EFFECT OF DIETARY OMEGA-3 AND OMEGA-6 POLYUNSATURATED FATTY ACIDS ON ALCOHOLIC LIVER DISEASE

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Preeti Purwaha

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Steven Qian

Chair

Dr. Jagdish Singh

Dr. Benedict Law

Dr. Chung Park

Approved:

13 March 2012

Date

Dr. Jagdish Singh

Department Chair

ABSTRACT

PUFAs have been shown to modulate ALD by several mechanisms, including free radical generation from hepatic lipid peroxidation. However, how they modulate lipid peroxidation and generation of bioactive metabolites in ALD is poorly understood and it is still not clear which PUFAs (ω-3 or ω-6) are beneficial or detrimental in ALD. Thus, our objective was to study the effect of ω-3/ω-6 PUFAs on lipid peroxidation and ethanol mediated steatosis and inflammation. Using standard liquid diet (LDC), LDC with fish oil (rich in ω-3) and safflower oil (rich in ω-6), we studied the generation of bioactive metabolites, such as eicosanoids and free radicals generated via lipid peroxidation. In addition, we determined the effect of PUFAs on several inflammatory and fibrotic factors, e.g. gene as well as protein expression, using western blot and RT-PCR, respectively. We also investigated the effect of PUFA diets on novel targets, such as hepatic membrane transporters with potential role in liver inflammation.

Our results suggest that ω-3 diet prevented while ω-6 based diets promoted the development of fatty liver and inflammation. ω-3 PUFA reduced AA-peroxidation by lowering hepatic AA concentration and expression of peroxidation enzymes, COX-2 and 5-LOX, resulting in lower generation of pro-inflammatory AA-derived PGs (Series-2), HETEs and free radicals, along with increase in anti-inflammatory EPA and DHA-derived PGs (Series-3). ω-3 diet might also reduce liver inflammation by preventing activation of NF-κB and induction of TNF-α. Rats fed with ω-3 diet showed high protein expression of efflux transporters, MRP-2 and ABCA1, indicating elimination of peroxidation metabolites and triglycerides from the liver and decreased inflammation. In contrast, ω-6 diets led to

increase in AA-peroxidation and generation of AA-derived pro-inflammatory metabolites. ω-6 based diets also promoted fatty liver and inflammation by activating NF-κB, inducing TNF-α and downregulation of efflux transporters, MRP-2 and ABCA1.

This study not only provides new insights into the effects and possible mechanisms by which ω-3 and ω-6 PUFAs may alter hepatic steatosis and inflammation, but also put forward new targets of research, such as hepatic membrane transporters in relation to liver pathology in ALD.

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AA…….....	arachidonic acid
ABCA1……..	ATP-binding cassette transporter A1
ALD……	alcoholic liver disease
ALT……	alanine amino transferase
AST……	aspartate amino transaminase
COX……	Cyclooxygenase
CYP2E1…….	cytochrome P2E1
DHA……	docosahexaenoic acid
EIC……..	extracted ion current chromatogram
EPA…….	eicosapentaenoic acid
ESR…….	electron spin resonance
FATP……..	fatty acid transport proteins
Fish-LDC……	lieber-di-carli diet modified with fish oil
GSH……	reduced glutathione
GSSG……..	oxidized glutathione
HPLC……..	high performance liquid chromatography
4-HNE…….	4-hydroxynonenal
4-HHE……	4-hydroxyhexenal
HETE……..	hydroxyeicosatetraenoic acid
IG…….	intra-gastric
i-NOS……..	inducible nitric oxide synthase
IL……	Interleukin

LA…… linoleic acid

LC…… liquid chromatography

LDC…… lieber-di-carli

LPS…….. Lipopolysaccharide

LTB_4…… leukotriene B_4

MDA…… Malondialdehyde

MRM…… multiple reaction monitoring

MRP…… multi-drug resistance associated protein

MS…… mass spectrometry

NFκB…… nuclear factor κB

OATP…… organic anion transport proteins

PG…… Prostaglandins

$PGF_{2\alpha}$…… isoprostane 8-isoprostaglandin F_2 alpha

POBN…… α-[4-pyridyl-1-oxide]-N-tert-butyl nitrone

PPAR…… peroxisome proliferator receptor activator

PUFA…… polyunsaturated fatty acids

RT-PCR…… real time-polymerase chain reaction

ROS……...… reactive oxygen species

Saff-LDC…….. lieber-di-carli diet modified with safflower oil

SAMe…… S-adenosyl methionine

TGF-β…… transforming growth factor-β

TNF-α…….... tumor necrosis factor-α

CHAPTER 1. INTRODUCTION

1.1. Introduction

1.1.1. Alcoholic liver disease (ALD)

Alcoholic liver disease (ALD) is a major cause of illness and death in United States [1-3]. In the initial stages of the disease, fat accumulation in hepatocytes leads to the development of fatty liver (steatosis), a reversible condition. If alcohol consumption is continued, steatosis may progress to hepatitis and fibrosis, eventually leading to liver cirrhosis [1]. Decreasing the accumulation of fat within the liver during alcohol consumption may block or delay the progression of fatty liver to hepatitis and fibrosis [1-3]. Alcoholic fatty liver (steatosis) is thus considered a pathological condition that may predispose the liver to further injury (hepatitis and fibrosis) by several mechanisms, including cytochrome P450 2E1 (CYP2E1) induction [2, 3], increased free radical generation and lipid peroxidation [2-5], nuclear factor κB activation (NF-κB), and increased transcription of pro-inflammatory mediators, e.g. tumor necrosis factor-alpha (TNF-α), etc [2-4]. Some other factors can also affect susceptibility to ALD, including ethnicity, gender, age, poor nutrition, obesity, hepatitis C viral infection and polymorphisms in genes such as CYP2E1, glutathione S-transferases (GST) and TNF-α [5-11]. Our research is focused on the effect of alteration in lipid peroxidation and its related free radical generation, expression of hepatic membrane transporters on inflammation and development of alcoholic steatosis by dietary ω-3 and ω-6 PUFAs.

