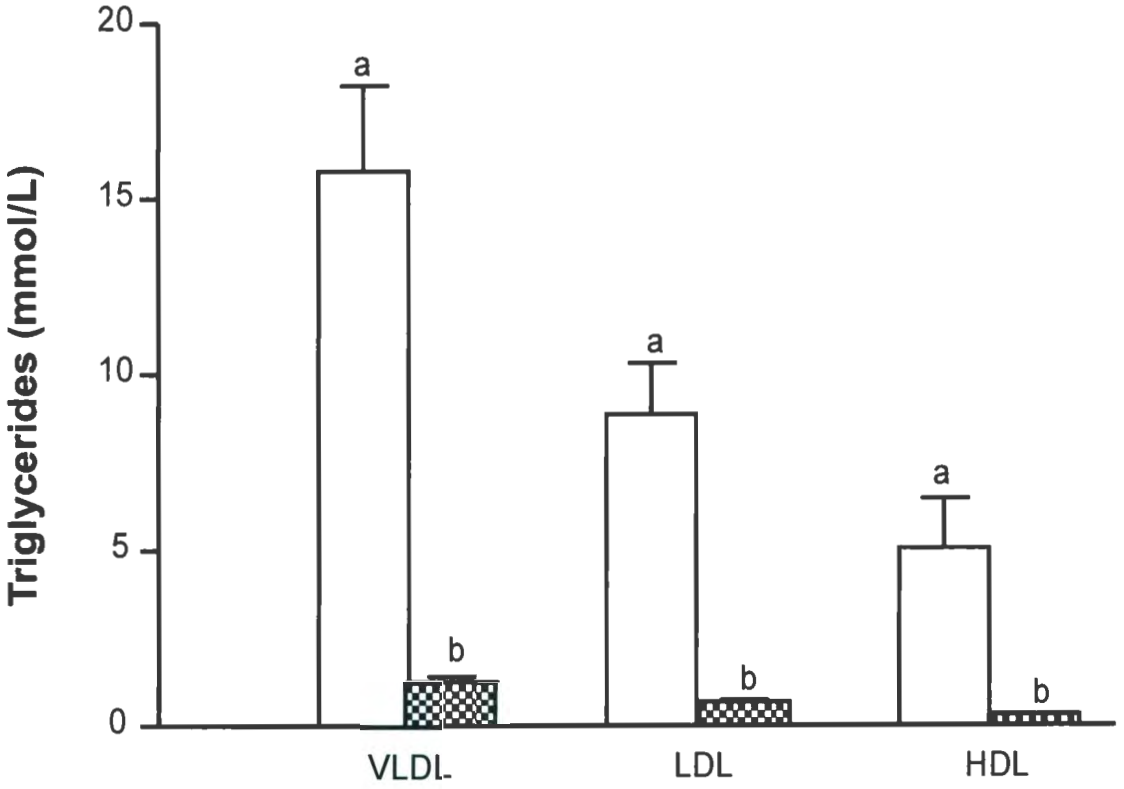


Figure 3.3 Triglyceride concentrations in lipoprotein fractions of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were collected; lipoprotein fractions were separated by density gradient centrifugation and assayed for triglycerides as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

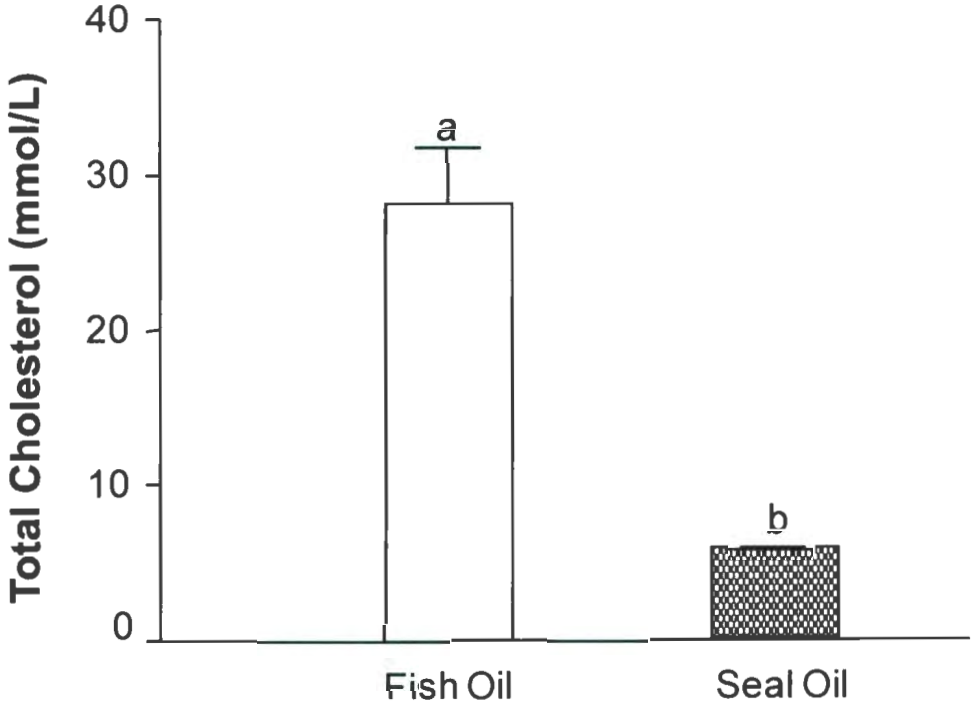


Figure 3.4 Whole plasma total cholesterol concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were assayed for total cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired *t*-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

cholesterol ($p < 0.0001$ for VLDL and LDL; $p = 0.005$ for HDL) as compared to seal oil fed animals (Figure 3.5).

Plasma cholesterol esters and free cholesterol concentrations in fish oil and seal oil fed BioF1B hamsters also followed a similar trend as the TG and total cholesterol concentrations. Whole plasma from fish oil fed BioF1B hamsters showed significantly higher levels of cholesterol esters ($p < 0.0001$) and free cholesterol ($p = 0.0002$) compared to seal oil fed hamsters (Figure 3.6 and Figure 3.8 respectively). Moreover, concentrations of cholesterol esters ($p < 0.0001$ for VLDL and LDL; $p = 0.005$ for HDL) (Figure 3.7) and free cholesterol ($p = 0.0003$ for VLDL; $p < 0.001$ for LDL; $p = 0.003$ for HDL) (Figure 3.9) were significantly higher in different lipoprotein fractions from fish oil fed BioF1B hamsters.

3.1.3 Hepatic lipids of hamsters fed fish oil and seal oil

Hepatic TG concentrations are shown in Figure 3.10. Fish oil feeding resulted in significantly higher TG concentrations as compared to seal oil feeding ($p = 0.004$). Hepatic total cholesterol, cholesterol esters and free cholesterol concentrations were also significantly different in response to different type of fat in the hamster diet (Figure 3.11 – Figure 3.13). For all three lipid parameters, fish oil fed BioF1B hamsters had significantly higher concentrations ($p < 0.0001$) for total cholesterol (Figure 3.11), cholesterol esters (Figure 3.12) and free cholesterol (Figure 3.13). While hepatic total cholesterol and cholesterol ester concentrations were about 50% higher in fish oil fed

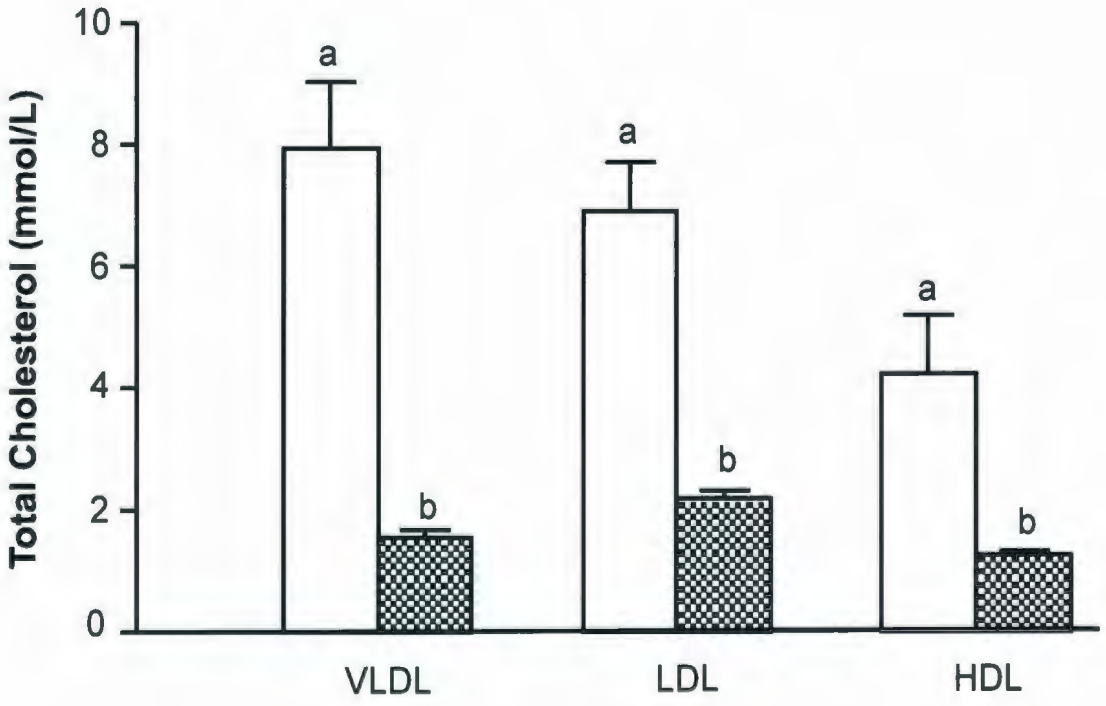


Figure 3.5 Total cholesterol concentrations in lipoprotein fractions of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were collected; lipoprotein fractions were separated by density gradient centrifugation and assayed for total cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

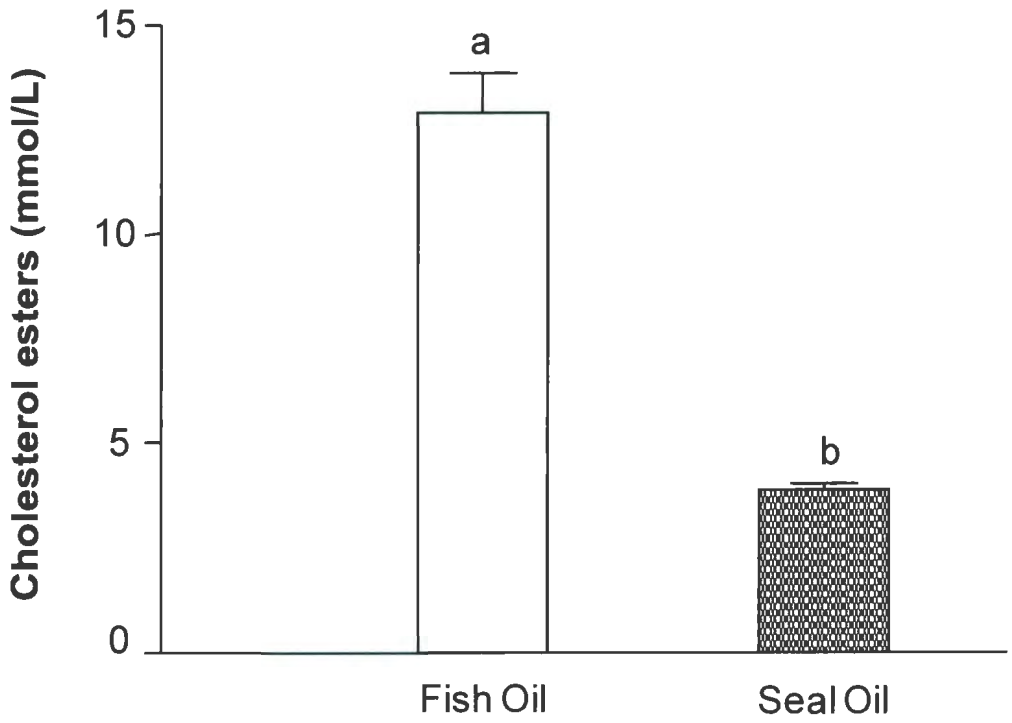


Figure 3.6 Whole plasma cholesterol ester concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were assayed for cholesterol esters as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

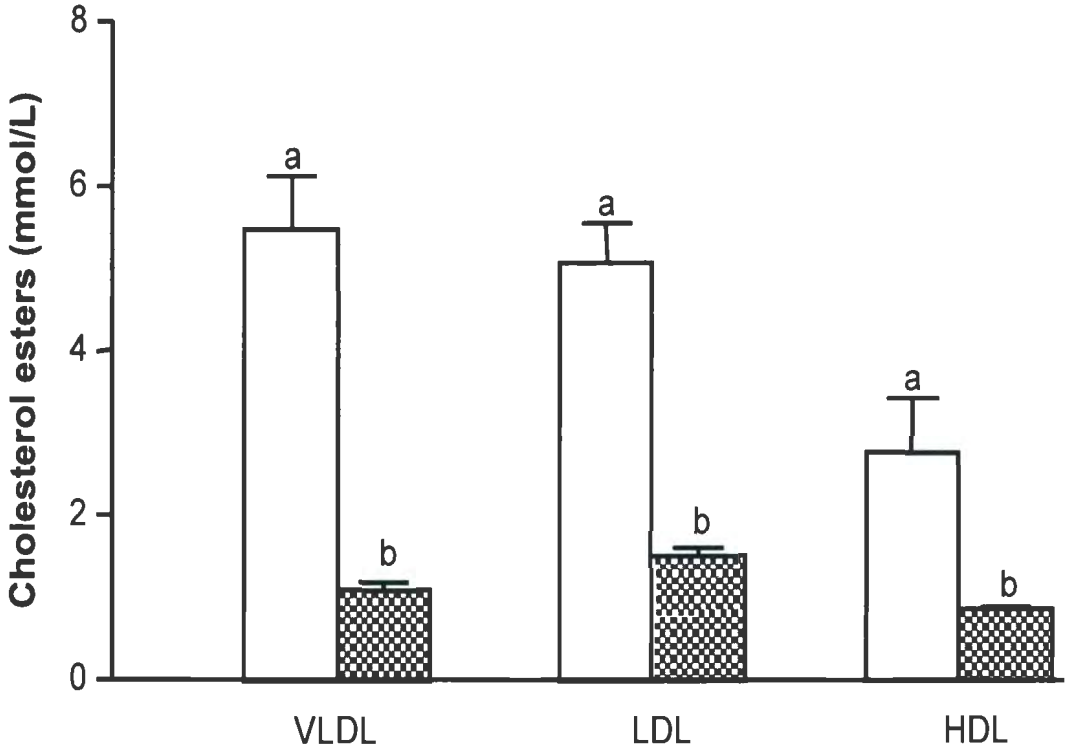


Figure 3.7 Cholesterol ester concentrations in lipoprotein fractions of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were collected; lipoprotein fractions were separated by density gradient centrifugation and assayed for cholesterol esters as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

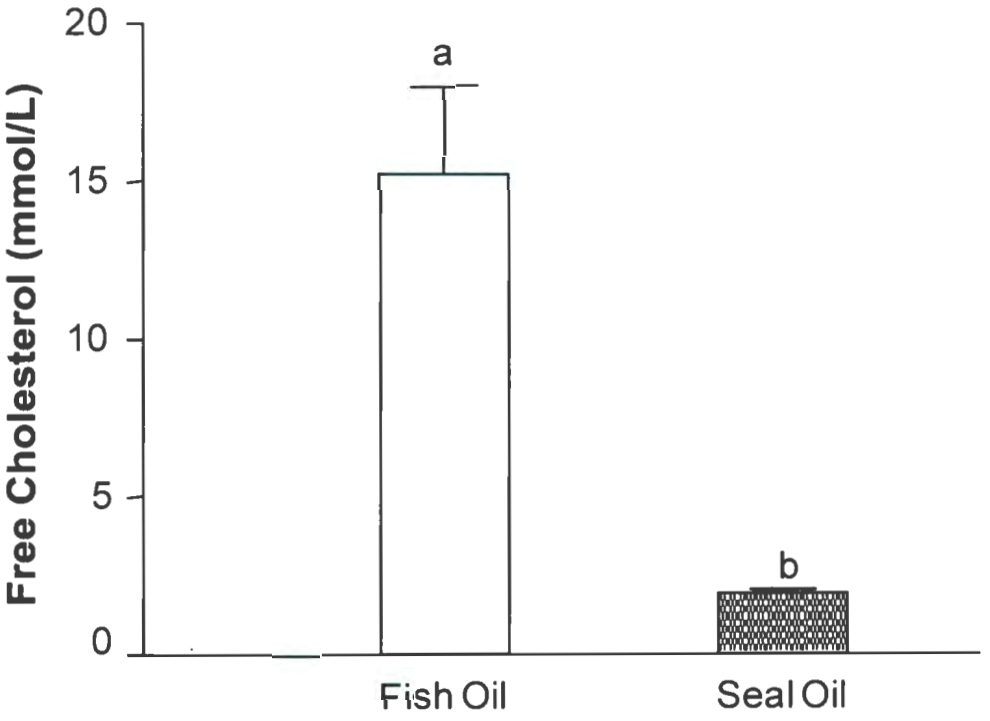


Figure 3.8 Whole plasma free cholesterol concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were assayed for free cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

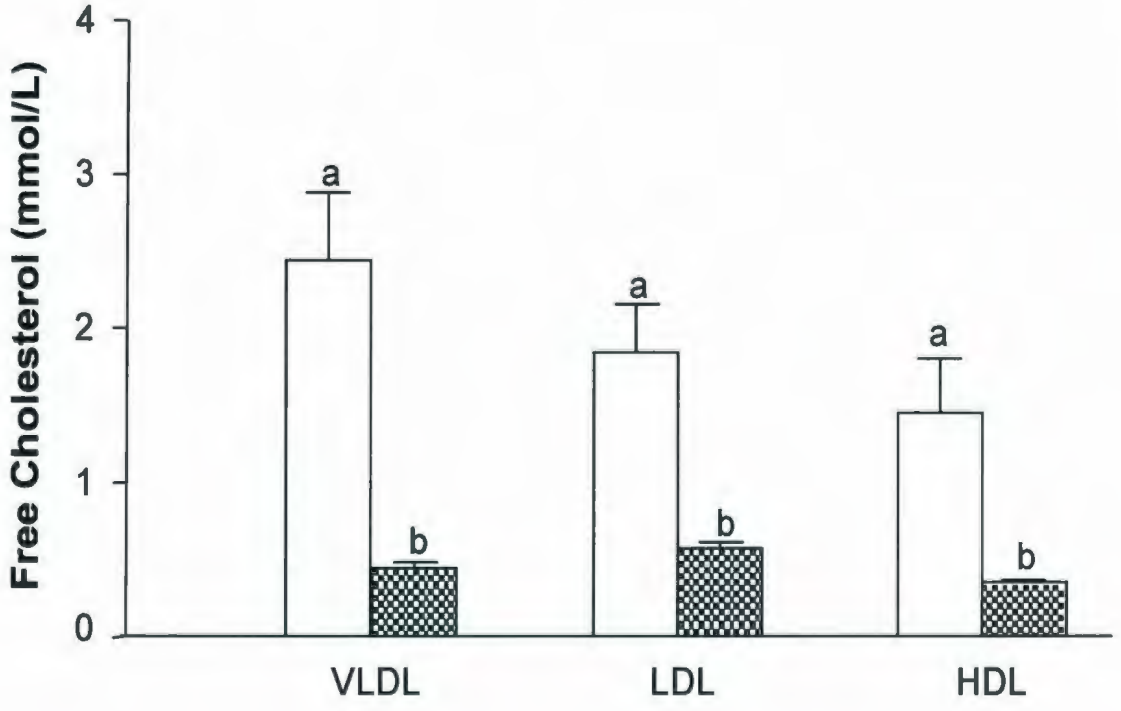


Figure 3.9 Free cholesterol concentrations in lipoprotein fractions of BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Fasting plasma samples were collected; lipoprotein fractions were separated by density gradient centrifugation and assayed for free cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