1.1.2. Role of hepatic-lipid peroxidation in ALD

It is now widely accepted that chronic alcohol ingestion initiates a pro-oxidant environment in the liver. Ethanol metabolism has been thought to involve oxidative stress

by generation of reactive oxygen species (ROS), such as hydroxyethyl radicals, peroxyl radicals and superoxide, etc [12-15]. These ROS are thought to further initiate hepatic lipid peroxidation and increase free-radical generation. Increased overall radical production during ethanol induced liver injury, including hydroxyethyl radicals derived from ethanol and the presumed lipid-derived radicals had previously been correlated with the degree of severity of liver injury [16-19].

Polyunsaturated fatty acids (PUFAs) are especially vulnerable to peroxidation because they contain weak carbon-hydrogen bonds [20]. The weakest carbon-hydrogen bonds that are highly susceptible to lipid peroxidation are those of the bis-allylic methylene positions [21]. In-vivo lipid peroxidation is a very complex free-radical event in which different kinds of free radicals could form from a variety of peroxidation pathways. Free radical mediated lipid peroxidation has three major components: initiation, propagation and termination reactions [21, 22]. The process begins when a free-radical abstracts a hydrogen atom from a PUFA, forming a lipid radical ($L^{\bullet}$) **(Figure 1)** which quickly reacts with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). Peroxyl radical ($LOO^{\bullet}$) can then abstract a hydrogen atom from another PUFA to form a new lipid radical and lipid hydroperoxide (LOOH) (Figure1). Through Fenton type reaction, lipid hydroperoxide (LOOH) leads to formation of lipid alkoxyl radical ($LO^{\bullet}$) that may undergo β-scission to form carbon-centered radicals, such as the pentyl radical (Figure1). Alkoxyl radical can also give rise to epoxyl radical ($^{\bullet}LO$) and epoxyperoxyl radical ($OLOO^{\bullet}$), which are considered to be the major propagating species along with peroxyl radical in this system [23-25].

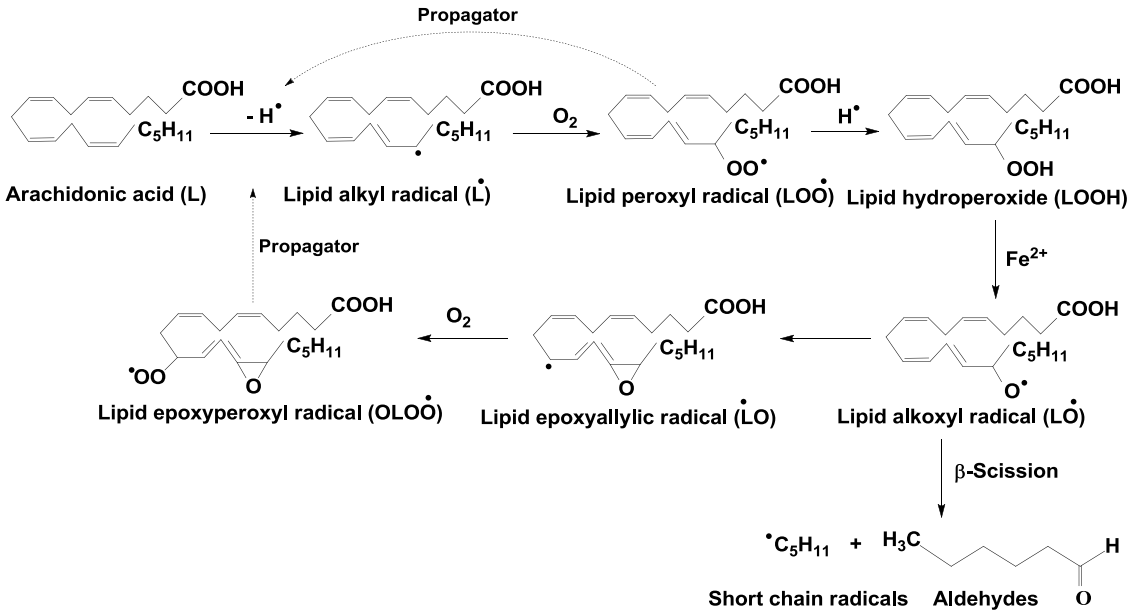


Figure 1. Pathway of initiation and propagation of lipid peroxidation. As shown in scheme, different types of lipid-derived free radicals and non-radical products, e.g, aldehydes are generated [24, 26-29]. Epoxyperoxyl radical ($OLOO^{\bullet}$) and peroxyl radical ($LOO^{\bullet}$) may be the major propagators in lipid peroxidation [23-25]. Lipid peroxidation could be mediated by both enzymatic (COX and LOX) and non-enzymatic (auto-oxidation) pathways.