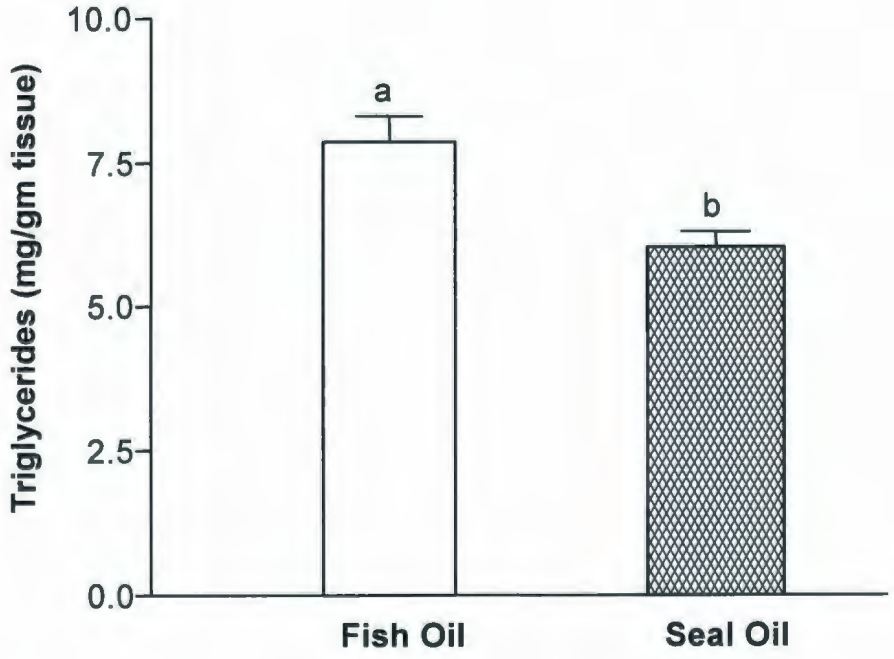


Figure 3.10: Hepatic triglyceride concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Liver lipids were isolated and assayed for triglycerides as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

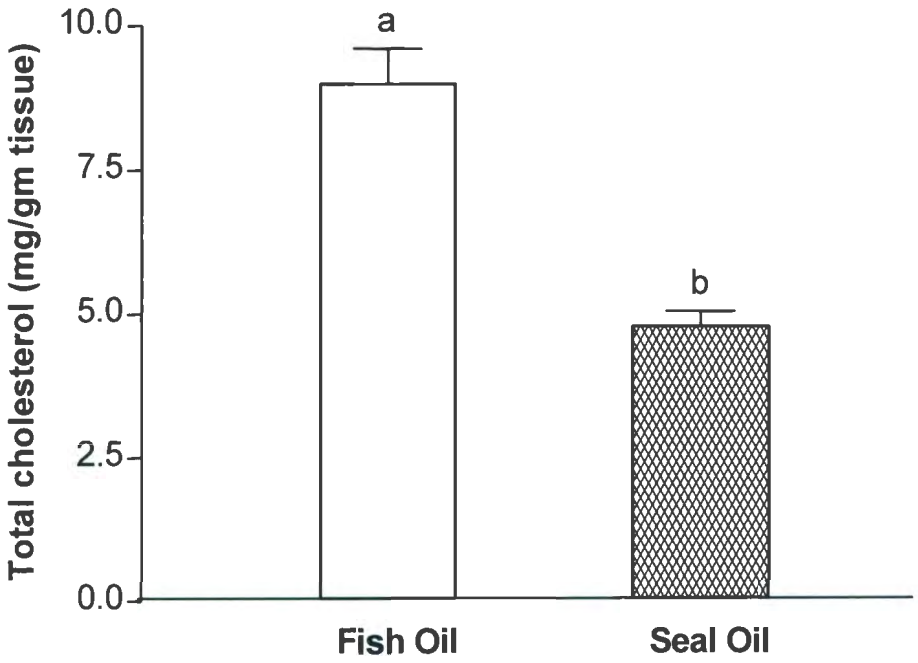


Figure 3.11: Hepatic total cholesterol concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Liver lipids were isolated and assayed for total cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

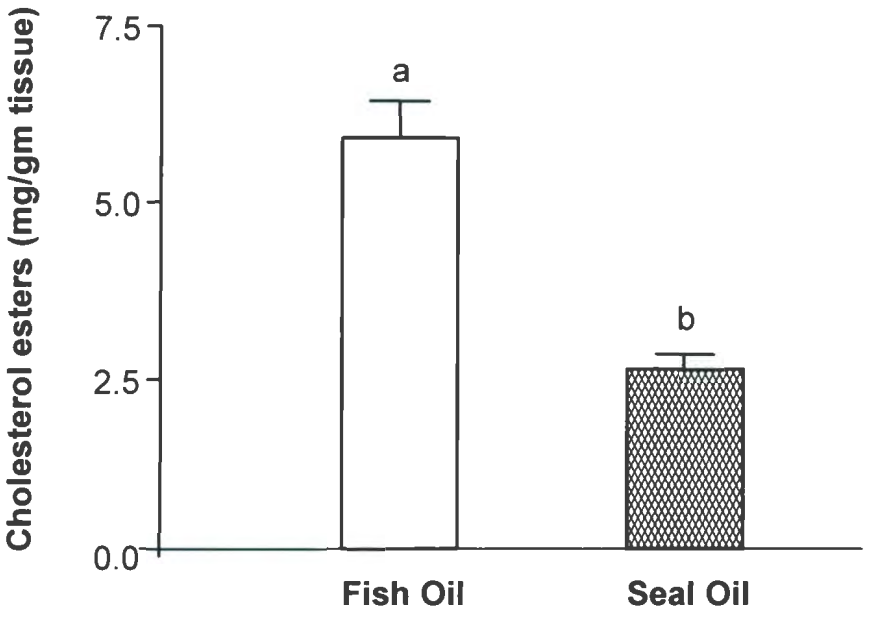


Figure 3.12: Hepatic cholesterol ester concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Liver lipids were isolated and assayed for cholesterol esters as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

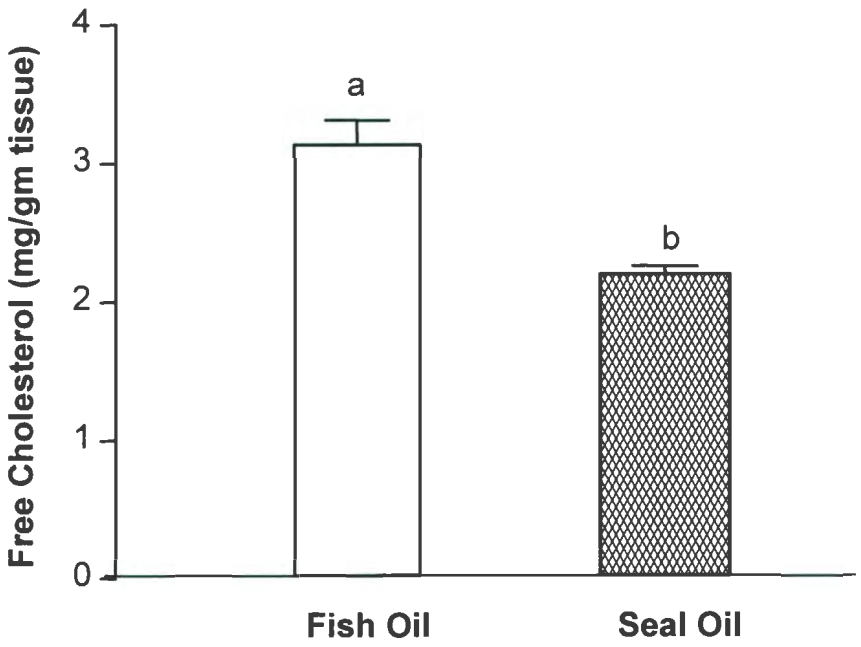


Figure 3.13: Hepatic free cholesterol concentrations of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Liver lipids were isolated and assayed for free cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

hamsters compared to hamsters on seal oil diet, relatively smaller differences (25%) were observed for liver TG and liver free cholesterol.

3.1.4 Hepatic mRNA expression of various transcription factors

The hepatic mRNA expression of SREBP-1c, SREBP-2, PPAR-α, and LXR-α was measured from hamsters fed fish oil and seal oil diets. SREBP-1c mRNA expression was significantly elevated in fish oil fed hamsters ($p= 0.04$) compared to seal oil fed hamsters (Figure 3.14). Expression levels of SREBP-2 mRNA showed a trend towards an increase in fish oil fed hamsters compared to seal oil fed hamsters, however, this was not statistically significant ($p= 0.08$) (Figure 3.15). There was no significant effect of fish oil or seal oil on the mRNA levels of PPAR-α and LXR-α (Figure 3.16 and Figure 3.17 respectively).

3.1.5 Plasma and liver TBARS of BioF1B hamsters

The extent of lipid peroxidation in fish oil and seal oil fed hamsters was evaluated using TBARS assay. Hamsters fed fish oil showed significantly higher levels of plasma lipid peroxides ($p< 0.001$) compared to seal oil fed hamsters (Figure 3.18). Liver TBARS also showed significant effect of diet (Figure 3.19), where fish oil fed hamsters had significantly elevated levels of liver TBARS compared to seal oil fed hamsters ($p< 0.01$).

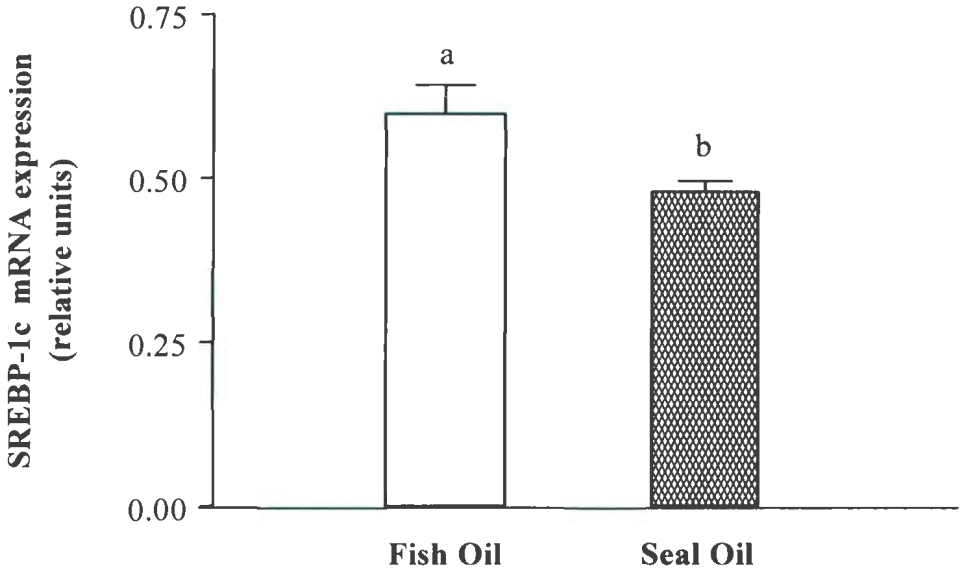


Figure 3.14: Hepatic SREBP-1c mRNA expression of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Total liver RNA was extracted, reverse transcribed and the cDNA template for SREBP-1c and β-actin was amplified as described in the methods section. SREBP-1c mRNA expression was normalized against β-actin mRNA expression and expressed as relative units. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

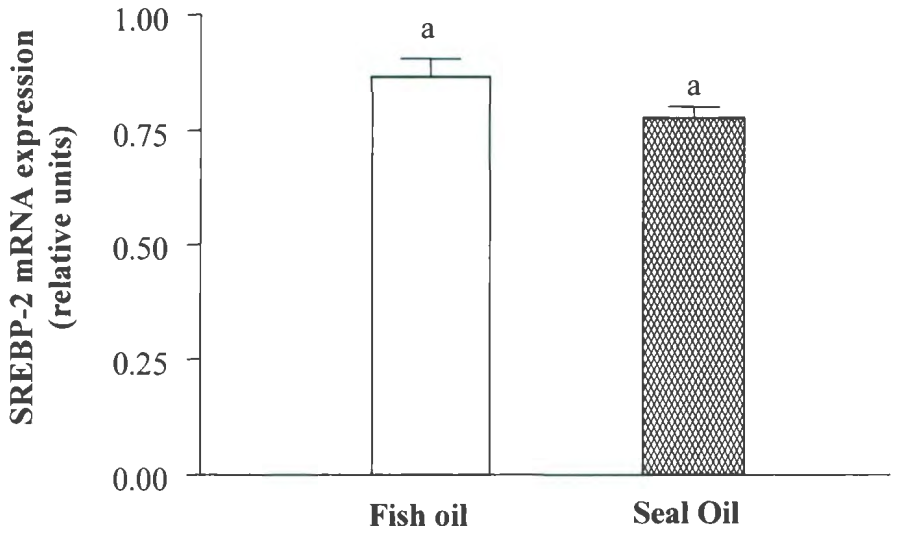


Figure 3.15: Hepatic SREBP-2 mRNA expression of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Total liver RNA was extracted, reverse transcribed and the cDNA template for SREBP-2 and β-actin was amplified as described in the methods section. The SREBP-2 mRNA expression was normalized against β-actin mRNA expression and expressed as relative units. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

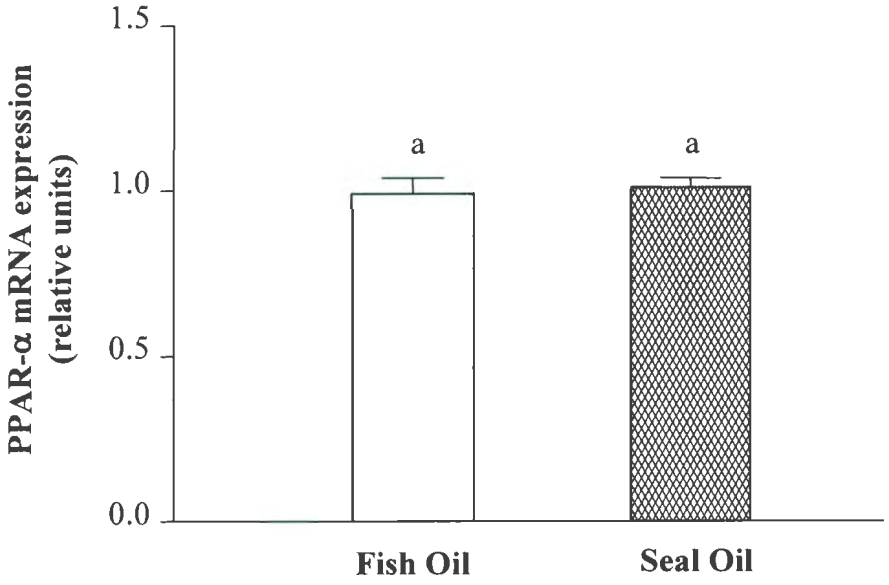


Figure 3.16: Hepatic PPAR-α mRNA expression of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Total liver RNA was extracted, reverse transcribed and the cDNA template for PPAR-α and β-actin was amplified as described in the methods section. The PPAR-α mRNA expression was normalized against β-actin mRNA expression and expressed as relative units. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

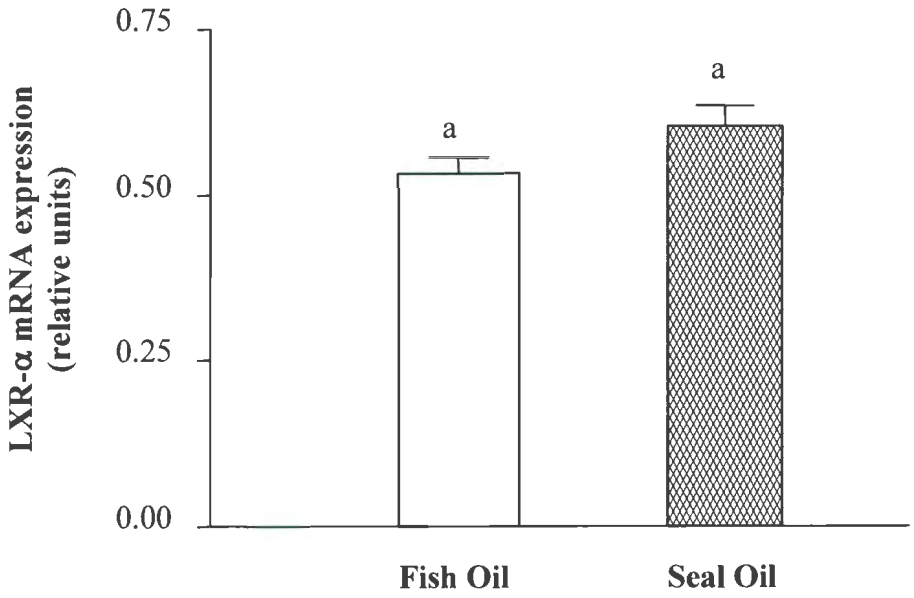


Figure 3.17: Hepatic LXR-α mRNA expression of BioF1B hamsters fed either fish oil (open) or seal oil (shaded), for a period of 4 weeks. Total liver RNA was extracted, reverse transcribed and the cDNA template for LXR-α and β-actin was amplified as described in the methods section. The LXR-α mRNA expression was normalized against β-actin mRNA expression and expressed as relative units. Values given are means ± SEM (n=6) analyzed by unpaired t-test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

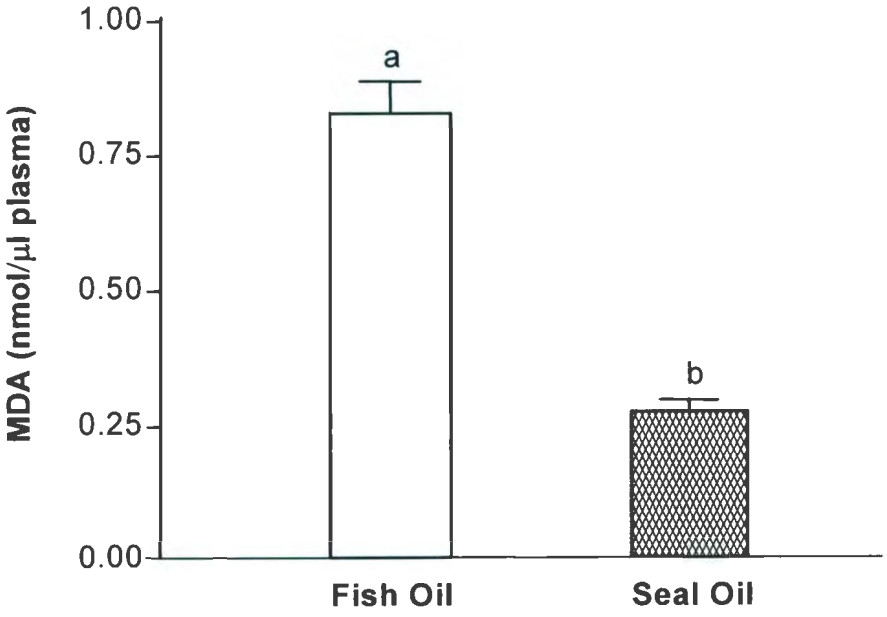


Figure 3.18: Plasma TBARS levels in BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Plasma samples were assayed for TBARS as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired-t test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

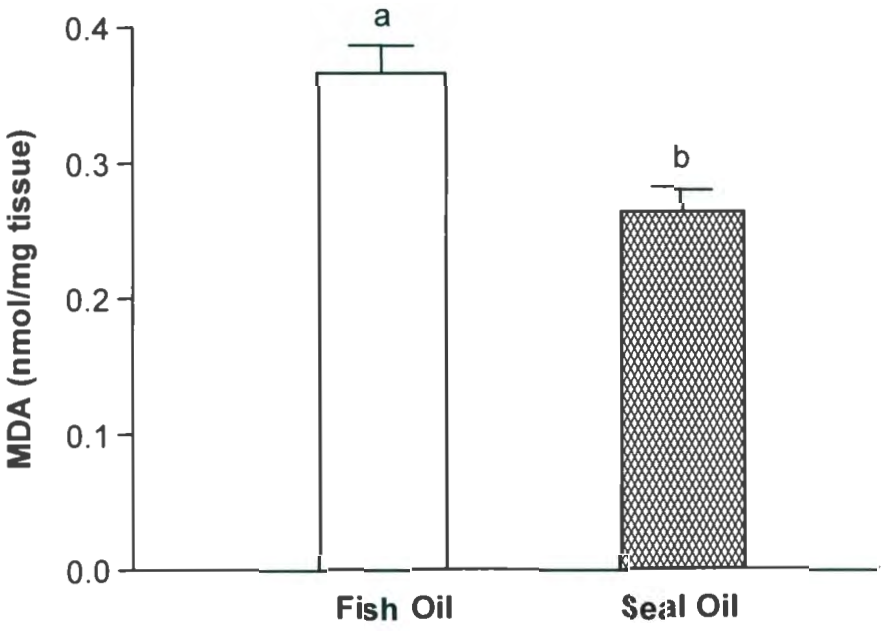


Figure 3.19: Hepatic TBARS levels in BioF1B hamsters fed either fish oil (open) or seal oil (shaded) for a period of 4 weeks. Liver samples were assayed for TBARS as described in the methods section. Values given are means ± SEM (n=6) analyzed by unpaired-t test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

Table 3.1 Body weight gain after 4-weeks of dietary treatments in Bio F1B hamsters fed either fish oil or seal oil alone or in combination with elderberry extract.