Decomposition of lipid hydroperoxides may also give rise to end products, such as alcohols, ketones, ethers and aldehydes. Some end products, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are biologically active and possess cytotoxic potential [30]. Possible mechanisms for formation of MDA from PUFAs involve the formation of bicyclo-endoperoxide intermediates which subsequently breakdown to MDA by auto or enzyme catalyzed reaction, such as those mediated by 5-LOX or 15-LOX [31, 32] **(Figure 2)**.

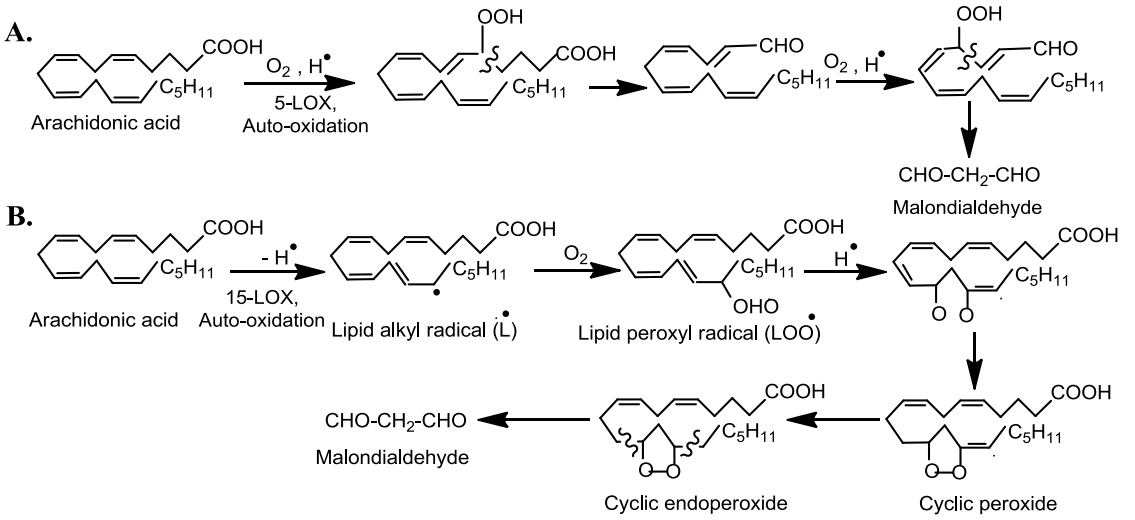


Figure 2. Proposed pathway of generation of malondialdehyde (MDA). MDA can be generated from AA via: (A) 5-LOX catalyzed peroxidation or auto-oxidation, and (B) 15-LOX catalyzed peroxidation or auto-oxidation [31, 32].

Suggested mechanism for formation of 4-HNE from ω-6 PUFA, such as AA, involve the formation of 11-hydroperoxide. Reduction of this hydroperoxide (e.g by iron) and further cyclization gives rise to a cyclic hydroperoxide which undergoes beta-scission and hydrolysis leading to generation of 4-HNE [33, 34] **(Figure 3)**.

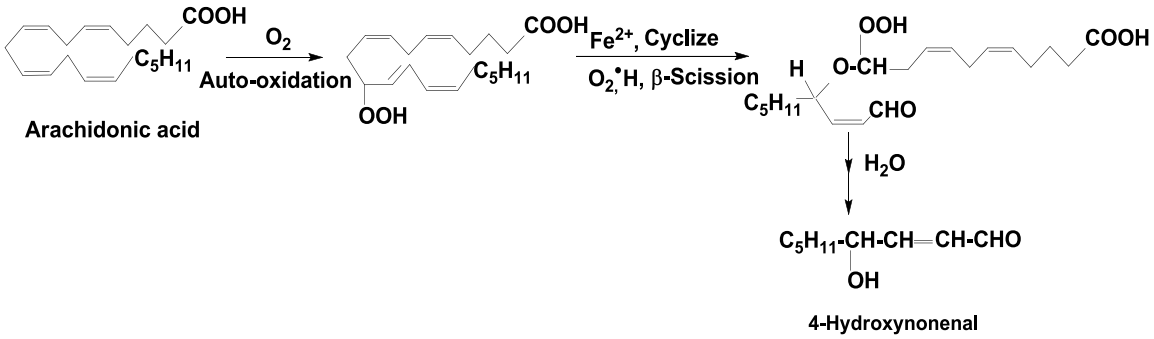


Figure 3. Proposed pathway of generation of 4-hydroxynonenal (4-HNE).

Lipid peroxidation can take place in the absence or presence of enzymes. Enzymatic lipid peroxidation is brought about by the action of two peroxidizing enzymes, e.g. cycloxygenase (COX) and lipoxygenase (LOX). It has been proposed that lipids are

important contributors to alcoholic liver injury, probably acting through the actions of the enzyme COX [35]. There are two major isoforms of COX, COX-1 and COX-2. COX-1 exists constitutively in several tissues and is responsible for normal physiological functions. COX-2 could be induced by lipid peroxidation and inflammatory stimuli, such as cytokines and endotoxins, the two important contributors to alcohol mediated liver injury [36-38]. COX catalyzes arachidonic acid (AA) to synthesize prostaglandin PGG_2 which gets converted to PGH_2 by the peroxidase activity of COX. PGH_2 is subsequently converted into a series of PGs, as well as thromboxanes (TX), such as TXA_2 **(Figure 4)** [35]. Reactive oxygen intermediates and lipid hydroperoxides have also been shown to regulate the expression of COX-2 [39]. TNF-α and COX-2 have been found to be co-expressed in the kupffer cells of ethanol fed rats as evidence of necro-inflammatory changes [40]. In this thesis, we studied the alteration in generation of COX-2 mediated AA-derived eicosanoid metabolites after dietary administration of ω-3 and ω-6 PUFA rich diets and its effect on ethanol induced liver inflammation.

Another enzyme implicated in lipid peroxidation is the lipoxygenase (LOX). Out of the various isoforms of LOX (5-, 15-, 12- and 8-LOX), 5-LOX has previously been implicated in the progression of chronic liver diseases [41-46]. 5-LOX catalyzes the oxygenation of AA to produce 5-hydroperoxyeicosatetraenoic acid (5-HpETE) followed by synthesis of leukotrienes, such as LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4, which display a variety of pro-inflammatory actions. LTB_4 has been shown to be one of the most potent chemotactic and activating factors for leukocytes [47, 48]. LTC_4 and LTD_4 are potent vasoactive mediators that regulate vascular permeability (Figure 4) [41, 45, 46].

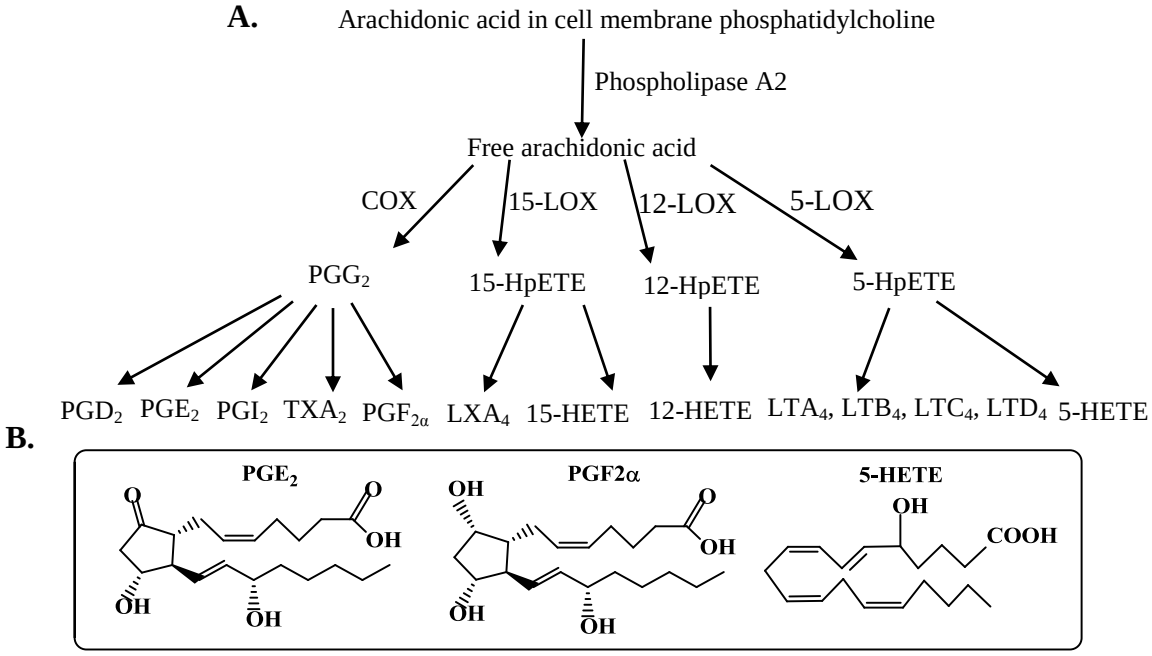


Figure 4. Eicosanoid biosynthesis from arachidonic acid. (A) Outline of pathway of eicosanoid biosynthesis from AA peroxidaton catalyzed by cyclooxygenase and lipoxygenase [49, 50], and (B) Chemical structures of metabolites (PGE_2, $PGF_{2\alpha}$ and 5-HETE) with pro-inflammatory effects, measured in this thesis to study their alteration after administration of different dietary PUFAs in ALD.

Various studies have indicated the correlation of eicosanoid production from AA (series-2 PGs and series-4 LTs, Figure 4), such as prostaglandins, thromboxanes and leukotrienes. For example, PGE_2, TXB_2, LTB_4 show pathological pro-inflammatory changes observed in ALD [39, 51]. In this thesis, we studied the alteration in generation of LOX mediated AA-derived eicosanoid metabolites, after dietary administration of ω-3 and ω-6 PUFA rich diets, in relation to ethanol induced inflammation. The generation of AA-derived free-radicals generated in vivo has never been studied mainly due to the lack of appropriate methodology. In this thesis, we tentatively identified the free radicals generated in vivo via peroxidation of AA and their possible source of generation, including both enzymatic and non-enzymatic lipid peroxidation pathways.

1.1.3. Effect of dietary PUFAs on liver inflammation

Several lines of investigation indicate that dietary fat, including both saturated and unsaturated fatty acids, can modulate the severity of ALD since ethanol potentially affects the metabolism of fatty acids [19, 52-62]. Free radical formation and the related peroxidation of various PUFAs have been implicated in the development and progression of ALD [18, 63, 64]. As described earlier in Sec 1.1.2, the effects of PUFAs in ALD are thought to be mediated through increased oxidative stress [15, 58-61, 65] by COX and LOX mediated lipid peroxidation [35, 39, 41, 43]. Both ethanol and dietary PUFAs may lead to generation of reactive species, such as free radicals and eicosanoids, contributing in alteration of liver injury in ALD **(Figure 5)**.