Diet Type	Weight gain (g)
Fish Oil	19.18±3.58 [a]
Fish Oil & Elderberry Extract	13.56±3.46 [b]
Seal Oil	15.2±2.21 [a]
Seal Oil & Elderberry Extract	10.32±1.56 [c]

Values are given as mean ± SEM (n=6)
Dietary groups were analyzed by 2- way ANOVA and the Newman-Keuls post-hoc test
Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

3.2 Effect of a combination of marine oils and elderberry extract in BioF1B hamsters

3.2.1 Body weight and diet consumption

The analysis of weight gain at the end of 4-week period revealed no significant differences in hamsters fed fish oil and seal oil diet alone. However, these high fat diets when cosupplemented with elderberry extract resulted in significantly lower body weight gain in hamsters fed the combination diet compared to hamsters on high fat diet alone (Table 3.1). While cosupplementation with elderberry extract in fish oil fed hamsters resulted in a 29% decrease in body weight gain as compared to hamsters fed fish oil diet alone ($p= 0.04$), a 32% decrease in weight gain was observed for hamsters fed a combination of seal oil and elderberry extract than animals fed seal oil alone ($p= 0.004$). Addition of elderberry extract to high fat diets however, did not result in significant differences in the average daily food intake (g/day) of BioF1B hamsters (fish oil: 5.41 $\pm$ 0.21; fish oil + elderberry extract: 5.30 $\pm$ 0.15; seal oil: 6.05 $\pm$ 0.56; seal oil + elderberry extract: 5.75 $\pm$ 0.64).

3.2.2 Plasma lipids in BioF1B hamsters fed a combination of marine oils and elderberry extract

Fasting plasma samples from hamsters fed fish oil and seal oil alone and those fed a combination of marine oils and elderberry extract were analyzed for TG (Figure 3.20), total cholesterol (Figure 3.21), cholesterol esters (Figure 3.22) and free cholesterol (Figure 3.23) concentrations. All lipid parameters showed significant differences when

elderberry extract was added. Two-way ANOVA revealed a significant effect of marine oils ($p< 0.0001$) as well as elderberry extract ($p< 0.0001$) on plasma TG, total cholesterol, cholesterol esters and free cholesterol. One-way ANOVA was also performed, as there was a significant interaction between marine oils and elderberry extract ($p< 0.0001$).

While fish oil fed BioF1B hamsters showed significantly higher levels of plasma TG compared to seal oil fed hamsters, addition of elderberry extract to fish oil diet resulted in a 90% decrease in the levels of plasma TG compared to BioF1B hamsters fed fish oil alone ($p< 0.001$) (Figure 3.20). These levels were comparable to plasma TG levels of BioF1B hamsters fed seal oil diet alone. Addition of elderberry extract to seal oil further led to a 70% decrease in plasma TG levels as compared to hamsters fed seal oil diet alone ($p< 0.0001$). There was a 80% decrease in plasma total cholesterol concentrations in hamsters fed a combination of fish oil and elderberry extract ($p = 0.0005$) (Figure 3.21) as compared to feeding fish oil alone. Total cholesterol levels of hamsters fed a combination of fish oil and elderberry extract were comparable to hamsters fed seal oil alone. Elderberry extract supplementation in the seal oil dietary group also showed a 70% lowering of total cholesterol levels as compared to seal oil fed hamsters ($p< 0.0001$).

Plasma cholesterol ester (Figure 3.22) and plasma free cholesterol (Figure 3.23) concentrations showed a similar trend to plasma TG and cholesterol concentrations. BioF1B hamsters fed a combination of fish oil and elderberry extract had significantly lower cholesterol ester ($p = 0.0002$) and free cholesterol ($p = 0.002$) concentrations compared to fish oil fed hamsters.

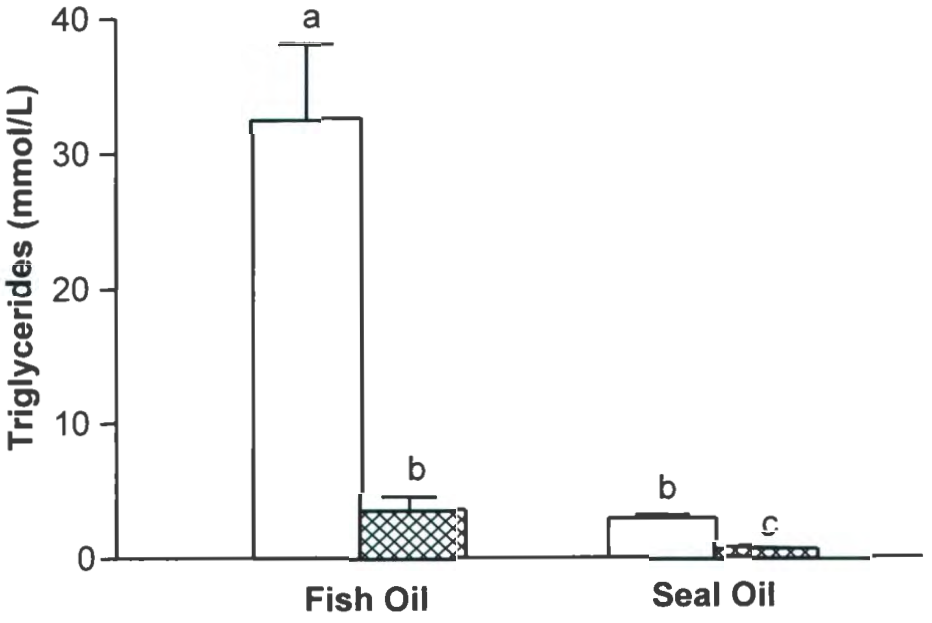


Figure 3.20: Plasma triglyceride concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Fasting plasma samples were collected and assayed for triglycerides as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman-Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

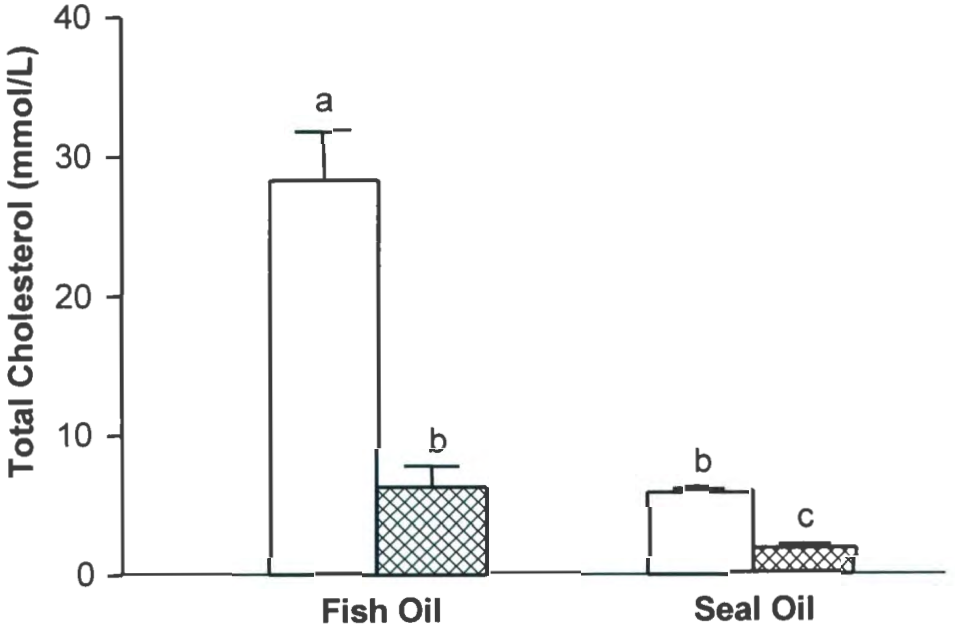


Figure 3.21: Plasma total cholesterol concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Fasting plasma samples were collected and assayed for total cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

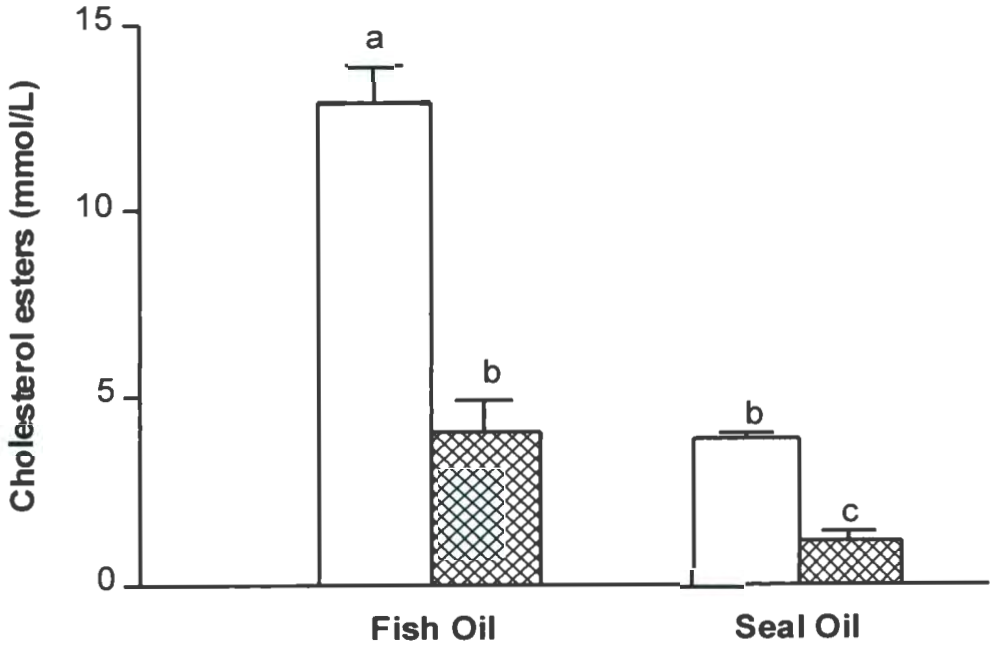


Figure 3.22: Plasma cholesterol ester concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Fasting plasma samples were collected and assayed for cholesterol esters as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

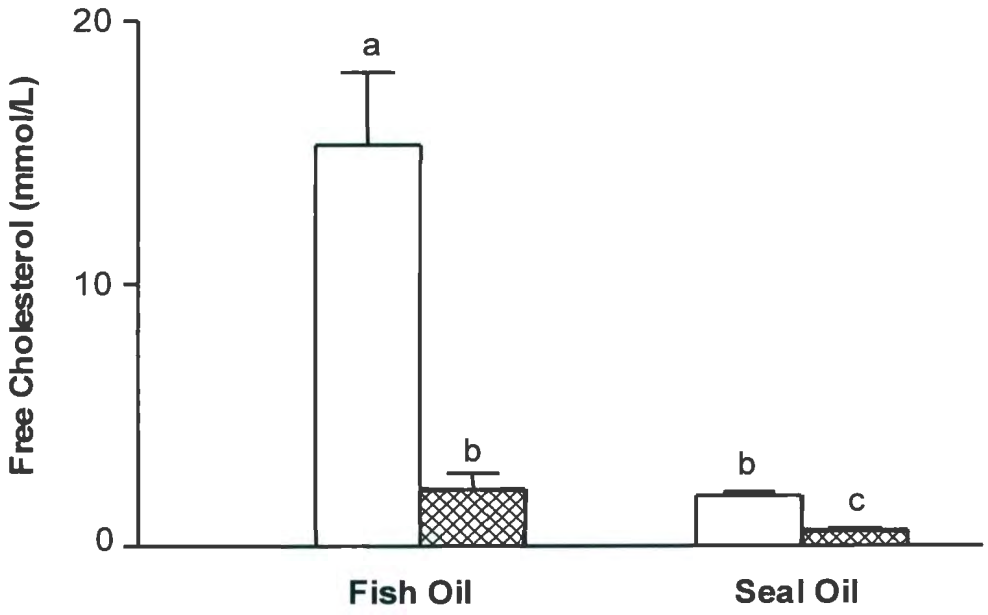


Figure 3.23: Plasma free cholesterol concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Fasting plasma samples were collected and assayed for free cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

The levels of cholesterol esters and free cholesterol of BioF1B hamsters fed a combination of fish oil and elderberry extract were similar to the levels observed for hamsters fed seal oil diet. Addition of elderberry extract to seal oil diet further reduced the levels of cholesterol esters ($p< 0.0001$) and free cholesterol ($p< 0.0001$) as compared to hamsters fed seal oil alone.

3.2.3 Liver lipids of hamsters fed a combination of marine oils and elderberry extract

Hepatic TG concentrations of hamsters fed either fish oil or seal oil alone or a combination of marine oils and elderberry extract are given in Figure 3.24. Two-way ANOVA revealed significant differences in different dietary groups in response to fat type ($p= 0.04$), elderberry extract ($p= 0.01$) and interaction between fat and elderberry extract (Fat*elderberry extract; $p= 0.01$). BioF1B hamsters fed a combination of fish oil and elderberry extract showed significantly lower TG concentrations ($p< 0.001$) as compared to hamsters fed fish oil alone. In contrast, the combination of seal oil and elderberry extract had no effect on hepatic TG levels as compared to hamsters fed seal oil alone.

Liver total cholesterol concentrations were significantly different in response to different marine oils and elderberry extract (Figure 3.25). Hepatic total cholesterol concentrations were significantly reduced when BioF1B hamsters were fed marine oils along with elderberry extract as compared to the respective fat diet alone ($p< 0.001$ for fish oil treated hamsters and $p< 0.05$ for the seal oil fed hamsters).

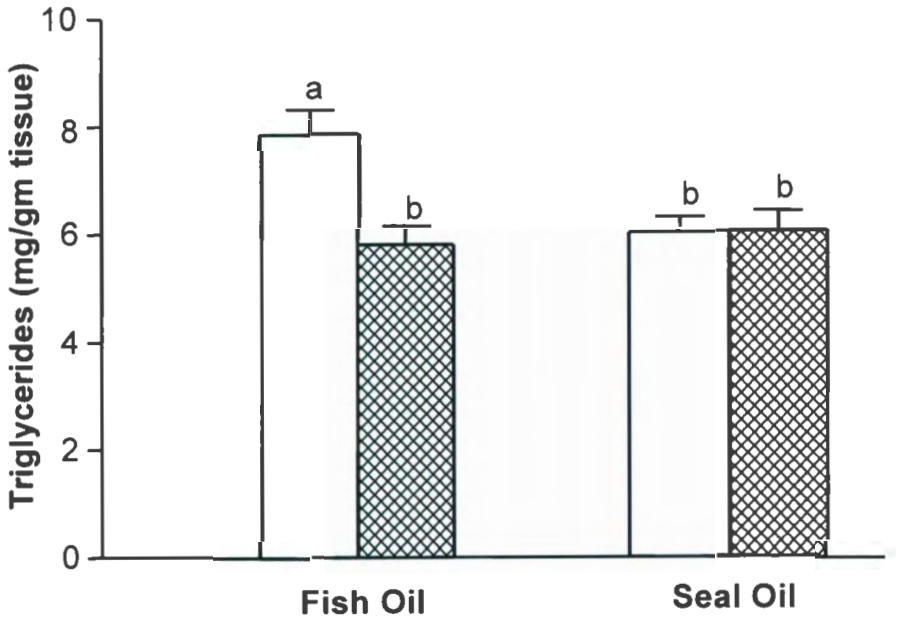


Figure 3.24: Hepatic triglyceride concentrations in BioF1B hamsters either fed fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Liver samples were assayed for triglycerides as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman-Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

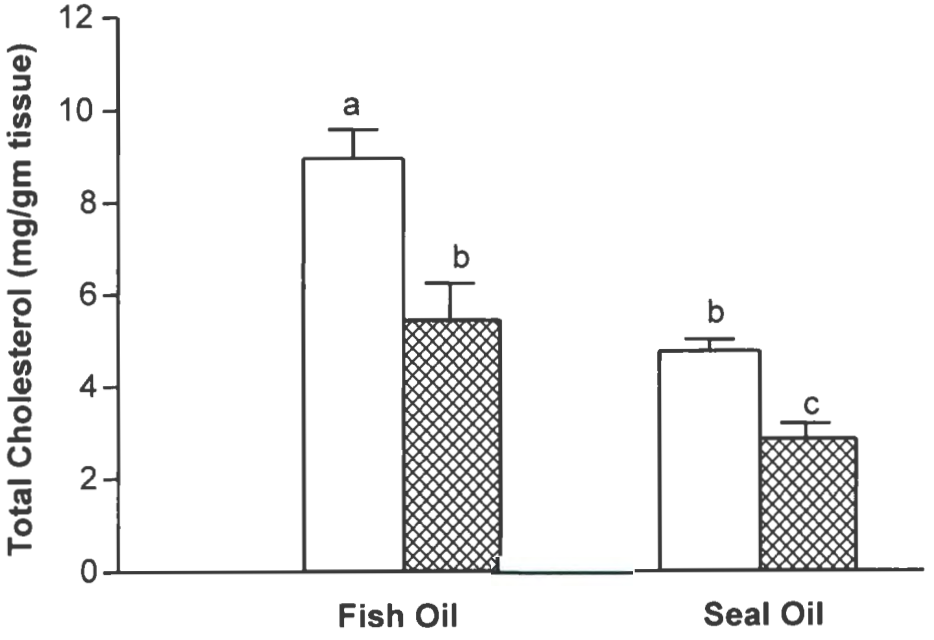


Figure 3.25: Hepatic total cholesterol concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Liver samples were assayed for total cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

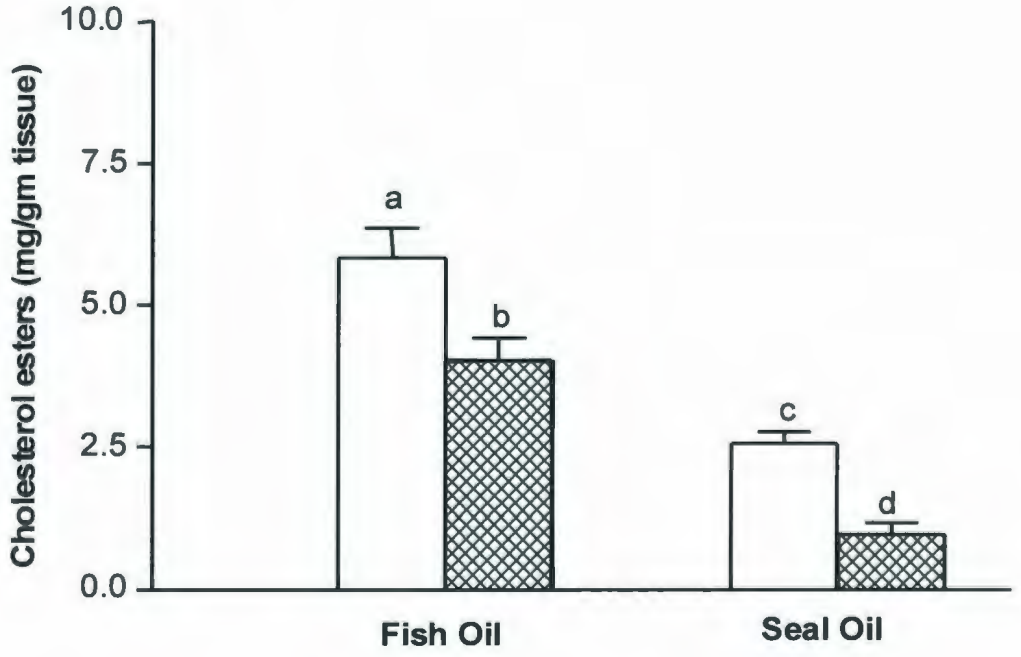


Figure 3.26: Hepatic cholesterol ester concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Liver samples were assayed for cholesterol esters as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

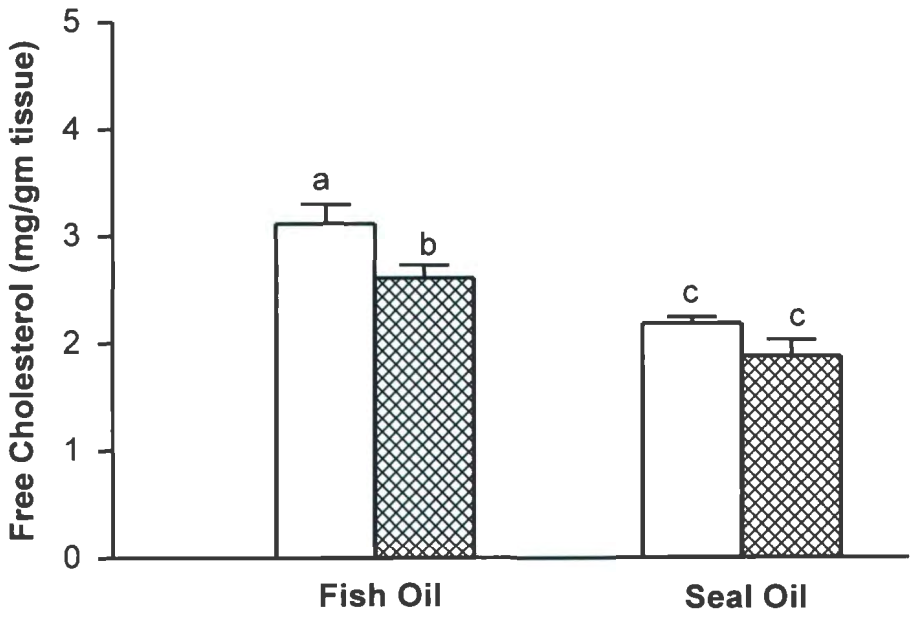


Figure 3.27: Hepatic free cholesterol concentrations in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Liver samples were assayed for free cholesterol as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

Analysis of hepatic cholesterol esters revealed significant differences in response to different dietary treatments (Figure 3.26). Similar to total cholesterol concentrations, cholesterol esters were also significantly reduced in BioF1B hamsters fed a combination of marine oils and elderberry extract as compared to hamsters fed the respective fat diet alone ($p< 0.001$ for fish oil and seal oil fed hamsters).