Ethanol administration has been shown to decrease the levels of antioxidant enzymes and enhance lipid peroxidation [19]. Some of the other mechanisms through which the fatty acids have been proposed to promote ALD include increase in oxidative stress, production of endotoxin and increased expression of pro-inflammatory cytokines (described in detail in section 1.1.4). Studies have also shown that dietary modifications with saturated fatty acids reduced liver damage significantly as compared to diets with polyunsaturated fatty acids [40, 58, 66]. Some investigators reported that ethanol administration led to decrease in tissue PUFA concentration because of increased fatty acid catabolism through lipid peroxidation and generation of ROS [12-14]. Thus, many factors may influence the ethanol mediated liver injury via alteration of liver PUFA concentration, e.g. the fatty acid composition of diet, amount and duration of alcohol consumption, and the balance of anabolic and catabolic processes (Figure 5).

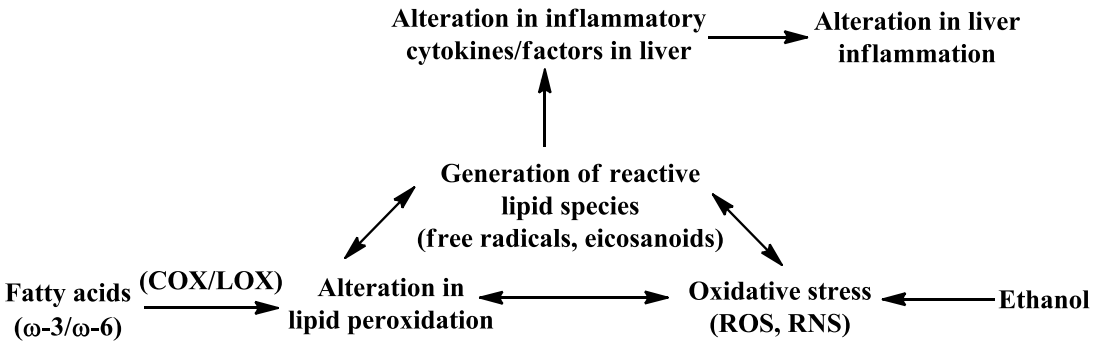


Figure 5. Proposed pathway of alteration in liver inflammation. Both ethanol and dietary PUFAs may alter the peroxidation of PUFAs via alteration of both expression of peroxidation enzymes and availability of substrates (PUFAs), thus affecting the generation of reactive lipid species having pro-or anti-inflammatory activities, thereby contributing in alteration of liver injury in ALD.

Conflicting evidence has been presented from several investigators regarding which type of PUFAs, e.g. ω-3 or ω-6, lead to exacerbation of inflammation and progression of liver injury in ALD [14, 19, 57, 67-71]. Some investigators reported that corn oil (rich in ω-6 PUFAs) caused less severe ethanol induced liver injury in comparison to fish oil (rich in ω-3 PUFAs) [19]. Fish oil led to development of more severe liver injury by increase in level of CYP2E1 [14]. However, the ingestion of fish oil on the day prior to ethanol treatment was found in another study to reduce ethanol induced fatty liver by 73% in mice [67]. Thus, it is still not clear whether ω-3 or ω-6 PUFAs exert beneficial or detrimental effect in alteration of ethanol induced liver injury.

While some studies have shown the deleterious effects of ω-3 PUFAs in ALD, several others indicate the anti-inflammatory effects of these PUFAs [57, 68-71]. Eicosapentaenoic acid (EPA), the most abundant ω-3 PUFA, has been shown to be a substrate for enzymes COX/LOX, limiting the net production of AA-derived pro-inflammatory compounds, such as PGs, TXs and LTs.

Lipoxygenase products of EPA, LTs of the 5-series have been shown to possess lower inflammatory activity than the corresponding LTs of the 4-series derived from AA **(Figure 6)** [56, 57, 72]. In addition to changes in AA-derived eicosanoid metabolism, EPA and docosahexaenoic acid (DHA, another major ω-3 PUFA) have been shown to be the precursors of the anti-inflammatory E-series and D-series of resolvins, respectively. Resolvins, including resolvin D and resolvin E series (Figure 6) are a family of novel endogenous lipid mediators which display potent anti-inflammatory and pro-resolution actions in animal models of inflammation [57, 68-71]. Unlike AA which is the precursor of pro-inflammatory series-2 PGs and series-4 LTs, EPA and DHA are the precursors of anti-inflammatory series-3 PGs, series-5 LTs resolvins E and D series, respectively, via COX and LOX pathways (Figure 6). Emerging evidence was found to support a potent anti-hyperalgesic role of resolvins in animal models of inflammatory pain [57, 68-71].

Despite the existing evidence of the anti-inflammatory effects of ω-3 PUFAs, it is still not clear how PUFAs exert beneficial or detrimental effects in ALD. In this thesis, we studied the effect of dietary ω-3 and ω-6 PUFAs on lipid peroxidation (of AA) and generation of eicosanoids and AA derived free radicals on ethanol induced liver inflammation. We found that diets rich in ω-3 PUFAs increased the generation of anti-inflammatory (series-3) eicosanoids by reduced AA peroxidation and lowered the generation of pro-inflammatory (series-2) eicosanoids, consequently reducing ethanol induced liver inflammation.

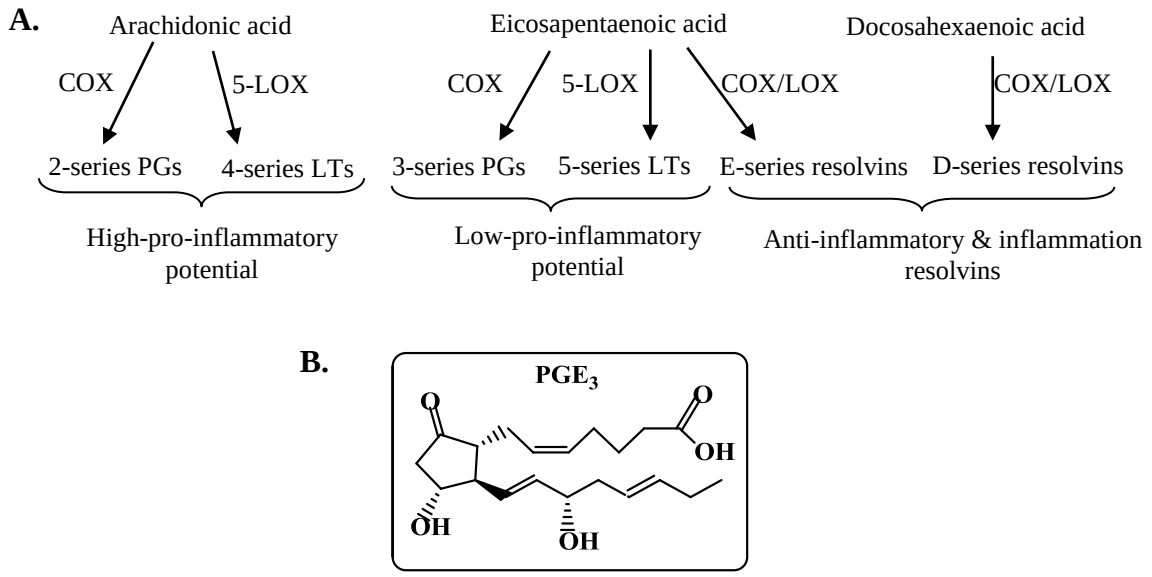


Figure 6. Generation of lipid mediators by AA, EPA and DHA via COX/LOX mediated peroxidation. (A) Lipid mediators derived from AA, EPA and DHA via COX/LOX mediated peroxidation with their inflammatory potential, and (B) Chemical structure of EPA metabolite, PGE_3 with anti-inflammatory effect, measured in this thesis to study its alteration by different dietary PUFAs in ethanol induced liver inflammation.

1.1.4. Alteration of cytokine generation in ALD by ω-3 and ω-6 PUFAs

The liver consists of several cell types, such as hepatocytes, endothelial cells, kupffer cells (liver macrophages) and stellate cells. Under normal circumstances these cells produce only minimal levels of cytokines. Cytokine production increases when the liver cells, particularly kupffer cells become activated by stimulants such as alcohol or dietary fatty acids [73-77]. Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), further activate kupffer cells to release more of the same cytokines. Clinical studies have demonstrated that ALD patients show increased levels of inflammatory cytokines IL-1, IL-6 and TNF-α, as well as the chemokine IL-8, etc [78-80]. These cytokines may be responsible for some of the symptoms associated with alcoholic hepatitis, such as fever, metabolic changes and

weight loss. TNF-α can trigger both apoptosis and necrosis [81]. Alcohol may directly or indirectly stimulate kupffer cells to produce and release TNF-α into small channels called sinusoids in which the blood flows through the liver [78, 82]. Secondly, alcohol may increase the sensitivity of hepatocytes to TNF-α [83]. Alcohol-mediated increase in gut-derived endotoxin (toxic bacterial products) from oxidative stress mechanisms sensitizes hepatic macrophages to release inflammatory cytokines, TNF-α, IL-1β, IL-1α and IL-6. These cytokines in turn activate stellate cells and hepatocytes **(Figure 7)** [84-86].

In addition, alcohol consumption increases permeability of the intestine, permitting endotoxin to pass through the intestinal wall into the bloodstream [87]. Upon reaching the liver, endotoxin stimulates the kupffer cells to produce cytokines which may contribute to liver inflammation (Figure 7) [88, 89]. Endotoxin also affects activation of stellate and endothelial cells which contributes to liver injury [90] (Figure 7). ROS and pro-inflammatory cytokines have also been implicated in abnormal activation of hepatocytes and stellate cells (HSC) and induction of fibrosis [91]. Thus, an impaired immune response mediated by alcohol contributes to increased apoptosis of both infected and non-infected cells, thereby contributing to liver damage.

In addition to ethanol, dietary ω-3 and ω-6 PUFAs have also been shown to affect the secretion of cytokines [56, 70, 71] in several inflammatory pathological conditions. When administered to healthy volunteers, ω-3 PUFAs have been shown to decrease bacterial endotoxin induced pro-inflammatory cytokines, e.g. IL-1 and TNF-α from peripheral blood lymphomonocytes [92-94]. ω-3 PUFAs have also been shown to modulate the activation of nuclear factor kappa B (NF-κB) in several inflammatory conditions [92, 94-98]. Some studies have indicated that linoleic acid (ω-6 PUFA) activated NF-κB and

TNF-α production in porcine peripheral blood mononuclear cells [99]. Fats rich in ω-6 PUFAs were also shown to enhance IL-1 production and tissue responsiveness to cytokines, while ω-3 PUFAs were shown to have the opposite effect [100].