Hepatic free cholesterol levels were also influenced by treatment with marine oils ($p< 0.0001$) as well as elderberry extract ($p< 0.01$) (Figure 3.27). A combination of fish oil and elderberry extract caused a significant reduction in hepatic free cholesterol concentrations as compared to hamsters fed fish oil alone ($p= 0.04$). However, no significant difference was observed in the seal oil fed hamsters in the presence or absence of elderberry extract.

3.2.4 Plasma and liver TBARS of hamsters fed marine oils and elderberry extract

Figure 3.28 shows plasma TBARS levels to evaluate the extent of lipid peroxidation in response to different diets. Two-way ANOVA revealed a significant effect of fat and elderberry extract ($p< 0.0001$ and 0.001 respectively) on plasma lipid peroxide concentrations in hamsters fed different diets. Seal oil fed BioF1B hamsters had significantly lower levels of lipid peroxides ($p< 0.001$) compared to fish oil fed hamsters. A combination of fish oil and elderberry extract significantly reduced plasma TBARS as compared to hamsters fed fish oil alone ($p< 0.001$). In contrast to fish oil fed hamsters, a combination of seal oil and elderberry extract had no significant effect on plasma TBARS as compared to hamsters fed seal oil alone.

Liver TBARS from hamsters fed different marine oils in the absence or presence of elderberry extract are shown in Figure 3.29. Fish oil fed hamsters had significantly higher levels of liver TBARS as compared to seal oil fed hamsters ($p< 0.01$). Addition of elderberry extract to fish oil fed BioF1B hamsters significantly reduced liver TBARS as compared to hamsters fed fish oil alone. On the other hand, addition of elderberry extract to seal oil had no significant effect on liver TBARS as compared to seal oil alone fed hamsters.

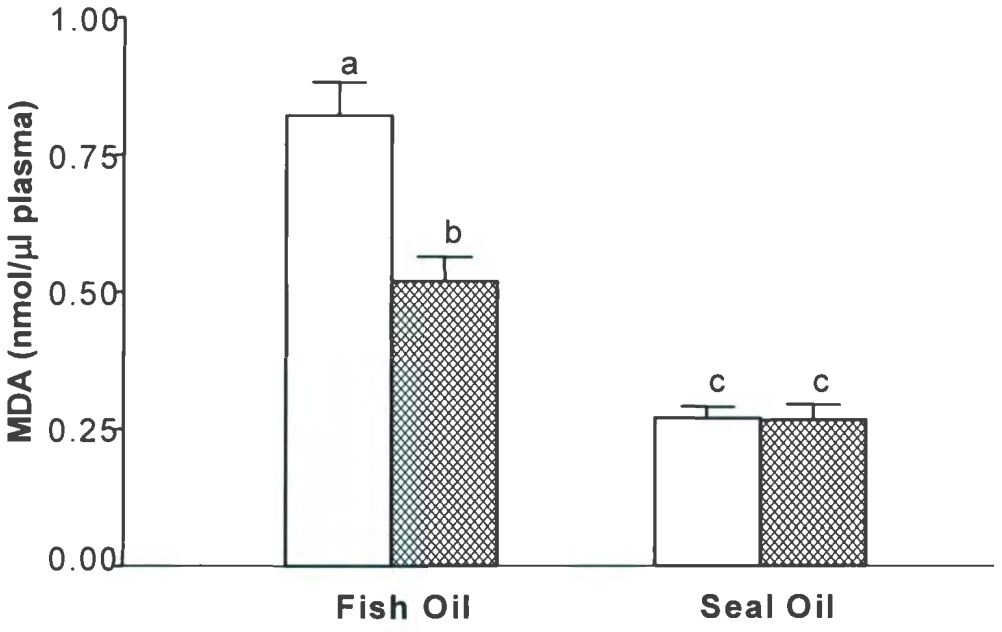


Figure 3.28: Plasma TBARS levels in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Plasma samples were assayed for TBARS as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

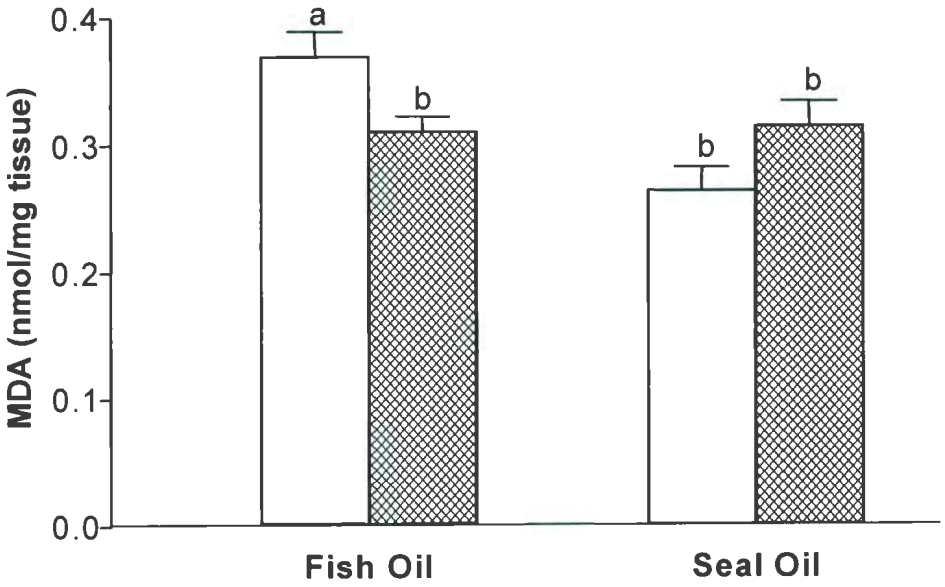


Figure 3.29: Hepatic TBARS levels in BioF1B hamsters fed either fish oil or seal oil in the presence (shaded) and absence (open) of elderberry extract for a period of 4 weeks. Liver samples were assayed for TBARS as described in the methods section. Values given are means ± SEM (n=6) analyzed by 2-way ANOVA and the Newman- Keuls post-hoc test. Mean values shown with different letters depict statistically significant differences ($p < 0.05$).

Chapter 4: Discussion

4.1 Effects of dietary fish oil in BioF1B hamsters

Previous studies from our laboratory have shown that high levels of fish oil cause hyperlipidemia in BioF1B hamsters (de Silva *et al.*, 2004). These hamsters showed milky plasma rich in CM like particles and significantly elevated plasma-, VLDL-, LDL-cholesterol and TG concentrations. While there is a possibility that fish oil induced hyperlipidemia could partly be contributed by VLDL fraction, however, CM appears to be the major candidate. Subsequent findings demonstrated that diet induced hyperlipidemia in BioF1B hamsters were specific to fish oil compared to a diet rich in MUFA or ω-6/ ω-3 diet (Cheema and Cornish, 2007). Presence of milky plasma, rich in CM like particles, in fish oil fed BioF1B hamsters indicated that fish oil feeding either affected the synthesis and secretion or clearance of TG-rich lipoproteins. Diet induced hyperlipidemia was partly explained by reduced post-heparin LPL activity and thus, appeared to have hindered the clearance of CM- like particles from circulation. For the present study, we hypothesized that BioF1B hamsters will be able to metabolize seal oil better than fish oil due to differences in fatty acid composition and the positional distribution of fatty acids in the TG molecule. We had previously established that fish oil feeding in BioF1B hamsters led to increased lipid peroxidation and compromised anti-oxidant status as compared to the other diets (Dubey and Cheema, unpublished data). Thus, we compared the effects of fish oil and seal oil on the regulation of lipid metabolism and oxidative stress in BioF1B hamsters.

4.2 Effect of dietary fish oil or seal oil on plasma lipids

4.2.1 Effect of dietary fish oil or seal oil on plasma TG

Feeding a high fat seal oil diet for 4 weeks to BioF1B hamsters did not cause milky plasma after an overnight fast (Figure 3.1). The whole plasma (Figure 3.2) and individual lipoprotein fractions (Figure 3.3) showed significantly lower TG concentrations in seal oil fed hamsters as compared to hamsters fed fish oil. Other studies have also reported significantly higher TG concentrations in fish oil fed rabbits and hamsters (Kristensen *et al.*, 1988; Hayes *et al.,* 1992; Kubow *et al.*, 1996). The absence of milky plasma and significantly lower plasma TG levels in BioF1B hamsters fed seal oil as compared to fish oil fed hamsters suggested differences in the clearance of TG rich lipoproteins. Hydrolysis of TG in circulating CM and VLDL to FFA and glycerol by LPL represents a rate-limiting step in the clearance of these TG-rich lipoproteins from the circulation. Reduced post-heparin LPL activity has been associated with an increased accumulation of CM in BioF1B hamsters fed a cholesterol supplemented diet (McAteer *et al.*, 2003). Significantly lower post-heparin LPL activity was observed in BioF1B hamsters compared to Golden Syrian hamsters which was further reduced by fish oil feeding (Cheema and Cornish, 2007). BioF1B hamsters also showed significantly reduced adipocyte LPL mRNA levels as compared to Golden Syrian hamsters indicating variations in genetic background between the two hamster strains. It appears that lower levels of LPL may be sufficient to clear lower density lipoproteins from seal oil fed hamsters as compared to fish oil fed hamsters, likely due to positional distribution of fatty acids in the triglyceride molecule.

Both fish oil and seal oil are rich sources of ω-3 PUFA with fundamental differences in the composition and intramolecular distribution of ω-3 PUFA. In addition to EPA and DHA found in fish oil, seal oil contains higher levels of DPA. Christensen *et al.* (1994) showed that while EPA and DHA are primarily located in *sn*-2 position in fish oil TG, these are distributed in *sn*-1 and *sn*-3 positions in seal oil TG. These structural differences may play an important role in the differential metabolism of these oils in the BioF1B hamsters. The pancreatic lipase and gastrointestinal lipase hydrolyze primary ester bonds in TG. While ω-3 PUFA from fish oil are primarily absorbed as *sn*-2 monoglycerols, these are absorbed as FFA upon seal oil consumption. It is suggested that differences in intramolecular distribution of ω-3 PUFA in the two marine oils can potentially alter their uptake and bioavailability. Previous studies have shown a faster clearance of seal oil CM compared to fish oil CM (Christensen *et al.*, 1995). Yoshida *et al.* (1999) have also demonstrated seal oil to be more effective than fish oil in reducing serum and liver TG levels. These findings are in support of our observations for the absence of CM like particle enriched plasma in seal oil fed BioF1B hamsters compared to fish oil fed hamsters.

4.2.2 Effect of dietary fish oil or seal oil on plasma cholesterol levels

In the present study, whole plasma total cholesterol (Figure 3.4), cholesterol esters (Figure 3.6) and free cholesterol (Figure 3.8) concentrations were significantly lower in seal oil fed BioF1B hamsters as compared to fish oil fed BioF1B hamsters. Additionally, significant reductions in total cholesterol, cholesterol esters and free cholesterol

concentrations were observed in the individual lipoprotein fractions namely VLDL, LDL and HDL, in BioF1B hamsters fed seal oil as compared to those fed fish oil.

While several studies on fish oil supplementation have reported reduced VLDL cholesterol levels, the effects of fish oil on plasma total and LDL-cholesterol concentrations are inconsistent. Though some studies show lower LDL-cholesterol levels in response to dietary fish oil, others reveal high plasma total- and LDL- cholesterol concentrations upon fish oil feeding (Nestel *et al.*, 1984; Sullivan *et al*, 1986; Hsu *et al.*, 2000; Rivellese *et al.*, 2003). Genetic susceptibility to dyslipidemia in certain populations can further result in elevated LDL-cholesterol levels upon fish oil supplementation. In fact, our laboratory has reported increased plasma VLDL and LDL- cholesterol concentrations in fish oil fed BioF1B hamsters as compared to hamsters fed a mixture of lard and safflower oil (1.5:1) (de Silva *et al.*, 2004). It was previously reported that both fish oil and seal oil significantly lowered plasma total cholesterol concentrations as compared to corn oil fed guinea pigs (Murphy *et al.*, 1999b). However, no significant differences in total cholesterol, LDL-cholesterol and HDL cholesterol were observed in fish oil and seal oil fed normocholesterolemic subjects as well as moderately hypercholesterolemic subjects (Vognild *et al.*, 1998; Conquer *et al.*, 1999; Brox *et al.*, 2001). Recently, a study on dietary response to ω-3 PUFA in hypertriglyceridemic volunteers revealed reductions of 7% and 14% in plasma triglyceride levels of fish oil and seal oil fed subjects respectively, compared to control group (Meyer *et al.*, 2009). Moreover, our findings show that dietary seal oil fed BioF1B hamsters had lower cholesterol concentrations as compared to fish oil fed BioF1B hamsters. These data

further suggest that genetic variability might be crucial in determining any differential response of ω-3 PUFA supplementation on lipid profile and other biological variables, based on the type of ω-3 PUFA fed.

Preliminary observations from our laboratory show that the CM fatty acid composition is different in fish oil fed and seal oil fed BioF1B hamsters (Banfield, 2008). Fatty acid analysis showed significantly higher EPA and DHA concentrations in CM from fish oil fed BioF1B hamsters compared to seal oil fed BioF1B hamsters. These findings suggest that the uptake and/or removal of these fatty acids might not be as efficient in fish oil fed BioF1B hamsters as compared to seal oil fed BioF1B hamsters. Fish oil feeding in BioF1B hamsters has also been shown to lower LPL activity compared to BioF1B hamsters fed MUFA and ω-6/ ω-3 diets (Cheema and Cornish, 2007). In addition to regulating energy distribution in the form of free fatty acids, LPL is also known to play an important role in lipoprotein metabolism by controlling the distribution of cholesterol to LDL and HDL (Tsutsumi, 2003). LPL-mediated hydrolysis of VLDL leads to the formation of LDL, which is considered to be an independent predictor of CVD. LPL is a rate-limiting enzyme in the formation of LDL particles; structural mutations in the LPL gene have revealed a genetic linkage to LDL particle size (Hokanson *et al.*, 1999). A clinical study reported by Ando *et al.* (2001) has shown genetic LPL deficiency to be associated with hypercholesterolemia due to increased VLDL and LDL levels. Over expression of LPL in transgenic Watanabe heritable hyperlipidemic rabbits significantly suppressed high fat diet induced hyperlipidemia, suggesting therapeutic potential of LPL genetic manipulation in human Familial

Hypercholesterolemia (Koike *et al.*, 2004). In our study, it appears that BioF1B hamsters with reduced LPL activity clear CM and VLDL more efficiently in response to dietary seal oil as compared to fish oil. Plasma LDL cholesterol levels have been negatively correlated with the rate of clearance of TG-rich lipoproteins from plasma (Chung *et al.*, 2004; Sposito *et al.*, 2002). This further supports our hypothesis that hindered TG-rich lipoprotein clearance in fish oil fed BioF1B hamsters contributes to elevated plasma LDL levels. However, to date there are no reports on the association of altered clearance of TG-rich lipoproteins and plasma cholesterol levels in seal oil and fish oil fed BioF1B hamsters as well as the molecular mechanisms involved. The possibility of fat malabsorption in differentially fed hamsters also needs to be explored. Thus, more elaborate studies to investigate the differential regulation of VLDL, LDL and HDL cholesterol levels in fish oil and seal oil fed BioF1B hamsters need to be carried out.

The differential regulation of lipoprotein metabolism by the two ω-3 rich PUFA sources in BioF1B hamsters could also involve LDL receptor, which is important in the clearance of lipoprotein cholesterol from the circulation. Theobald *et al.* (2004) reported elevated LDL cholesterol concentrations and reduced LDL receptor expression after DHA consumption (0.7g/day) in middle aged men and women for 3 months. Similarly, fish oil induced hyperlipidemia in BioF1B hamsters revealed significantly lower hepatic LDL-receptor mRNA levels as compared to ω-6/ ω-3 diet fed hamsters (de Silva *et al.*, 2004). It still remains to be explored whether ω-3 PUFA in seal oil exert any effect on LDL receptor gene expression and alter lipoprotein clearance.

Lecithin:cholesterol acyl transferase (LCAT) and CETP play a major role in "reverse cholesterol transport", a process which facilitates the removal of free cholesterol from extrahepatic tissues and transports cholesterol to liver. In the plasma, LCAT and CETP mediate the esterification of free cholesterol as well as reciprocal exchange of cholesterol esters and TG between cholesterol-rich lipoproteins (LDL and HDL) and TG-rich lipoproteins (CM and VLDL) (Chung *et al.*, 2004; Tall 1993; Glomset and Norum, 1973). Reduced CETP activity has been demonstrated in the plasma of patients with LPL deficiency (Bagdade *et al.*, 1996). Differential regulation of LCAT and CETP might partially account for differences in the rate of clearance of TG-rich lipoproteins as well as cholesterol levels in response to two marine oils in BioF1B hamsters. Thus, precise mechanisms involved in differential regulation of lipid metabolism by the two ω-3 PUFA rich marine oils, especially with regards to heterogeneity in genetic background need to be studied in detail.

4.2.3 Effect of dietary fish oil or seal oil on hepatic lipid profile

We observed significantly lower hepatic TG, total-cholesterol, cholesterol esters and free cholesterol levels in seal oil fed BioF1B hamsters as compared to fish oil fed BioF1B hamsters (Figure 3.10 - Figure 3.13). Elevated hepatic lipid levels have previously been shown from our laboratory in fish oil fed BioF1B hamsters (de Silva *et al.*, 2004, Cheema and Cornish, 2007). While all the hepatic lipid parameters were significantly lower in seal oil fed BioF1B hamsters as compared to the fish oil fed BioF1B hamsters, the differences for hepatic total- cholesterol and cholesterol esters were

more dramatic as compared to the hepatic TG and free cholesterol concentrations in BioF1B hamsters fed dietary seal oil. Seal oil fed BioF1B hamsters showed almost 50% lower hepatic total cholesterol and cholesterol ester concentrations, which suggests that seal oil specifically alters the regulation of metabolic pathways involved in cholesterol metabolism in the liver.

Liver is believed to be the most important organ in cholesterol regulation and the metabolic mechanisms that influence hepatic free cholesterol and cholesterol ester levels are critical in maintaining total body cholesterol homeostasis. Since cholesterol primarily contributes to the development of CVD by accumulating in the arterial plaques as cholesterol esters (Rudel and Shelness, 2000), it is important to study in detail the transport and storage of cholesterol in BioF1B hamsters fed marine oils. A key cholesterol esterification enzyme, acyl CoA: cholesterol acyltransferase 2 (ACAT-2) is expressed in the liver and intestine. Cholesterol esters generated by ACAT-2 in the intestine are incorporated into CM and are rapidly and selectively removed from the circulation by the liver (Klein and Rudel, 1983, Goodman, 1962). ACAT-2 further regulates hepatic cholesterol metabolism as well as the secretion and transport in plasma lipoproteins (Rudel and Shelness, 2000). ACAT also regulates biliary cholesterol secretion by influencing the availability of "metabolically active" free cholesterol for transport into bile (Turley and Dietschy, 1988). ACAT-2 deficient mice have been shown to be resistant to diet-induced hypercholesterolemia with reduced capacity to absorb cholesterol (Buhman *et al.*, 2000). These authors reported that disruption of cholesteryl ester formation in the intestine by ACAT-2 prevents cholesteryl ester accumulation in the

liver, within lipoprotein particles as well as in the bile. Since we observed drastic differences in the cholesterol levels of fish oil and seal oil fed BioF1B hamsters with more pronounced changes in total cholesterol and cholesteryl ester levels, it will be interesting to study any effects of different diets on ACAT expression and activity.

Contrary to the role of ACAT, cholesteryl ester hydrolase (CEH) hydrolyses cholesteryl esters to generate free cholesterol. When cellular free cholesterol levels are depressed, cytosolic CEH releases free cholesterol from the intracellular cholesteryl ester storage, thus contributing to maintaining a constant cellular free cholesterol pool (Ghosh and Grogan, 1989; Ghosh *et al.*, 1990; Lee and Carr, 2004). Regulation of CEH in the liver mainly occurs at the transcriptional level (Grogan *et al.*, 1991; Ghosh *et al.*, 1998). ACAT and CEH are likely to be coordinately regulated since they act together in a cyclic and opposite manner (Lee and Carr, 2004). It remains to be ascertained whether fish oil and seal oil differently regulate CEH expression and activity in BioF1B hamsters.

Interestingly, gas chromatography analysis of the two ω-3 PUFA rich diets revealed that the MUFA content in seal oil was more than double of that found in fish oil (22.2% in fish oil and 50.6% in seal oil). These observations lead us to believe that increased MUFA content of seal oil diet might partially account for significantly lower hepatic total cholesterol and cholesterol ester levels in seal oil fed BioF1B hamsters as compared to fish oil fed BioF1B hamsters, along with differential regulation of transcription factors as discussed in the following sections.

4.2.4 Effect of dietary fish oil or seal oil on mRNA expression of transcription factors

Dramatic effects of ω-3 PUFA rich marine oils, namely fish oil and seal oil, on plasma and liver lipid profile suggest that the two marine oils might be differentially regulating the molecular mechanisms involved in lipid and lipoprotein metabolism. Thus we investigated whether the differences in gene regulation by fish oil and seal oil were evident at the level of transcription factors and nuclear receptors, i.e. SREBP-1c, SREBP-2, LXR-α and PPAR-α.

4.2.4.1 Effect of dietary fish oil or seal oil on SREBP mRNA expression

Regulation of lipid metabolism involves SREBPs, helix-loop-helix transcription factors, which bind to the sterol regulatory elements in promoters of genes involved in lipogenesis and cholesterol metabolism (Sampath and Ntambi, 2005). While SREBP-1c preferentially activates lipogenesis, SREBP-2 preferentially enhances the transcription of genes involved in cholesterogenesis (Le Jossic-Corcos *et al.*, 2005). In our study, seal oil fed BioF1B hamsters showed significantly reduced hepatic SREBP-1c mRNA expression as compared to the fish oil fed BioF1B hamsters (Figure 3.14). The suppression of lipogenesis in seal oil fed BioF1B hamsters can be partially explained by reduced SREBP-1c mRNA levels. On the other hand, fish oil and seal oil fed hamsters did not show any significant differences in hepatic SREBP-2 mRNA levels (Figure 3.15), though there was a trend towards reduced SREBP-2 mRNA expression in seal oil fed BioF1B hamsters compared to the fish oil fed BioF1B hamsters.

Dietary ω-3 PUFA can exert an inhibitory effect on lipogenesis either by inhibition of SREBP maturation or repression of SREBP mRNA. In addition to regulation of SREBP-1 proteolytic degradation by ω-3 PUFA *in vivo* (Xu *et al.*, 1999b; Kim *et al.*, 1999), some studies have reported lower intracellular levels of SREBP-1c mRNA in response to dietary ω-3 PUFA (Kim *et al.*, 1999; Yahagi *et al.*, 1999). These suppressive effects of dietary fish oil on the expression of lipogenic enzymes at the level of SREBP-1 mRNA expression as well as proteolytic processing of mature SREBP-1 have been reported in rodent liver and human cell lines (Kim *et al.*, 1999; Hannah *et al.*, 2001). Similar to our findings with seal oil supplementation in BioF1B hamsters, EPA and DHA treatment in H4IIEC3 cells showed reduced levels of SREBP-1c mRNA expression, with no changes seen with SREBP-1a and SREBP-2 levels (Le Jossic-Corcos *et al.*, 2005).

Thus, lower SREBP-1c mRNA levels in seal oil fed BioF1B hamsters indicate a potential role of SREBP-1c in differential regulation of lipogenesis in response to the marine oils in BioF1B hamsters. However, it needs to be elucidated whether the regulation of SREBP-1c levels occurs at the level of mRNA synthesis or mRNA stability. Moreover, no significant differences in SREBP-2 mRNA expression were seen in fish oil and seal oil fed BioF1B hamsters. This may suggest that either SREBP-2 is not involved in regulating lipid levels in response to the high fat diets or the diets might exert differential effects on lipid metabolism via SREBP-2 activation at translational or post-translational level. Further studies are needed to elucidate precise downstream effectors

of differential SREBP-1c expression that contribute to the anti-hyperlipidemic outcome in seal oil fed BioF1B hamsters.

4.2.4.2 Effect of dietary fish oil or seal oil on PPAR-α mRNA expression

PPAR-α is predominantly found in the liver and regulates genes of lipid transport and oxidation. Since the two different ω-3 PUFA rich sources in our study showed drastic alterations in plasma and liver lipid profile, we investigated whether there were alterations in the regulation of hepatic PPAR- α expression levels in fish oil and seal oil fed BioF1B hamsters. We did not observe any significant differences in PPAR-α expression in response to fish oil or seal oil feeding in BioF1B hamsters (Figure 3.16). Nakatani *et al.* (2003) has previously reported an activation of PPAR-α in rodents fed fish oil. Increased hepatic expression of PPAR-α in fish oil fed mice also demonstrated increased expression of peroxisomal and microsomal fatty acid oxidation genes (Larter *et al.*, 2008). The expression of several PPAR dependent enzymes mediating fatty acid oxidation was also significantly increased upon menhaden oil supplementation in the livers of corpulent JCR rats (Deng *et al.*, 2004). Structural analysis had demonstrated that EPA is an endogenous ligand for PPARs while DHA needs to be converted to EPA to activate PPARs (Xu *et al.*, 1999a; Sprecher 2000). Though the two marine oils in our study differ in their ω-3 PUFA composition and MUFA content, these changes did not seem to significantly alter the hepatic PPAR-α expression. Thus, the observed changes in plasma and hepatic lipids by seal oil and fish oil feeding in the BioF1B hamsters were not due to alterations in the mRNA expression of PPAR- α.

4.2.4.3 Effect of dietary fish oil or seal oil on LXR-α mRNA expression

LXR -α and –β are believed to be involved in the regulation of several genes of lipid metabolism, e.g. Cyp7a, FAS, LPL, ACC. The transcription of SREBP-1c has also been shown to be under the positive control of LXR- α (Repa *et al.*, 2000). While some studies suggest that SREBP-mediated repression of lipogenesis by PUFA is LXR dependent (Yoshikawa *et al.*, 2002b), others propose that repression of SREBP-1c mRNA levels by PUFA is LXR-independent (Pawar *et al.*, 2003). Since we observed reduced SREBP-1c mRNA levels in seal oil fed BioF1B hamsters as compared to fish oil fed BioF1B hamsters, we decided to elucidate the effects of marine oils on LXR-α expression in BioF1B hamsters. Our results did not show any significant differences in hepatic LXR-α mRNA expression in the fish oil and seal oil fed BioF1B hamsters (Figure 3.17). Thus, the effect of seal oil on SREBP-1c inhibition is likely due to LXR-independent pathways.

4.2.5 Effect of dietary fish oil or seal oil on plasma and liver lipid peroxidation

The consumption of long chain PUFA, i.e. ω-3 PUFA in marine oils, can confer increased susceptibility to oxidative stress. Dietary ω-3 PUFA has been shown to displace ω-6 PUFA and get efficiently incorporated into membrane phospholipids. Leibovitz *et al.* (1990) reported that membranes rich in ω-3 PUFA were more prone to oxidative stress. Previous studies from our laboratory demonstrated significantly higher liver lipid peroxidation levels in BioF1B hamsters as compared to Golden Syrian hamsters (Dubey and Cheema, unpublished data). BioF1B hamsters fed fish oil showed

significant elevations in liver lipid peroxidation levels as compared to BioF1B hamsters on MUFA and ω-6/ ω-3 diets. We observed reduced catalase activity in fish oil fed BioF1B hamsters as compared to ω-6/ ω-3 diet fed BioF1B hamsters. These findings support several reports in which fish oil supplementation has been shown to enhance *in-vivo* lipid peroxidation and compromise anti-oxidant enzyme status and potentially contribute to atherogenesis (Kaasgaard *et al.*,1992; Cho *et al.*, 1995; Ando *et al.*, 1998; Ando *et al.*, 2000; Dommels *et al.*, 2003; Yuan and Kitts, 2003). Fish oil induced lipid peroxidation has been associated with fish oil induced hyperlipidemia (Kubow, 1998). Hence, the elevations in lipid peroxide levels and compromised anti-oxidant defenses in fish oil fed BioF1B hamsters can also potentially contribute to fish oil induced hyperlipidemia in BioF1B hamsters. In contrast to most studies that propose detrimental effects of dietary fish oil on oxidative stress, other studies report beneficial effects of fish oil against oxidation (Erdogan *et al.*, 2004; Wang *et al.*, 2004).

In the present study, we compared the effects of dietary fish oil and seal oil on the extent of oxidative stress in BioF1B hamsters. It was observed that both plasma (Figure 3.18) and liver (Figure 3.19) lipid peroxidation levels were significantly lower in seal oil fed hamsters as compared to fish oil fed BioF1B hamsters. However, the plasma TBARS levels showed more dramatic differences as compared to the liver TBARS. The plasma malondialdehyde levels in seal oil fed BioF1B hamsters were almost one-third of that observed for fish oil fed BioF1B hamsters. The possible explanation for differences in oxidation levels in response to the two ω-3 PUFA rich marine oils could be due to the fact that ω-3 PUFA from seal oil are relatively more stable and less prone to lipid

oxidation than fish oil (Nakhla, 1997). Furthermore, while high levels of MUFA in seal oil diet compared to fish oil can also potentially confer resistance to oxidation, there is a possibility that an as yet unknown antioxidant in seal oil might be responsible for its resistance to oxidation. Our findings suggest that seal oil feeding does not induce oxidative stress as compared to dietary fish oil in BioF1B hamsters. The effect of two different sources of ω-3 PUFA on anti-oxidant enzyme activities in seal oil and fish oil fed BioF1B hamsters remains to be investigated. More studies need to be undertaken to gain a better understanding of the potential beneficial effects of seal oil as an alternative source of ω-3 PUFA compared to fish oil in BioF1B hamsters.

4.3 Effects of a combination of marine oils and elderberry extract

Recent studies have focused on the investigation of biological anti-oxidants such as anthocyanins. Several plant anthocyanins have been reported to be more potent anti-oxidants when compared to traditional vitamins such as vitamin E and vitamin C (Tsao and Deng, 2004; Bakowska-Barczak *et al.*, 2007). The potential health benefits of anthocyanins have mainly been attributed to their anti-atherogenic and anti-oxidant properties. Since our preliminary findings showed higher oxidative stress in fish oil BioF1B hamsters as compared to MUFA and ω-6/ ω-3 diets, we investigated the effects of co-supplementation of anthocyanin-rich elderberry extract (25% w/w) along with high fat diets rich in fish oil or seal oil.

There was no significant effect on the average food intake upon dietary supplementation with anthocyanin-rich elderberry extract when given with either fish oil

or seal oil. At the end of the 4-week treatment period, there was no significant change in the weight gain data for BioF1B hamsters fed fish oil or seal oil alone. However, cosupplementation with elderberry extract in BioF1B hamsters fed high fish oil and seal oil diets resulted in significant reductions in weight gain after 4-weeks treatment (29% decrease in fish oil group and 32% decrease in seal oil group (Table 3.1). These observations support the existing reports about the possible anti-obesity effects of anthocyanins. Tsuda *et al.* (2003) demonstrated that dietary cyanidin-3 glucoside rich purple corn color significantly suppressed the high-fat diet induced increase in body weight gain as well as white and brown adipose tissue weights in mice. Anthocyanin treatment in C57BL/6 mice fed high fat diet showed about 24% decrease in body weight gain (Jayaprakasam *et al.*, 2006). Similarly, rats fed high fat diet and black soybean anthocyanins showed significantly lower weight gain than the rats fed high fat diet alone (Kwon *et al.*, 2007). Oral administration of anthocyanins-rich Hibiscus sabdariffa resulted in significantly reduced body weight gain in obese mice (Alarcon-Aguilar *et al.*, 2007). Overall, these reports suggest that anthocyanins might influence various metabolic pathways and thus exert potential beneficial effects in ameliorating obesity.

4.3.1 Effect of marine oils and elderberry extract on plasma lipid and lipoprotein profile

Anthocyanins might have preventative effects on the progression of CVD due to their lipid-lowering properties. We investigated the role of anthocyanin-rich elderberry extract supplementation on plasma lipid levels in marine oil fed BioF1B hamsters. An

observation of great significance was that BioF1B hamsters fed high fat fish oil diet along with elderberry extract did not show milky plasma. All the lipid parameters namely plasma TG, total-cholesterol, cholesteryl esters and free cholesterol concentrations were significantly reduced upon addition of elderberry extract to the marine oils in BioF1B hamsters (Figure 3.20 – Figure 3.23). These observations are in support of earlier reports on hypolipidemic effects of anthocyanin supplementation in several experimental animals (Kwon *et al.*, 2007; Valcheva *et al.*, 2007 (a, b). The hypolipidemic effects of anthocyanins have further been reported to retard the progression of atherogenesis. Supplementation of anthocyanin rich black rice to rabbits significantly improved the lipid profile and inhibited atherosclerotic plaque formation (Ling *et al.,* 2001; Ling *et al.,* 2002). Xia *et al.* (2006) also showed that anthocyanin rich extract from black rice improved lipid profile and enhanced plaque stabilization in ApoE deficient mice.

Our experimental model for increased susceptibility to diet induced hyperlipidemia, BioF1B, showed lipid lowering effects of anthocyanin rich elderberry extract in both fish oil and seal oil fed hamsters. The reductions in plasma lipid levels were more pronounced in the fish oil fed hamsters as compared to the seal oil fed hamsters. Interestingly, for all the studied lipid parameters, we noted that the lipid levels for the fish oil fed hamsters along with elderberry extract were comparable to those seen for the seal oil alone fed Bio F1B hamsters. Moreover, plasma lipid concentrations in seal oil fed hamsters were similar to MUFA supplemented BioF1B hamsters. Our results suggest that supplementing anthocyanin rich elderberry extract in BioF1B hamsters fed

fish oil more effectively lowered lipid levels due to the inherent susceptibility of BioF1B hamsters to high dietary fish oil induced hyperlipidemia.

The mechanism of action of elderberry extract on the regulation of lipid and lipoprotein metabolism in experimental animals or humans is not known. Berry extract can potentially regulate numerous pathways involved in the lipoprotein metabolism, i.e. the synthesis or clearance of TG-rich lipoproteins or cholesterol transporting lipoproteins. Absence of milky plasma in BioF1B hamsters fed fish oil and elderberry extract strongly suggests a role of elderberry extract in promoting lipoprotein clearance, which is inhibited in high fish oil fed BioF1B hamsters. The hypolipidemic action of dietary blueberry extract has been suggested to be due to improved TG-rich lipoprotein clearance in ethanol-treated normolipidemic and in genetically hyperlipidemic Yoshida rats (Cignarella *et al.*, 1996). These authors concluded that supplementing blueberry extract might prove to be beneficial for treating dyslipidemias associated with impaired TG-rich lipoprotein clearance. Since BioF1B hamsters are an experimental model with reduced LPL activity, and thus impaired TG-rich lipoprotein clearance, the precise effects of elderberry extract on lipoprotein catabolism need to be explored in future studies.

4.3.2. Effect of marine oils and elderberry extract on liver lipids

Supplementing elderberry extract in fish oil fed BioF1B hamsters effectively lowered hepatic TG (Figure 3.24) and free cholesterol levels (Figure 3.27) in contrast to seal oil fed BioF1B hamsters, which did not show any significant change in hepatic TG and free cholesterol levels upon elderberry extract supplementation. Previous studies

have reported significant reductions in the hepatic TG levels and decreased hepatic lipid accumulation in high fat and anthocyanin fed C57BL/6 mice (Jayaprakasam *et al.,* 2006). It appears that elderberry extract was more effective in lowering hepatic TG and free cholesterol levels under hyperlipidemic conditions as observed in fish oil fed hamsters. Effects of elderberry extract on hepatic TG synthesis and/or the uptake of TG-rich lipoproteins by liver need to be explored to understand the mechanisms involved.

Both fish oil and seal oil fed BioF1B hamsters showed significantly lower hepatic total cholesterol (Figure 3.25) and cholesterol ester (Figure 3.26) concentrations upon cosupplementation with elderberry extract. These findings indicate that anthocyanin rich elderberry extract might specifically regulate the cholesterol synthesis and metabolic pathways in exerting its anti-hyperlipidemic effects in fish oil and seal oil fed BioF1B hamsters. Since we are the first to report lipid lowering effects of elderberry extract in fish oil and seal oil fed BioF1B hamsters, there is no existing data on the precise metabolic pathways involved in the regulation of cholesterol metabolism by elderberry extract. However, a recent report suggested that anthocyanin-rich extract might have greater potential against atherosclerosis progression as compared to simvastatin in ApoE-deficient mice model (Xia *et al.,* 2006). It is well established that statin class of drugs lower plasma cholesterol levels by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Interestingly, ApoE-deficient mice on anthocyanin-rich extract demonstrated lower total cholesterol and non-HDL cholesterol with elevated HDL-cholesterol as compared to those on simvastatin

(Xia *et al.,* 2006). Such findings emphasize the need to conduct more studies to evaluate the metabolic pathways affected by anthocyanins.

In addition to affecting cholesterol biosynthesis, anthocyanins might also lower hepatic cholesterol ester concentrations by inhibiting ACAT activity thereby affecting cholesterol absorption and its incorporation into lipoproteins. Inhibition of ACAT activity in response to anthocyanin rich elderberry extract might be yet another mechanism for the dramatic decrease in hepatic cholesterol ester levels observed in marine oil fed BioF1B hamsters. Cranberry extract induced significantly higher LDL-receptor expression and increased intracellular cholesterol uptake in hepatocytes in a dose dependent manner (Chu and Liu, 2005). This study proposed the beneficial effects of berry extract in the clearance of excessive plasma cholesterol from the circulation. Anthocyanin rich extract from black rice has been reported to suppress cholesterol accumulation in the liver and aorta and significantly ameliorate hypercholesterolemia, suggesting a potential role in cholesterol efflux from the tissues (Xia *et al.*, 2003). Xia *et al.* (2005) recently showed that anthocyanins can induce cholesterol efflux from mouse peritoneal macrophages and macrophage derived foam cells by increasing gene expression of ATP binding cassette A1, a key mediator of reverse cholesterol transport. This study further showed that stimulation of cholesterol efflux by anthocyanins in these cells was dependent on the activation of nuclear factors PPAR-γ and LXR-α thereby suggesting that differential regulation of various nuclear receptors and transcription factors by anthocyanins might also play an important role in the regulation of lipid and lipoprotein metabolism in BioF1B hamsters. These reports further emphasize the need to

investigate the anti-hyperlipidemic effects of anthocyanin rich elderberry extract on various metabolic mediators as well as nuclear receptors and transcription factors involved in lipid and lipoprotein metabolism in marine oil fed BioF1B hamsters.