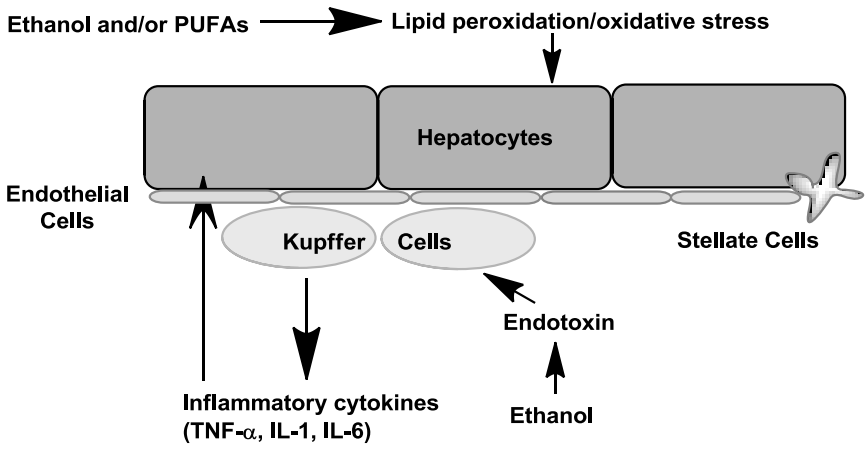


Figure 7. Cell types with the immune mechanisms involved in alcoholic liver injury. Ethanol may exert its effects via several inter-related pathways. Ethanol mediated alteration in lipid peroxidation may activate the hepatocytes, kupffer cells and stellate cells. Activation of kupffer cells stimulates the release of inflammatory cytokines. In another pathway, ethanol by mediating endotoxin activates the kupffer cells, endothelial cells and stellate cells, secretion of pro-inflammatory cytokines, e.g. TNF-α, IL-1 and IL-6 resulting in liver injury [90].

TNF-α can also increase the metabolism of the hepatocytes leading to increased production of ROS in the mitochondria [101, 102]. These ROS in turn could activate a regulatory protein, NF-κB, transcription factor which can control the activities of numerous genes (e.g. genes encoding for TNF-α and one of its receptors) [2-4, 103, 104]. Thus, a "feedback cycle" is established in the hepatocytes, TNF-α promotes ROS production, ROS production activates NF-κB which leads to enhanced production of more TNF-α and its receptors, e.g. TNF receptor 1 (TNFR1) as well as production of factors that promote apoptosis **(Figure 8)**. This cycle eventually alters the structure of hepatocytes, impairs their function and leads to hepatocyte apoptosis [90].

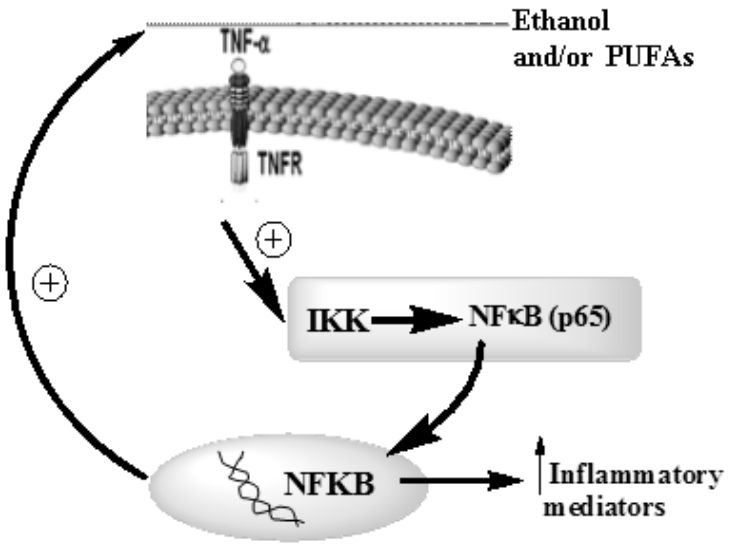


Figure 8. Feedback cycle for activation of NF-κB by TNF-α. TNF-α activates the TNF receptor type-I, and subsequently causes the disintegration of the inhibitor complex IKK and activation of transcription factor NF-κB which further leads to generation of inflammatory mediators including TNF-α [105, 106].

Studies from several investigators have reported that blocking of NF-κB reduced alcohol induced inflammation and necrosis thereby preventing the development of ALD. NF-kB exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to an inhibitor protein, IkB. The currently known subunit members of the NF-kB family in mammals are p50, p65 (RelA), c-Rel, p52, and RelB, [107, 108]. Most research on NF-kB has been focused on the p50/p65 dimer, and its phosphorylated p65 subunit being the predominant form of activated NF-kB in many cells [2]. In contrast to pro-inflammatory cytokines, immunoregulatory cytokines, e.g. IL-10 reduce production of pro-inflammatory cytokines and promote secretion of antibodies, [80]. Activation of kupffer cells has been shown to produce IL-10, which thus may play a protective role in ALD [109].

Although some studies have investigated the role of dietary PUFAs in alteration of pro- and anti-inflammatory cytokines, their modulation in alcohol induced liver steatosis has not been studied. In this thesis, we observed that diets rich in ω-3 PUFAs reduced the activation of NF-κB and pro-inflammatory cytokine TNF-α but increased expression of anti-inflammatory cytokine IL-10, thus reducing ethanol induced liver inflammation. In contrast, diets rich in ω-6 PUFAs acted as 'double hit', i.e. the second stimulant for inflammation, while ethanol being the first stimulant of hepatic inflammation [73-77]. By increasing the activation of NF-κB and induction of TNF-α, ω-6 PUFAs further promoted liver inflammation.