4.3.3 Effect of marine oils and elderberry extract on plasma and liver lipid peroxidation

Anthocyanins have gained much attention due to their ability to act as potent anti-oxidants and reactive oxygen species scavengers, thus preventing CVD progression (Ramirez-Tortosa *et al.*, 2001; Stintzing *et al.*, 2002; Kähkönen and Heinonen, 2003, Wu *et al.*, 2004, Bell and Gochenaur, 2006). Vinson *et al.* (1995) showed that single anthocyanin cyanidin-3-O-glucose chloride was more effective in protecting against oxidant degradation of LDLs as compared to vitamin C, E and beta-carotene combined. On examining the effects of anthocyanin enriched elderberry supplementation on plasma TBARS in marine oil fed BioF1B hamsters, we noted significant reductions in the levels of plasma lipid peroxidation in fish oil fed BioF1B hamsters in response to elderberry extract (Figure 3.28). Supplementation of seal oil fed BioF1B hamsters with elderberry extract did not show significant differences in the extent of lipid peroxidation when compared to hamsters fed seal oil alone. Similarly, we noted significant lowering of liver TBARS for BioF1B hamsters fed elderberry extract and fish oil as compared to hamsters fed fish oil alone (Figure 3.29). No significant changes for liver TBARS were seen in BioF1B hamsters fed either seal oil alone or a combination of seal oil and elderberry extract. Hence, our findings suggest that elderberry extract supplementation proved more

effective in lowering the extent of lipid peroxidation in fish oil fed BioF1B hamsters partly due to an increased susceptibility to oxidative stress in fish oil fed BioF1B hamsters. Seal oil fed BioF1B hamsters, however being relatively resistant to diet induced oxidative stress, showed no significant differences on plasma and lipid peroxidation levels upon supplementing with elderberry extract.

The ability of anthocyanins to inhibit oxidation of lipids (Ramirez-Tortosa *et al.,* 2001) and LDLs (Vinson *et al.,* 1995; Kano *et al.,* 2005; Chang *et al.,* 2006) has mostly been reported based on *in vitro* studies. Youdim *et al.* (2000) demonstrated increased protection against oxidative stress due to increased incorporation of elderberry anthocyanins into the membrane and cytosol of vascular endothelial cells. However, there are a few reports on the beneficial effects of anthocyanins against oxidative stress *in-vivo.* Acylated anthocyanins from dietary cabbage proved their anti-oxidant potential by preventing paraquat induced oxidative stress in rats (Igarashi *et al.,* 2000). Moreover, rats fed anthocyanin-rich purple potato flakes showed improved anti-oxidant potential with significantly reduced serum lipid peroxidation and elevated mRNA expression of anti-oxidant enzymes (Han *et al.,* 2006; Han *et al.,* 2007). In this study, we report the protective effects of anthocyanin-rich elderberry extract in fish oil fed BioF1B hamsters, with significantly reduced levels of plasma and liver peroxidation levels, as compared to fish oil fed BioF1B hamsters. In the future, it will be relevant to study the mechanisms of action of anthocyanin rich elderberry extract by measuring enzyme activity and mRNA expression of different anti-oxidant enzymes.

4.4 Conclusions and future directions

Previous studies from our laboratory have shown severe hyperlipidemia with increased TG and cholesterol concentrations in fish oil fed BioF1B hamsters (de Silva *et al.*, 2004). Diet induced hyperlipidemia was further shown to be specific for fish oil when compared to MUFA and ω-6/ ω-3 diets, and specific to BioF1B hamsters when compared to Golden Syrian hamsters. Molecular studies to compare the two strains of hamsters showed significantly lower LPL activity, adipocyte LPL mRNA levels and elevated ApoB expression in BioF1B hamsters compared to Golden Syrian hamsters (Cheema and Cornish, 2007). These differences partially attribute the fish oil induced hyperlipidemia to genetic diversity in the hamster strains.

In the current study, we showed that an alternative ω-3 PUFA-rich source, seal oil, did not cause hyperlipidemia at high doses as seen with fish oil feeding in BioF1B hamsters. Seal oil feeding showed significantly lower plasma and liver lipid levels in comparison to the fish oil diet. In the future, it will be interesting to investigate the regulation of LPL expression and activity in the BioF1B hamsters fed seal oil and fish oil. The differences in the positional distribution of ω-3 PUFA in the TG of the two marine oils might potentially confer better TG clearing capacity in seal oil fed BioF1B hamsters as compared to fish oil fed BioF1B hamsters. It further needs to be investigated whether the anti-hypertriglyceridemic effects of seal oil are exclusively due to the differences in ω-3 PUFA composition and positional distribution of fatty acids in the triglyceride molecule of two oils or is it partly also due to increased MUFA levels or an as yet unidentified antioxidant in seal oil. *In vitro* assays to react commercial LPL with CM

from marine oil fed hamsters will help elucidate whether there is greater resistance to LPL in case of fish oil CM compared to seal oil CM. Transgenic studies targeting LPL gene expression in fish oil and seal oil fed animals will help shed more light on the importance of the differences in the positional distribution of ω-3 PUFA in the two oils on the regulation of lipid metabolism.

Supplementation of dietary fish oil and seal oil, along with elderberry extract in BioF1B hamsters, resulted in significant reductions in weight gain at the end of the 4-week study period as compared to BioF1B hamsters on fish oil or seal oil alone. Moreover, co-supplementation of fish oil and seal oil with elderberry extract showed significantly improved plasma lipid profile for both dietary fats in BioF1B hamsters. Further, comparing the effects of elderberry supplementation on lipid peroxidation levels in plasma and liver revealed significant reductions in oxidative stress in fish oil fed hamsters, while no significant changes were observed for seal oil fed BioF1B hamsters. Thus, anthocyanins showed immense potential in improving lipid profile as well as the extent of lipid peroxidation in fish oil fed BioF1B hamsters. However, the mechanisms by which anthocyanins, alone or in combination with marine oils, elicit these effects are not known, which should be undertaken in the future.

This study shows that BioF1B hamsters fed high seal oil diet resist hyperlipidemia and oxidative stress as compared to fish oil fed BioF1B hamsters. While we believe that differences in ω-3 PUFA composition and positional differences in TG from two marine oils are likely responsible for the differences in the regulation of lipid and lipoprotein

metabolism, the differences in fatty acid composition of the two marine oils might also play an important role, which could be a topic for future investigations.

Overall, findings from the present study suggest that seal oil may be better than fish oil to lower plasma lipid levels and to prevent oxidative stress under genetically variable conditions. Our findings further suggest that co-supplementation of fish oil with anthocyanin rich berry extract may be beneficial under these conditions than fish oil alone.

Chapter 5: Bibliography

Aas, V., Rokling-Andersen, M.H., Kase, E.T., Thoresen, G.H. and Rustan, A.C. (2006) Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J. Lipid Res.* **47(2)**: 366-374.

Accinni, R., Rosina, M., Bamonti, F., Della Noce, C., Tonini, A., Bernacchi, F., Campolo, J., Caruso, R., Novembrino, C., Ghersi, L., Lonati, S., Grossi, S., Ippolito, S., Lorenzano, E., Ciani, A. Gorini M. (2006) Effects of combined dietary supplementation on oxidative and inflammatory status in dyslipidemic subjects. *Nutr. Metab. Cardiovasc. Dis.* **16(2)**: 121-127.

Ahn, Y.S., Smith, D. E., Osada, J., Li, Z., Schaefer, R.J. and Ordovas, J.M. (1994) Dietary fat saturation affects apolipoprotein gene expresssion and high density lipoprotein size distribution in golden syrian hamsters. *J. Nutr.* **124**: 2147-2155.

Alarcon-Aguilar, F.J., Zamilpa, A., Perez-Garcia, M.D., Almanza-Perez, J.C., Romero-Nuñez, E., Campos-Sepulveda, E.A., Vazquez-Carrillo, L.I. and Roman-Ramos, R. (2007) Effect of Hibiscus sabdariffa on obesity in MSG mice. *J. Ethnopharmacol.* **114(1)**: 66 -71.

Alexander Aguilera, A., Diaz, G.H., Barcelata, M.L., Guerrero, O.A. and Ros, R.M. (2006) Induction of Cd36 expression elicited by fish oil PUFA in spontaneously hypertensive rats. *J. Nutr. Biochem.* **17(11)**: 760-765.

Amorini, A.M., Fazzina, G., Lazzarino, G., Tavazzi, B., Di Pierro, D., Santucci, R., Sinibaldi, F., Galvano, F. and Galvano, G. (2001) Activity and mechanism of the antioxidant properties of cyanidin-3-O-beta-glucopyranoside. *Free Radic. Res.* **35(6)**: 953-966.

Ando, H., Nishimura, Y., Nemoto, T., Takamura, T., Nagai, Y. and Kobayashi, K. (2001) Severe hypercholesterolemia in a double heterozygote for lipoprotein lipase deficiency (LPL (Arita)) and apolipoprotein epsilon4: a report of a family with LPL(Arita). *Endocr. J.* **48(1)**:113-118.

Ando, K., Nagata, K., Beppu, M., Kikugawa, K., Kawabata, T., Hasegawa, K. and Suzuki, M. (1998) Effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation in rat erythrocyte membranes. *Lipids* **33(5)**: 505-512.

Ando, K., Nagata, K., Yoshida, R., Kikugawa, K. and Suzuki, M. (2000) Effect of n-3 polyunsaturated fatty acid supplementation on lipid peroxidation of rat organs. *Lipids* **35(4)**: 401- 407.

Atalay, M., Laaksonen, D.E., Khanna, S., Kaliste-Korhonen, E., Hänninen, O. and Sen, C.K. (2000) Vitamin E regulates changes in tissue antioxidants induced by fish oil and acute exercise. *Med. Sci. Sports Exerc.* **32(3)**: 601-607.

Auwerx, J., Schoonjans, K., Fruchart, J.C. and Staels, B. (1996) Regulation of triglyceride metabolism by PPARs: fibrates and thiazolidinediones have distinct effects. *J. Atheroscler. Thromb.* **3(2)**: 81-89.

Aviram, M. and Fuhrman, B. (2002) Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. N. Y. Acad. Sci.* **957**:146-161.

Bagchi, D., Sen, C.K., Bagchi, M. and Atalay, M. (2004) Anti-angiogenic, antioxidant, and anti carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry (Mosc).* **69(1)**:75-80.

Bagdade, J.D., Ritter, M.C., Lithell, H., Bassett, D., Mailly, F., Talmud, P. and Hayden, M.R. (1996) Reduced cholesteryl ester transfer in plasma of patients with lipoprotein lipase deficiency. *J. Lipid Res.* **37(8)**:1696-1703.

Bakowska-Barczak, A.M., Marianchuk, M. and Kolodziejczyk, P. (2007) Survey of bioactive components in Western Canadian berries. *Can. J. Physiol. Pharmacol.* **85(11)**: 1139-1152.

Banfield, H.J. (2008) Comparison of plasma and tissue fatty acid composition of BioF1B hamsters fed fish oil and seal oil. Memorial University of Newfoundland, Canada.

Bang, H.O., Dyerberg, J. and Hjoorne, N. (1976) The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.* **200(1-2)**: 69-73.

Bell, D.R. and Gochenaur, K. (2006) Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. *J. Appl. Physiol.* **100(4)**: 1164-1170.

Bennett, A.J., Billett, M.A., Salter, A.M., Mangiapane, H.E., Bruce, J.S., Anderson, K.L., Marenah, C.B. and Lason, N. (1995) Modulation of hepatic apolipoprotein B, HMG-coA reductase and low density lipoprotein receptor mRNA and plasma lipoprotein concentrations by defined dietary fats. *Biochem. J.* **311**: 167-173.

Berger, A., Mutch, D.M., German, J.B. and Roberts, M.A. (2002) Unraveling lipid metabolism with microarrays: effects of arachidonate and docosahexaenoate acid on murine hepatic and hippocampal gene expression. *Genome Biol.* **3(7)**: 1-4.

Bonefeld-Jørgensen, E.C., Møller, S.M. and Hansen, J.C. (2001) Modulation of atherosclerotic risk factors by seal oil: a preliminary assessment. *Int. J. Circumpolar Health* **60(1)**: 25-33.

Bonen, A., Campbell, S.E., Benton, C.R., Chabowski, A., Coort, S.L., Han, X.X., Koonen, D.P., Glatz, J.F. and Luiken, J.J. (2004) Regulation of fatty acid transport by fatty acid translocase/CD36. *Proc. Nutr. Soc.* **63(2)**: 245-249.

Bordin, P., Bodamer, O.A., Venkatesan, S., Gray, R.M., Bannister, P.A. and Halliday, D. (1998) Effects of fish oil supplementation on apolipoprotein B100 production and lipoprotein metabolism in normolipidaemic males. *Eur. J. Clin. Nutr.* **52(2)**: 104-109.

Botolin, D., Wang, Y., Christian, B. and Jump, D.B. (2006) Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. *J. Lipid Res.* **47(1)**: 181-192.

Brox, J., Olaussen, K., Osterud, B., Elvevoll, E.O., Bjørnstad, E., Brattebøg, G. and Iversen, H. (2001) A long-term seal- and cod-liver-oil supplementation in hypercholesterolemic subjects. *Lipids* **36(1)**: 7-13.

Buhman, K.K., Accad, M., Novak, S., Choi, R.S., Wong, J.S., Hamilton, R.L., Turley, S. and Farese, R.V. Jr. (2000) Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat. Med.* **6(12)**: 1341-1347.

Chambrier, C., Bastard, J.P., Rieusset, J., Chevillotte, E., Bonnefont-Rousselot, D., Therond, P., Hainque, B., Riou, J.P., Laville, M. and Vidal, H. (2002) Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor gamma. *Obes. Res.* **10(6)**: 518-525.

Chan, D.C., Barrett, P.H. and Watt, G.F. (2004) Lipoprotein transport in the metabolic syndrome: methodological aspects of stable isotope kinetic studies. *Clin. Sci.* **107 (3)**: 221-232.

Chan, D.C., Watts, G.F., Mori, T.A., Barrett, P.H., Redgrave, T.G. and Beilin, L.J. (2003) Randomized controlled trial of the effect of n-3 fatty acid supplementation on the metabolism of apolipoprotein B-100 and chylomicron remnants in men with visceral obesity. *Am. J. Clin. Nutr.* **77(2)**: 300-307.

Chang, Y.C., Huang, K.X., Huang, A.C., Ho, Y.C. and Wang, C.J. (2006) Hibiscus anthocyanins-rich extract inhibited LDL oxidation and oxLDL-mediated macrophages apoptosis. *Food Chem. Toxicol.* **44(7)**: 1015-1023.

Chatelain, F., Kohl, C., Esser, V., Mc- Garry, J.D., Girard, J. and Pegorier, J.P. (1996) Cyclic AMP and fatty acids increase carnitine palmitoyltransferase I gene transcription in cultured fetal rat hepatocytes. *Eur. J. Biochem.* **235(3)**: 789–798.

Cheema, S.K. and Cornish, M.L. (2007) Bio F1B hamster: a unique animal model with reduced lipoprotein lipase activity to investigate nutrient mediated regulation of lipoprotein metabolism. *Nutr. Metab. (London)* **4**: 27.

Chen, J., Song, W. and Redinger, R.N. (1996) Effects of dietary cholesterol on hepatic production of lipids and lipoproteins in isolated hamster liver. *Hepatology* **24(2)**: 424-434.

Cho, S.H. and Choi, Y.S. (1994) Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. *Lipids* **29(1)**: 47-52.

Cho, S.H., Im, J.G., Choi, Y.S., Son, Y.S. and Chung, M.H. (1995) Lipid peroxidation and 8-hydroxydeoxyguanosine formation in rats fed fish oil with different levels of vitamin E. *J. Nutr. Sci. Vitaminol. (Tokyo)* **41(1)**: 61-72.

Christensen, M.S., Høy, C.E. and Redgrave, T.G. (1994) Lymphatic absorption of n - 3 polyunsaturated fatty acids from marine oils with different intramolecular fatty acid distributions. *Biochim. Biophys. Acta* **1215(1-2)**:198-204.

Christensen, M.S., Mortimer, B.C., Hoy, C.E. and Redgrave, T.G. (1995) Clearance of chylomicrons following fish oil and seal oil feeding. *Nutr. Res.* **15 (3)**: 359-368.

Chu, Y.F. and Liu, R.H. (2005) Cranberries inhibit LDL oxidation and induce LDL receptor expression in hepatocytes. *Life Sci.* **77(15)**: 1892-1901.

Chung, B.H., Cho, B.H., Liang, P., Doran, S., Osterlund, L., Oster, R.A., Darnell, B. and Franklin, F. (2004) Contribution of postprandial lipemia to the dietary fat-mediated changes in endogenous lipoprotein-cholesterol concentrations in humans. *Am. J. Clin. Nutr.* **80(5)**: 1145-1158.

Chupukcharoen, N., Komaratat, P. and Wilairat, P. (1985) Effects of vitamin E deficiency on the distribution of cholesterol in plasma lipoproteins and the activity of cholesterol 7 alpha-hydroxylase in rabbit liver. *J. Nutr.* **115(4)**: 468-472.

Cignarella, A., Nastasi, M., Cavalli, E. and Puglisi, L. (1996) Novel lipid-lowering properties of Vaccinium myrtillus L. leaves, a traditional antidiabetic treatment, in several models of rat dyslipidaemia: a comparison with ciprofibrate. *Thromb. Res.* **84(5)**: 311-322.

Clarke, S.D., Armstrong, M.K. and Jump, D.B. (1990) Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. *J. Nutr.* **120(2):** 225–231.

Clarke, S.D. (2000) Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. *Br. J. Nutr.* **83(1)**: S59-S66.

Clifford, M.N. (2000) Anthocyanins - nature, occurrence and dietary burden. *J. Sci. Food Agricul.* **80**: 1063-1072.

Connor, W.E., Prince, M.J., Ullmann, D., Riddle, M., Hatcher, L., Smith, F.E. and Wilson, D. (1993) The hypotriglyceridemic effect of fish oil in adult-onset diabetes without adverse glucose control. *Ann. N. Y. Acad. Sci.* **683**: 337–340.

Conquer, J.A., Cheryk, L.A., Chan, E., Gentry, P.A. and Holub, B.J. (1999) Effect of supplementation with dietary seal oil on selected cardiovascular risk factors and hemostatic variables in healthy male subjects. *Thromb. Res.* **96(3)**: 239-250.

De Silva, P.P., Agarwal-Mawal, A., Davis, P.J. and Cheema, S.K. (2005) The levels of plasma low density lipoprotein are independent of cholesterol ester transfer protein in fish-oil fed F1B hamsters. *Nutr. Metab. (London)* **2(1)**: 8.

De Silva, P.P., Davis, P.J. and Cheema, S.K. (2004) Hyperlipidaemic effect of fish oil in Bio F1B hamsters. *Br. J. Nutr.* **91(3)**: 341-349.

Deng, X., Elam, M.B., Wilcox, H.G., Cagen, L.M., Park, E.A., Raghow, R., Patel, D., Kumar, P., Sheybani, A. and Russell, J.C. (2004) Dietary olive oil and menhaden oil mitigate induction of lipogenesis in hyperinsulinemic corpulent JCR: LA-cp rats: microarray analysis of lipid-related gene expression. *Endocrinology* **145(12)**: 5847-5861.

Dommels, Y.E., Heemskerk, S., van den Berg, H., Alink, G.M., van Bladeren, P.J. and van Ommen, B. (2003) Effects of high fat fish oil and high fat corn oil diets on initiation of AOM-induced colonic aberrant crypt foci in male F344 rats. *Food Chem. Toxicol.* **41(12)**: 1739-1747.

Dorfman, S.E., Smith, D.E., Osgood, D.P. and Lichtenstein, A.H. (2003) Study of diet induced changes in lipoprotein metabolism in two strains of golden syrian hamsters. *J. Nutr.* **133**: 4183-4188.

Dubey, P. and Cheema, S.K. (2008) Seal oil has greater benefits to lower plasma lipid levels as compared to fish oil. 12th World Congress on Clinical Nutrition: Nutritional factors in health and disease. *Nutrition Bulletin* **33**: 67-71.

Erdogan, H., Fadillioglu, E., Ozgocmen, S., Sogut, S., Ozyurt, B., Akyol, O. and Ardicoglu, O. (2004) Effect of fish oil supplementation on plasma oxidant/antioxidant status in rats. *Prostaglandins Leukot. Essent. Fatty Acids* **71(3)**: 149-152.

Farmer, A., Montori, V., Dinneen, S. and Clar, C. (2001) Fish oil in people with type 2 diabetes mellitus. *Cochrane Database Syst. Rev.* **3**: CD003205.

Farwer, S.R., der Boer, B.C., Haddeman, E., Kivits, G.A., Wiersma, A. and Danse, B.H. (1994) The vitamin E nutritional status of rats fed on diets high in fish oil, linseed oil or sunflower seed oil. *Br. J. Nutr.* **72(1)**: 127-145.

Field, F.J., Born, E. and Mathur, S.N. (2003) Fatty acid flux suppresses fatty acid synthesis in hamster intestine independently of SREBP-1 expression. *J. Lipid Res.* **44**: 1199–1208.

Finné Nielsen, I.L., Elbøl Rasmussen, S., Mortensen, A., Ravn-Haren, G., Ma, H.P., Knuthsen, P., Hansen, B.F., McPhail, D., Freese, R., Breinholt, V., Frandsen, H. and Dragsted, L.O. (2005) Anthocyanins increase low-density lipoprotein and plasma cholesterol and do not reduce atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits. *Mol. Nutr. Food Res.* **49(4)**: 301-308.

Fisher, E.A., Pan, M., Chen, X., Wu, X., Wang, H., Jamil, H., Sparks, J.D. and Williams, K.J. (2001) The triple threat to nascent apolipoprotein B. Evidence for multiple, distinct degradative pathways. *J. Biol. Chem.* **276(30)**: 27855-27863.

Fisher, W.R., Zech, L.A. and Stacpoole, P.W. (1998) Apolipoprotein B metabolism in hypertriglyceridemic diabetic patients administered either a fish oil- or vegetable oil-enriched diet. *J Lipid Res.* **39(2)**: 388-401.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226(1)**: 497-509.

Frémont, L., Gozzélino, M.T., Franchi, M.P. and Linard, A. (1998) Dietary flavonoids reduce lipid peroxidation in rats fed polyunsaturated or monounsaturated fat diets. *J. Nutr.* **128(9)**: 1495-1502.

Frenoux, J.M., Prost, E.D., Belleville, J.L. and Prost, J.L. (2001) A polyunsaturated fatty acid diet lowers blood pressure and improves antioxidant status in spontaneously hypertensive rats. *J. Nutr.* **131(1)**: 39-45.

Frolov, A. and Hui, D.Y. (2007) The modern art of atherosclerosis: a picture of colorful plants, cholesterol, and inflammation. *Arterioscler. Thromb. Vasc. Biol.* **27(3)**: 450-452.

Fukumoto, L.R. and Mazza, G. (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **48(8)**: 3597-3604.

Galvano, F., La Fauci, L., Lazzarino, G., Fogliano, V., Ritieni, A., Ciappellano, S., Battistini, N.C., Tavazzi, B. and Galvano, G. (2004) Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.* **15(1)**: 2-11.

Ghosh, S. and Grogan, W.M. (1989) Activation of rat liver cholesterol ester hydrolase by cAMP-dependent protein kinase and protein kinase C. *Lipids* **24(8)**: 733-736.

Ghosh, S., Kounnas, M.Z. and Grogan, W.M. (1990) Separation and differential activation of rat liver cytosolic cholesteryl ester hydrolase, triglyceride lipase and retinyl palmitate hydrolase by cholestyramine and protein kinases. *Lipids* **25(4)**: 221-225.

Ghosh, S., Natarajan, R., Pandak, W.M., Hylemon, P.B. and Grogan, W.M. (1998) Regulation of hepatic neutral cholesteryl ester hydrolase by hormones and changes in cholesterol flux. *Am. J. Physiol.* **274**: G662-668.

GISSI Prevenzione Investigators. (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and Vitamin E after myocardial infarction: results of the GISSI Prevenzione trial. *Lancet* **354 (9177)**: 447-455.

Glomset, J.A. and Norum, K.R. (1973) The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology. *Adv. Lipid Res.* **11**: 1-65.

Goodman, D.S. (1962) The metabolism of chylomicron cholesterol ester in the rat. *J. Clin. Invest.* **41**: 1886-1896.

Grogan, W.M., Bailey, M.L., Heuman, D.M. and Vlahcevic, Z.R. (1991) Effects of perturbations in hepatic free and esterified cholesterol pools on bile acid synthesis, cholesterol 7 alpha hydroxylase, HMG-CoA reductase, acyl-CoA:cholesterol acyltransferase and cytosolic cholesteryl ester hydrolase. *Lipids* **26(11)**: 907-914.

Ha, Y.C. and Barter, P.J. (1982) Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol. B.* **71(2)**: 265-269.

Haglund, O., Luostarinen, R., Wallin, R., Wibell, L. and Saldeen, T. (1991) The effects of fish oil on triglycerides, cholesterol, fibrinogen and malondialdehyde in humans supplemented with vitamin E. *J. Nutr.* **121(2)**: 165-169.

Han, K.H., Sekikawa, M., Shimada, K., Hashimoto, M., Hashimoto, N., Noda, T., Tanaka, H. and Fukushima, M. (2006) Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats. *Br. J. Nutr.* **96(6)**: 1125-1133.

Han, K.H., Shimada, K., Sekikawa, M. and Fukushima, M. (2007) Anthocyanin-rich red potato flakes affect serum lipid peroxidation and hepatic SOD mRNA level in rats. *Biosci. Biotechnol. Biochem.* **71(5)**: 1356-1359.

Hannah, V.C., Ou, J., Luong, A., Goldstein, J.L. and Brown, M.S. (2001) Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J. Biol. Chem.* **276(6)**: 4365-4372.

Harris, W.S., Hustvedt, B.E., Hagen, E., Green, M.H., Lu, G. and Drevon, C.A. (1997a) N-3 fatty acids and chylomicron metabolism in the rat. *J. Lipid Res.* **38(3)**: 503-515.

Harris, W.S., Lu, G., Rambjør, G.S., Wålen, A.I., Ontko, J.A., Cheng, Q. and Windsor, S.L. (1997b) Influence of n-3 fatty acid supplementation on the endogenous activities of plasma lipases. *Am. J. Clin. Nutr.* **66(2)**: 254-260.

Harris, W.S. (1997) N-3 fatty acids and serum lipoproteins: human studies. *Am. J. Clin. Nutr.* **65(5 Suppl)**: 1645S-1654S.

Hassimotto, N.M., Genovese, M.I. and Lajolo, F.M. (2005) Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. *J. Agric. Food Chem.* **53(8)**: 2928-2935.

Hayes, K.C., Khosla, P., Kaiser, A., Yeghiazarians, V. and Pronczuk, A. (1992) Dietary fat and cholesterol modulate the plasma lipoprotein distribution and production of pigment or cholesterol gallstones in hamsters. *J. Nutr.* **122(2)**: 374-384.

Hayes, K.C., Pronczuk, A., Stephan, Z.F. and Lanzkron, S. (1990) Comparative lipemias in hamsters and three monkey species fed fish oil, in *Proceedings of the AOCS short course on polyunsaturated fatty acids and eicosanoids (Lands, W.E.M., ed.)*, American Oil Chemists' Society, Champaign: 334-339.

He, Y.H., Zhou, J., Wang, Y.S., Xiao, C., Tong, Y., Tang, J.C., Chan, A.S. and Lu, A.P. (2006) Anti-inflammatory and anti-oxidative effects of cherries on Freund's adjuvant-induced arthritis in rats. *Scand. J. Rheumatol.* **35(5)**: 356-358.

Hirai, A., Hamazaki, T., Terano, T., Nishikawa, T., Tamura, Y., Kumagai, A. and Sajiki, J. (1980) Eicosapentaenoic acid and platelet function in Japanese. *Lancet* **2(8204)**: 1132-1133.

Hokanson, J.E. and Austin, M.A. (1996) Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J. Cardiovasc. Risk* **3(2)**: 213-219.

Hokanson, J.E., Brunzell, J.D., Jarvik, G.P., Wijsman, E.M. and Austin, M.A. (1999) Linkage of low-density lipoprotein size to the lipoprotein lipase gene in heterozygous lipoprotein lipase deficiency. *Am. J. Hum. Genet.* **64(2)**: 608-618.

Hooper, L., Thompson, R.L., Harrison, R.A., Summerbell, C.D., Ness, A.R., Moore, H.J., Worthington, H.V., Durrington, P.N., Higgins, J.P., Capps, N.E., Riemersma, R.A., Ebrahim, S.B. and Davey Smith, G. (2006) Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *Br. Med. J.* **332(7544)**: 752-760.

Hsu, H.C., Lee, Y.T. and Chen, M.F. (2000) Effect of n-3 fatty acids on the composition and binding properties of lipoproteins in hypertriglyceridemic patients. *Am. J. Clin. Nutr.* **71(1)**: 28-35.

Hsu, J.M., Wang, P.H., Liu, B.H. and Ding, S.T. (2004) The effect of dietary docosahexaenoic acid on the expression of porcine lipid metabolism-related genes. *J. Anim. Sci.* **82(3)**: 683-689.

Hu, F.B., Bronner, L., Willett, W.C., Stampfer, M.J., Rexrode, K.M., Albert, C.M., Hunter, D. and Manson, J.E. (2002) Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. JAMA **287(14)**: 1815-1821.

Igarashi, K., Kimura, Y. and Takenaka, A. (2000) Preventive effects of dietary cabbage acylated anthocyanins on paraquat-induced oxidative stress in rats. *Biosci. Biotechnol. Biochem.* **64(8)**: 1600-1607.

Illingworth, D.R., Harris, W.S. and Connor WE. (1984) Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. *Arteriosclerosis* **4(3)**: 270-275.

Jayaprakasam, B., Olson, L.K., Schutzki, R.E., Tai, M.H. and Nair, M.G. (2006) Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (Cornus mas). *J. Agric. Food. Chem.* **54(1)**: 243-248.

Jenkins, D.J., Josse, A.R., Beyene, J., Dorian, P., Burr, M.L., LaBelle, R., Kendall, C.W. and Cunnane, S.C. (2008) Fish-oil supplementation in patients with implantable cardioverter defibrillators: a meta-analysis. *CMAJ* **178(2)**: 157-164.

Jones, P.J. and Lau, V.W. (2002) Effect of n-3 polyunsaturated fatty acids on risk reduction of sudden death. *Nutr. Rev.* **60(12)**: 407-409.

Jump, D.B., Clarke, S.D., Thelen, A. and Liimatta, M. (1994) Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids. *J. Lipid Res.* **35(6)**: 1076-1084.

Jump, D.B. (2002) The biochemistry of n-3 polyunsaturated fatty acids. *J. Biol. Chem.* **277(11)**: 8755-8758.

Kaasgaard, S.G., Hølmer, G., Høy, C.E., Behrens, W.A. and Beare-Rogers, J.L. (1992) Effects of dietary linseed oil and marine oil on lipid peroxidation in monkey liver *in vivo* and *in vitro*. *Lipids* **27(10)**: 740-745.

Kähkönen, M.P. and Heinonen, M. (2003) Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **51(3)**: 628-633.

Kano, M., Takayanagi, T., Harada, K., Makino, K. and Ishikawa, F. (2005) Antioxidative activity of anthocyanins from purple sweet potato, Ipomoera batatas cultivar Ayamurasaki. *Biosci. Biotechnol. Biochem.* **69(5)**: 979-988.

Katan, M.B., Beynen, A.C., de Vries, J.H. and Nobels, A. (1986) Existence of consistent hypo- and hyperresponders to dietary cholesterol in man. *Am. J. Epidemiol.* **123(2)**: 221-234.

Kendrick, J.S. and Higgins, J.A. (1999) Dietary fish oils inhibit early events in the assembly of very low density lipoproteins and target apoB for degradation within the rough endoplasmic reticulum of hamster hepatocytes. *J. Lipid Res.* **40(3)**: 504-514.

Khan, S., Minihane, A.M., Talmud, P.J., Wright, J.W., Murphy, M.C., Williams, C.M. and Griffin, B.A. (2002) Dietary long-chain n-3 PUFAs increase LPL gene expression in adipose tissue of subjects with an atherogenic lipoprotein phenotype. *J. Lipid Res.* **43(6)**: 979-985.

Kikugawa, K., Yasuhara, Y., Ando, K., Koyama, K., Hiramoto, K. and Suzuki, M. (2003) Protective effect of supplementation of fish oil with high n-3 polyunsaturated fatty acids against oxidative stress-induced DNA damage of rat liver *in vivo*. *J. Agric. Food Chem.* **51(20)**: 6073-6079.

Kim, H.J., Takahashi, M. and Ezaki, O. (1999) Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic gene mRNAs. *J. Biol. Chem.* **274(36)**: 25892–25898.

Klein, R.L. and Rudel, L.L. (1983) Cholesterol absorption and transport in thoracic duct lymph lipoproteins of nonhuman primates. Effect of dietary cholesterol level. *J. Lipid Res.* **24(4)**: 343-356.

Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., Hosoyama, H., Kataoka, S. and Ariga, T. (1999) Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *J. Agric. Food Chem.* **47(5)**: 1892-1897.

Koike, T., Liang, J., Wang, X., Ichikawa, T., Shiomi, M., Liu, G., Sun, H., Kitajima, S., Morimoto, M., Watanabe, T., Yamada, N. and Fan, J. (2004) Overexpression of lipoprotein lipase in transgenic Watanabe heritable hyperlipidemic rabbits improves hyperlipidemia and obesity. *J. Biol. Chem.* **279(9)**: 7521-7529.

Kowala, M.C., Nunnari, J.J., Durham, S.K. and Nicolosi, R.J. (1991) Doxazosin and cholestyramine similarly decrease fatty streak formation in the aortic arch of hyperlipidemic hamsters. *Atherosclerosis* **91(1-2)**: 35-49.

Kowalczyk, E., Kopff, A., Niedworok, J., Kopff, M. and Jankowski, A. (2002) Anthocyanins--an adjunct to cardiovascular therapy? *Kardiol. Pol.* **57(10)**: 332-336.

Kristensen, S.D., Roberts, K.M., Lawry, J. and Martin, J.F. (1988) The effect of fish oil on atherogenesis and thrombopoiesis in rabbits on high cholesterol diet. *Artery* **15(5)**: 250-258.

Kromann, N. and Green, A. (1980) Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med. Scand.* **208(5)**: 401-406.

Kromhout, D., Feskens, E.J. and Bowles, C.H. (1995) The protective effect of small amount of fish on coronary heart disease mortality in an elderly population. *Inter. J. Epidemiol.* **24**: 340-345.

Kubo, K., Saito, M., Tadokoro, T. and Maekawa, A. (1997) Changes in susceptibility of tissues to lipid peroxidation after ingestion of various levels of docosahexaenoic acid and vitamin E. *Br. J. Nutr.* **78(4)**: 655-669.

Kubow, S. (1998) The Role of Oxidative Stress and Antioxidant Supplementation on Lipoprotein Metabolism, *Rec. Res. Dev. Lipids* **2**: 81–99.

Kubow, S., Goyette, N., Kermasha, S., Stewart-Phillip, J. and Koski, K.G. (1996) Vitamin E inhibits fish oil-induced hyperlipidemia and tissue lipid peroxidation in hamsters. *Lipids* **31**: 839-847.

Kwon, S.H., Ahn, I.S., Kim, S.O., Kong, C.S., Chung, H.Y., Do, M.S. and Park, K.Y. (2007) Anti-obesity and hypolipidemic effects of black soybean anthocyanins. *J. Med. Food* **10(3)**: 552-556.

Lapillonne, A., Clarke, S.D. and Heird, W.C. (2004) Polyunsaturated fatty acids and gene expression. *Curr. Opin. Clin. Nutr. Metab. Care.* **7(2)**: 151-156.

Larter, C.Z., Yeh, M.M., Cheng, J., Williams, J., Brown, S., dela Pena, A., Bell-Anderson, K.S. and Farrell, G.C. (2008) Activation of peroxisome proliferator-activated receptor alpha by dietary fish oil attenuates steatosis, but does not prevent experimental steatohepatitis because of hepatic lipoperoxide accumulation. *J. Gastroenterol. Hepatol.* **23(2)**: 267-275.

Le Jossic-Corcos, C., Gonthier, C., Zaghini, I., Logette, E., Shechter, I. and Bournot, P. (2005) Hepatic farnesyl diphosphate synthase expression is suppressed by polyunsaturated fatty acids. *Biochem. J.* **385**: 787-794.

Lee, J.Y. and Carr, T.P. (2004) Dietary fatty acids regulate acyl-CoA:cholesterol acyltransferase and cytosolic cholesteryl ester hydrolase in hamsters. *J. Nutr.* **134(12)**: 3239-3244.

Leibovitz, B.E., Hu, M.L. and Tappel, A.L. (1990) Lipid peroxidation in rat tissue slices: effect of dietary vitamin E, corn oil-lard and menhaden oil. *Lipids* **25(3)**: 125-129.

Lemaitre, R.N., King, I.B., Mozaffarian, D., Kuller, L.H., Tracy, R.P. and Siscovick, D.S. (2003) N-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study. *Am. J. Clin. Nutr.* **77(2)**: 319-325.

Lin, M.H., Lu, S.C., Hsieh, J.W. and Huang, P.C. (1995) Lipoprotein responses to fish, coconut and Soybean oil diets with and without cholesterol in the Syrian hamster. *J. Formos Med. Assoc.* **94**: 724-731.

Lindi, V., Schwab, U., Louheranta, A., Laakso, M., Vessby, B., Hermansen, K., Storlien, L., Riccardi, G., A Rivellese, A: KANWU Study Group. (2003) Impact of the Pro12Ala polymorphism of the PPAR-gamma2 gene on serum triacylglycerol response to n-3 fatty acid supplementation. *Mol. Genet. Metab.* **79(1)**: 52-60.

Ling, W.H., Cheng, Q.X., Ma, J. and Wang, T. (2001) Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. *J. Nutr.* **131(5)**: 1421-1426.

Ling, W.H., Wang, L.L. and Ma, J. (2002) Supplementation of the black rice outer layer fraction to rabbits decreases atherosclerotic plaque formation and increases antioxidant status. *J. Nutr.* **132(1)**: 20-26.

Lu, S.H., Lin, M.H. and Huang, P.L. (1996) A high cholesterol (n-3) polyunsaturated fatty acid diet induces hypercholesterolemia more than a high cholesterol (n-6) polyunsaturated fatty acid diet in hamsters. *J. Nutr.* **126**: 1759-1765.

Martin, G., Schoonjans, K., Lefebvre, A.M., Staels, B. and Auwerx, J. (1997) Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *J. Biol. Chem.* **272(45)**: 28210–28217.

Mazza, G., Kay, C.D., Cottrell, T. and Holub, B.J. (2002) Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **50(26)**: 7731-7737.

Mazza, G.J. (2007) Anthocyanins and heart health. *Ann. Ist Super Sanita.* **43(4)**: 369-374.

McAteer, M.A., David, C., Grimsditch, Vidgeon-Hart, M., Benson, G.M. and Salter, M. (2003) Dietary cholesterol reduces lipoprotein lipase activity in the atherosclerosis-susceptible Bio F_1B hamster. *Brit. J. Nutr.* **89**: 341-350.

Mehta, R.S., Gunnett, C.A., Harris, S.R., Bunce, O.R. and Hartle, D.K. (1994) High fish oil diet increases oxidative stress potential in mammary gland of spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* **21(11)**: 881-889.

Menuet, R., Lavie, C.J. and Milani, R.V. (2005) Importance and management of dyslipidemia in the metabolic syndrome. *Am. J. Med. Sci.* **330(6)**: 295-302.

Meyer, B.J., Lane, A.E. and Mann, N.J. (2009) Comparison of seal oil to tuna oil on plasma lipid levels and blood pressure in hypertriglyceridaemic subjects. *Lipids* 44(9): 827-835.

Morazzoni, P. and Magistretti, M.J. (1990) Activity of Myrtocyan®, an anthosyanoside complex from *Vaccinium myrtillus* (VMA), on platelet aggregation and adhesiveness. *Fitoterapia* **61**: 13–21.

Morris, C.D. and Carson, S. (2003) Routine vitamin supplementation to prevent cardiovascular disease: a summary of the evidence for the U.S. Preventive Services Task Force. *Ann. Intern. Med.* **139(1)**: 56-70.

Murkovic, M., Abuja, P.M., Bergmann, A.R., Zirngast, A., Adam, U., Winklhofer-Roob, B.M. and Toplak, H. (2004) Effects of elderberry juice on fasting and postprandial serum lipids and low-density lipoprotein oxidation in healthy volunteers: a randomized, double-blind, placebo-controlled study. *Eur. J. Clin. Nutr.* **58(2)**: 244-249.

Murphy, M.C., Zampelas, A., Puddicombe, S.M., Furlonger, N.P., Morgan, L.M. and Williams, C.M. (1993) Pretranslational regulation of the expression of the lipoprotein lipase (EC 3.1.1.34) gene by dietary fatty acids in the rat. *Br. J. Nutr.* **70(3)**: 727-736.

Murphy, M.C., Brooks, C.N., Rockett, J.C., Chapman, C., Lovegrove, J.A., Gould, B.J., Wright, J.W. and Williams, C.M. (1999a) The quantitation of lipoprotein lipase mRNA in biopsies of human adipose tissue, using the polymerase chain reaction, and the effect of increased consumption of n-3 polyunsaturated fatty acids. *Eur. J. Clin. Nutr.* **53(6)**: 441-447.

Murphy, M.G., Wright, V., Scott, J., Timmins, A. and Ackman, R.G. (1999b) Dietary menhaden, seal, and corn oils differentially affect lipid and ex vivo eicosanoid and

thiobarbituric acid-reactive substances generation in the guinea pig. *Lipids* **34(2)**: 115-124.

Nakajima, J.I., Tanaka, I., Seo, S., Yamazaki, M. and Saito, K. (2004) LC/PDA/ESI-MS Profiling and Radical Scavenging Activity of Anthocyanins in Various Berries. *J. Biomed. Biotechnol.* **2004(5)**: 241-247.

Nakatani, T., Kim, H.J., Kaburagi, Y., Yasuda, K. and Ezaki, O. (2003) A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity. *J. Lipid Res.* **44(2)**: 369-379.

Nakhla, A. (1997) The Potential of Seal Oil as w-3 Polyunsaturated Fatty Acid Supplements. PhD thesis, Memorial University of Newfoundland.

Napolitano, M., Bravo, E., Avella, M., Chico, Y., Ochoa, B., Botham, K.M. and Rivabene, R. (2004) The fatty acid composition of chylomicron remnants influences their propensity to oxidate. *Nutr. Metab. Cardiovasc. Dis.* **14(5)**: 241-247.

Narayan, M.S., Naidu, K.A., Ravishankar, G.A., Srinivas, L. and Venkataraman, L.V. (1999) Antioxidant effect of anthocyanin on enzymatic and non-enzymatic lipid peroxidation. *Prostaglandins Leukot. Essent. Fatty Acids* **60(1)**: 1-4.

Natella, F., Belelli, F., Gentili, V., Ursini, F. and Scaccini, C. (2002) Grape seed proanthocyanidins prevent plasma postprandial oxidative stress in humans. *J. Agric. Food Chem.* **50(26)**: 7720-7725.

Nestel, P.J., Connor, W.E., Reardon, M.F., Connor, S., Wong, S. and Boston, R. (1984) Suppression by diets rich in fish oil of very low density lipoprotein production in man. *J. Clin. Invest.* **74(1)**: 82-89.

Nistor, A., Bulla, A., Filip, D.A. and Radu, A. (1987) The hyperlipidemic hamster as a model of experimental atherosclerosis. *Atherosclerosis* **68(1-2)**: 159-173.

Nozaki, S., Garg, A., Vega, G.L. and Grundy, S.M. (1991) Postheparin lipolytic activity and plasma lipoprotein response to omega-3 polyunsaturated fatty acids in patients with primary hypertriglyceridemia. *Am. J. Clin. Nutr.* **53(3)**: 638-642.

Nyby, M.D., Matsumoto, K., Yamamoto, K., Abedi, K., Eslami, P., Hernandez, G., Smutko, V., Berger, M.E. and Tuck, M.L. (2005) Dietary fish oil prevents vascular dysfunction and oxidative stress in hyperinsulinemic rats. *Am. J. Hypertens.* **18**: 213-219.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95(2)**: 351-358.

Ohtani, H., Hayashi, K., Hirata, Y., Dojo, S., Nakashima, K., Nishio, E., Kurushima, H., Saeki, M. and Kajiyama, G. (1990) Effects of dietary cholesterol and fatty acids on plasma cholesterol level and hepatic lipoprotein metabolism. *J. Lipid Res.* **31**: 1413–1422.

O'Malley, P.G. (2004) Review: vitamin E, vitamin C, and possibly coenzyme Q10 are ineffective for preventing or treating cardiovascular disease. *ACP J Club* **140(3)**: 73.

Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R.A., Bashmakov, Y., Goldstein, J.L. and Brown, M.S. (2001) Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. U.S.A.* **98(11)**: 6027-6032.

Overturf, M.L., Smith, S.A., Gotto, A.M. Jr., Morrisett, J.D., Tewson, T., Poorman, J., Loose-Mitchell, D.S. (1990) Dietary cholesterol absorption, and sterol and bile acid excretion in hypercholesterolemia-resistant white rabbits. *J. Lipid Res.* **31(11)**: 2019-2027.

Park, Y. and Harris, W.S. (2003) Omega-3 fatty acid supplementation accelerates chylomicron triglyceride clearance. *J. Lipid Res.* **44(3)**: 455-463.

Pawar, A., Botolin, D., Mangelsdorf, D.J. and Jump, D.B. (2003) The role of liver X receptor-alpha in the fatty acid regulation of hepatic gene expression. *J. Biol. Chem.* **278(42)**: 40736–40743.

Pergola, C., Rossi, A., Dugo, P., Cuzzocrea, S. and Sautebin, L. (2006) Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide* **15(1)**: 30-39.

Peyron-Caso, E., Quignard-Boulange, A., Laromiguiere, M., Feing-Kwong-Chan, S., Véronèse, A., Ardouin, B., Slama, G. and Rizkalla, S.W. (2003) Dietary fish oil increases lipid mobilization but does not decrease lipid storage-related enzyme activities in adipose tissue of insulin-resistant, sucrose-fed rats. *J. Nutr.* **133(7)**: 2239-2243.

Putadechakum, S., Tanphaichitr, V., Leelahagul, P., Pakpeankitvatana, V., Surapisitchart, T. and Komindr, S. (2005) Long-term treatment of N-3 PUFAS on plasma lipoprotein levels and fatty acid composition of total serum and erythrocyte lipids in hypertriglyceridemic patients. *J. Med. Assoc. Thai.* **88(2)**: 181-186.

Rambjor, G.S., Walen, A.I., Windsor, S.L. and Harris, W.S. (1996) Eicosapentaenoic acid is primarily responsible for hypotriglyceridemic effect of fish oil in humans. *Lipids* **31**: S45–S49.

Ramirez-Tortosa, C., Andersen, O.M., Gardner, P.T., Morrice, P.C., Wood, S.G., Duthie, S.J., Collins, A.R. and Duthie, G.G. (2001) Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radic. Biol. Med.* **31(9)**: 1033-1037.

Rémillard, P., Shen, G., Milne, R. and Maheux, P. (2001) Induction of cholesteryl ester transfer protein in adipose tissue and plasma of the fructose-fed hamster. *Life Sci.* **69(6)**: 677-687.

Ren, B., Thelen, A.P., Peters, J.M., Gonzalez, F.J. and Jump, D.B. (1997) Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.* **272(43)**: 26827-26832.

Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L. and Mangelsdorf, D.J. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* **14(22)**: 2819-2830.

Rivellese, A.A., Maffettone, A., Vessby, B., Uusitupa, M., Hermansen, K., Berglund, L., Louheranta, A., Meyer, B.J. and Riccardi, G. (2003) Effects of dietary saturated, monounsaturated and n-3 fatty acids on fasting lipoproteins, LDL size and post-prandial lipid metabolism in healthy subjects. *Atherosclerosis* **167(1)**: 149-158.

Roche, H.M. and Gibney, M.J. (1996) Postprandial triacylglycerolaemia: the effect of low-fat dietary treatment with and without fish oil supplementation. *Eur. J. Clin. Nutr.* **50(9)**: 617-624.

Rossi, A., Serraino, I., Dugo, P., Di Paola, R., Mondello, L., Genovese, T., Morabito, D., Dugo, G., Sautebin, L., Caputi, A.P. and Cuzzocrea, S. (2003) Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. *Free Radic. Res.* **37(8)**: 891-900.

Rudel, L.L. and Shelness, G.S. (2000) Cholesterol esters and atherosclerosis-a game of ACAT and mouse. *Nat. Med.* **6(12)**:1313-1314.

Salati, L.M. and Clarke, S.D. (1986) Fatty acid inhibition of hormonal induction of acetyl-coenzymeA carboxylase in hepatocyte monolayers. *Arch. Biochem. Biophys.* **246(1)**: 82–89.

Sampath, H. and Ntambi, J.M. (2005) Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu. Rev. Nutr.* **25**: 317-340.

Sanders, T.A., Oakley, F.R., Miller, G.J., Mitropoulos, K.A., Crook, D. and Oliver, M.F. (1997) Influence of n-6 versus n-3 polyunsaturated fatty acids in diets low in saturated fatty acids on plasma lipoproteins and hemostatic factors. *Arterioscler. Thromb. Vasc. Biol.* **17(12)**: 3449-3460.

Satue-Gracia, M.T., Heinonen, M. and Frankel, E.N. (1997) Anthocyanins as antioxidants on human low density lipoprotein and lecithin-liposome systems. *J. Agr. Food Chem.* **45**: 3362-3367.

Sealls, W., Gonzalez, M., Brosnan, M.J., Black, P.N. and DiRusso, C.C. (2008) Dietary polyunsaturated fatty acids (C18:2 omega6 and C18:3 omega3) do not suppress hepatic lipogenesis. *Biochim. Biophys. Acta.* **1781(8)**: 406-414.

Shekelle, P.G., Morton, S.C., Jungvig, L.K., Udani, J., Spar, M., Tu, W.J., Suttorp, M., Coulter, I., Newberry, S.J. and Hardy, M. (2004) Effect of supplemental vitamin E for the prevention and treatment of cardiovascular disease. *J. Gen. Intern. Med.* **19(4)**: 380-389.

Shidfar, F., Keshavarz, A., Jallali, M., Miri, R. and Eshraghian, M. (2003) Comparison of the effects of simultaneous administration of vitamin C and omega-3 fatty acids on lipoproteins, apo A-I, apo B, and malondialdehyde in hyperlipidemic patients. *Int. J. Vitam. Nutr. Res.* **73(3)**: 163-170.

Shimomura, I., Bashmakov, Y., Shimano, H., Horton, J. D., Goldstein, J. L. and Brown, M. S. (1997) Cholesterol feeding reduces nuclear forms of sterol regulatory element binding proteins in hamster liver. *Proc. Natl. Acad. Sci. USA.* **94:** 12354–12359.

Simopoulos, A.P. (1999) Essential fatty acids in health and chronic disease. *Am. J. Clin. Nutr.* **70(3 Suppl)**: 560S-569S.

Spady, D.K. and Dietschy, J.M. (1988) Interaction of dietary cholesterol and triglycerides in the regulation of hepatic low density lipoprotein transport in the hamster. *J. Clin. Invest.* **81**: 300–309.

Sposito, A.C., Santos, R.D., Hueb, W., Ventura, L.I., Vinagre, C.C., Ramires, J.A. and Maranhão, R.C. (2002) LDL concentration is correlated with the removal from the plasma of a chylomicron-like emulsion in subjects with coronary artery disease. *Atherosclerosis* **161(2)**: 447-453.

Sprecher, H. (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochim. Biophys. Acta* **1486 (2-3)**: 219-231.

Stintzing, F.C., Stintzing, A.S., Carle, R., Frei, B. and Wrolstad, R.E. (2002) Color and antioxidant properties of cyanidin-based anthocyanin pigments. *J. Agric. Food Chem.* **50(21)**: 6172-6181.

Suarez', A., Ramírez-Tortosa, M., Gil, A. and Faus, M.J. (1999) Addition of vitamin E to long-chain polyunsaturated fatty acid-enriched diets protects neonatal tissue lipids against peroxidation in rats. *Eur. J. Nutr.* **38(4)**: 169-176.

Sullivan, D.R., Sanders, T.A., Trayner, I.M. and Thompson, G.R. (1986) Paradoxical elevation of LDL apoprotein B levels in hypertriglyceridaemic patients and normal subjects ingesting fish oil. *Atherosclerosis* **61(2)**: 129-134.

Sullivan, M.P., Cerda, J.J., Robbins, F.L., Burgin, C.W. and Beatty, R.J. (1993) The gerbil, hamster, and guinea pig as rodent models for hyperlipidemia. *Lab. Anim. Sci.* **43(6)**: 575-578.

Surai, P.F. and Sparks, N.H. (2000) Tissue-specific fatty acid and alpha-tocopherol profiles in male chickens depending on dietary tuna oil and vitamin E provision. *Poult. Sci.* **79(8)**: 1132-1142.

Tall, A.R. (1993) Plasma cholesteryl ester transfer protein. *J. Lipid Res.* **34(8)**: 1255-1274.

Teissedre, P.L., Frankel, E.N., Waterhouse, A.L., Peleg, H. and German, J.B. (1996) Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. *J. Sci-Food Agric.* **70 (1)**: 55-61.

Theobald, H.E., Chowienczyk, P.J., Whittall, R., Humphries, S.E. and Sanders, T.A. (2004) LDL cholesterol-raising effect of low-dose docosahexaenoic acid in middle-aged men and women. *Am. J. Clin. Nutr.* **79 (4)**: 558-563.

Thiery, J. and Seidel, D. (1987) Fish oil feeding results in an enhancement of cholesterol-induced atherosclerosis in rabbits. *Atherosclerosis* **63(1)**: 53-56.

Tinker, L.F., Parks, E.J., Behr, S.R., Schneeman, B.O. and Davis, P.A. (1999) (n-3) fatty acid supplementation in moderately hypertriglyceridemic adults changes postprandial lipid and apolipoprotein B responses to a standardized test meal. *J. Nutr.* **129(6)**: 1126-1134.

Trautwein, E.A, Liang, J. and Hayes, K.C. (1993) Plasma lipoproteins, biliary lipids and bile acid profile differ in various strains of Syrian hamsters, mesocritus auratus. *Comp. Biochem. Physiol.* **104A:** 829-835.

Tsao, R. and Deng, Z. (2004) Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* **812(1-2)**: 85-99.

Tsuda, T., Horio, F., Uchida, K., Aoki, H. and Osawa, T. (2003) Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J. Nutr.* **133(7)**: 2125-2130.

Tsutsumi, K. (2003) Lipoprotein lipase and atherosclerosis. *Curr. Vasc. Pharmacol.* **1(1)**: 11-17.

Turley, S.D. and Dietschy, J.M. (1988) The metabolism and excretion of cholesterol by the liver. The Liver: Biology and Pathobiology (Chapter-34) Second Ed. Raven press Ltd., New York.

Valcheva-Kuzmanova, S., Kuzmanov, K., Tancheva, S. and Belcheva, A. (2007a) Hypoglycemic and hypolipidemic effects of Aronia melanocarpa fruit juice in streptozotocin-induced diabetic rats. *Methods Find Exp. Clin. Pharmacol.* **29(2)**: 101-105.

Valcheva-Kuzmanova, S., Kuzmanov, K., Mihova, V., Krasnaliev, I., Borisova, P. and Belcheva, A. (2007b) Antihyperlipidemic effect of Aronia melanocarpa fruit juice in rats fed a high-cholesterol diet. *Plant Foods Hum. Nutr.* **62(1)**: 19-24.

Valeille, K., Férézou, J., Amsler, G., Quignard-Boulangé, A., Parquet, M., Gripois, D., Dorovska-Taran, V. and Martin, J.C. (2005) A cis-9,trans-11-conjugated linoleic acid-rich oil reduces the outcome of atherogenic process in hyperlipidemic hamster. *Am. J. Physiol. Heart Circ. Physiol.* **289(2)**: H652-H659.

Vinson, J.A., Dabbagh, Y.A., Serry, M.M. and Jang, J. (1995) Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. *J. Agric. Food Chem.* **43**: 2800-2802.

Vivekananthan, D.P., Penn, M.S., Sapp, S.K., Hsu, A. and Topol, E.J. (2003) Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet* **361(9374)**: 2017-2023.

Vognild, E., Elvevoll, E.O., Brox, J., Olsen, R.L., Barstad, H., Aursand, M. and Osterud, B. (1998) Effects of dietary marine oils and olive oil on fatty acid composition, platelet membrane fluidity, platelet responses, and serum lipids in healthy humans. *Lipids* **33(4)**: 427-436.

Wang, H., Lu, S., Du, J., Yao, Y., Berschneider, H.M. and Black, D.D. (2001) Regulation of apolipoprotein secretion by long-chain polyunsaturated fatty acids in newborn swine enterocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280(6)**: G1137- G1144.

Wang, H., Cao, G. and Prior, R.L. (1997) Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **45**: 304-309.

Wang, H.H., Hung, T.M., Wei, J. and Chiang, A.N. (2004) Fish oil increases antioxidant enzyme activities in macrophages and reduces atherosclerotic lesions in apoE-knockout mice. *Cardiovasc. Res.* **61(1)**: 169-176.

Wang, J. and Mazza, G. (2002) Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **50(4)**: 850-857.

Wilkinson, J., Higgins, J.A., Fitzsimmons, C. and Bowyer, D.E. (1998) Dietary fish oils modify the assembly of VLDL and expression of the LDL receptor in rabbit liver. *Arterioscler. Thromb. Vasc. Biol.* **18(9)**: 1490-1497.

Williamson, K.S., Hensley, K. and Floyd, R.A. (2003) Fluorometric and colorimetric assessment of thiobarbituric acid-reactive lipid aldehydes in biological matrices. Methods in Pharmacology and Toxicology: Methods in Biological Oxidative Stress; Hensley, K., Floyd, R. A., Eds. Humana Press: Totowa, NJ. 57-65.

Woollett, L.A., Spady, D.K. and Dietschy, J.M. (1989) Mechanisms by which saturated triacylglycerols elevate the plasma low density lipoprotein-cholesterol concentration in hamsters. Differential effects of fatty acid chain length. *J. Clin. Invest.* **84(1)**: 119-128.

Wu, X., Gu, L., Prior, R.L. and McKay, S. (2004) Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity. *J. Agric. Food Chem.* **52(26)**: 7846-7856.

Xia, M., Ling, W.H., Ma, J., Kitts, D.D. and Zawistowski, J. (2003) Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein E deficient mice. *J. Nutr.* **133(3)**: 744-751.

Xia, M., Hou, M., Zhu, H., Ma, J., Tang, Z., Wang, Q., Li, Y., Chi, D., Yu, X., Zhao, T., Han, P., Xia, X. and Ling, W. (2005) Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages: the role of the peroxisome proliferator-activated receptor {gamma}-liver X receptor {alpha}- ABCA1 pathway. *J. Biol. Chem.* **280(44)**: 36792-36801.

Xia, X., Ling, W., Ma, J., Xia, M., Hou, M., Wang, Q., Zhu, H. and Tang, Z. (2006) An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *J. Nutr.* **136(8)**: 2220-2225.

Xu, H.E., Lambert, M.H., Montana, V.G., Parks, D.J., Blanchard, S.G., Brown, P.J., Sternbach, D.D., Lehmann, J.M., Wisely, G.B., Willson, T.M., Kliewer, S.A. and

Milburn, M.V. (1999a) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* **3(3)**: 397-403.

Xu, J., Nakamura, M.T., Cho, H.P. and Clarke, S.D. (1999b) Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J. Biol. Chem.* **274(33)**: 23577-23583.

Yahagi, N., Shimano, H., Hasty, A.H., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S. and Yamada, N. (1999) A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. *J. Biol. Chem.* **274(50)**: 35840–35844.

Yamakoshi, J., Kataoka, S., Koga, T. and Ariga, T. (1999) Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **142(1)**: 139-149.

Yokode, M., Hammer, R.E., Ishibashi, S., Brown, M.S. and Goldstein, J.L. (1990) Diet-induced hypercholesterolemia in mice: prevention by overexpression of LDL receptors. *Science* **250(4985)**: 1273-1275.

Yoshida, H., Ikeda, I., Tomooka, M., Mawatari, M., Imaizumi, K., Seto, A. and Tsuji, H. (2001) Effect of dietary seal and fish oils on lipid metabolism in hamsters. *J. Nutr. Sci. Vitaminol. (Tokyo)* **47(3)**: 242-247.

Yoshida, H., Mawatari, M., Ikeda, I., Imaizumi, K., Seto, A. and Tsuji, H. (1999) Effect of dietary seal and fish oils on triacylglycerol metabolism in rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* **45(4)**: 411-421.

Yoshikawa, M., Shimoda, H., Nishida, N., Takada, M. and Matsuda, H. (2002a) Salacia reticulata and its polyphenolic constituents with lipase inhibitory and lipolytic activities have mild antiobesity effects in rats. *J. Nutr.* **132(7)**: 1819-1824.

Yoshikawa, T., Shimano, H., Yahagi, N., Ide, T., Amemiya-Kudo, M., Matsuzaka, T., Nakakuki, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Takahashi, A., Sone, H., Osuga Ji, J., Gotoda, T., Ishibashi, S. and Yamada, N. (2002b) Polyunsaturated fatty acids suppress sterol regulatory element-binding protein1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J. Biol. Chem.* **277(3)**: 1705–1711.

Youdim, K.A., Martin, A. and Joseph, J.A. (2000) Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic. Biol. Med.* **29(1)**: 51-60.

Yuan, Y.V. and Kitts, D.D. (2003) Dietary (n-3) fat and cholesterol alter tissue antioxidant enzymes and susceptibility to oxidation in SHR and WKY rats. *J. Nutr.* **133(3)**: 679-688.

Zern, T.L., Wood, R.J., Greene, C., West, K.L., Liu, Y., Aggarwal, D., Shachter, N.S. and Fernandez, M.L. (2005) Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *J. Nutr.* **135(8)**: 1911-1917.