The pathogenesis of ALD involves the interaction of several other intracellular signaling pathways in different cell types in liver. It has been proposed that bacterial endotoxin (LPS) recognition by toll like receptors (TLR_4) on macrophages and other cell types in the liver [110-112] and activation of downstream signaling pathways results in activation of transcription factors, such as NF-κB, leading to increased inflammatory cytokine production in ALD [3, 4]. In addition to the enhanced pro-inflammatory cytokine production in ALD, ethanol also induces the release of pro-fibrogenic cytokines, such as transforming growth factor β (TGFβ-1) from hepatic stellate cells (HSCs) [113, 114]. Ethanol also upregulates the gene expression of collagen I and enhances type I collagen protein production by HSCs [113, 114]. TGF-β further contributes to liver damage by activation of hepatic stellate cells and collagen synthesis, resulting in scar tissue formation and development of liver fibrosis [115-118]. Ethanol further perpetuates an activated HSC phenotype through extracellular matrix remodeling, thus leading to liver fibrosis. In this thesis, we studied the alteration in expression of pro-fibrotic factors, e.g. TGF-β, collagen I

and smooth muscle actin (SMA) by dietary ω-3 and ω-6 PUFAs and their relationship with progression of ethanol induced steatosis to fibrosis.

1.1.5. Fatty acid beta-oxidation and lipid peroxidation

In addition to alteration of lipid peroxidation, the amount of dietary fat intake has been shown to be the principal determinant of the degree of fatty infiltration in animals and humans ingesting alcohol [53, 54, 58]. Long term ethanol consumption has been shown to inhibit the enzymes involved in beta-oxidation of fatty acids [119-123]. Ethanol has also been shown to increase the rate of fatty acid and triglyceride synthesis in liver by inducing the expression of key enzymes involved in lipid synthesis, leading to fat accumulation and mitochondrial dysfunction [119-123].

Peroxisome proliferator activated receptor-alpha (PPAR-α) is the transcription factor involved in fatty acid metabolism (beta-oxidation) and a member of the nuclear hormone receptor superfamily. PPAR-α is a key transcriptional regulator of many genes involved in free fatty acid (FFA) oxidation system in liver, including acyl-coA oxidase, liver fatty acid-binding protein and lipoprotein lipase [124]. PPAR-α controls transcription by binding to peroxisome proliferator response elements (PPREs) present in several genes involved in FFA transport and oxidation, including membrane transporters like carnitine palmitoyl transferase-I (CPT-I), apolipoprotein genes and several components of the mitochondrial and peroxisomal fatty acid oxidation pathways [125]. PPAR-α can be activated upon binding of FFA, thus the expression of PPAR-α plays significant role in determining the intracellular concentration of fatty acids in the liver. [125]. Several studies have investigated the role of PPAR-α in the pathogenesis of alcoholic fatty liver.

The mRNA level of PPAR-α was found to be decreased by ethanol feeding which led to increased concentration of FFA in the liver [126, 127]. This increase in FFA concentration in the liver may in turn alter the rate and extent of lipid peroxidation since these FFAs, including several PUFAs act as substrates for lipid peroxidation. Thus, alteration in the expression of PPAR-α may alter the rate of lipid peroxidation and may also affect the type of PUFAs available in the liver to undergo lipid peroxidation.

The alteration in expression of PPAR-α by administration of diets rich in ω-3 and ω-6 PUFAs in relation to disease severity in ALD has still not been investigated. In this thesis, we observed that ω-6 PUFA rich diet led to significant downregulation of PPAR-α gene expression. Downregulation of PPAR-α may possibly cause the dysregulation of fatty acid metabolism in the liver, contributing in the development of ethanol induced fatty liver or hepatic steatosis. These observations provide a good starting point for further studies involving the role of PPAR-α in alteration of lipid peroxidation and ethanol induced liver injury.

1.1.6. Hepatic membrane transporters in ALD

Recent studies suggest that the extent of liver damage may also be associated with changes in the various elimination mechanisms operating in the liver. Administration of lipopolysaccharide (LPS, *Escherichia coli*), a potent inflammatory mediator has been shown to result in cholestasis which was found to be associated with change in the expression of several liver transporters [47, 48]. Pro-inflammatory cytokines are shown to be the major mediators in transcriptional regulation of liver transporter genes, including Na^+-taurocholate co-transporting polypeptide (Ntcp), organic anion transporting polypeptides (Oatp1a1/Oatp1, Oatp1a4/Oatp2), multidrug resistant associated proteins,

MRP-2, MRP-3, bile salt export pump (BSEP), MDR1A and MDR1B **(Figure 9)** [128, 129]. Multidrug resistant associated proteins (MRPs) are efflux transporters known to play a major role in hepato-biliary and renal elimination of many structurally diverse xenobiotics, including glutathione conjugates and endogenous molecules, such as LTC_4 and PGs (products of lipid peroxidation) [130].

Out of the several isoforms of MRP, MRP-1 and MRP-3 are localized on the basolateral membrane of epithelial cells in the tissues and pump their substrates, e.g. GSH conjugates into the interstitial space (bloodstream) rather than excreting them into the bile [131, 132]. MRP-3, together with MRP-2, is called the GS-X pump, which also transports glutathione conjugates [133]. MRP-2 is mainly expressed on the canalicular membrane of hepatocytes and plays a vital role in the defense against oxidative stress by exporting glutathione conjugates of 4-HNE [134]. Decreased rat MRP-2 expression has been found in various cholestatic models [135, 136], however its alteration in ethanol mediated steatohepatitis has not been studied. It has also been reported that MRP-3 overexpression compensates for the impaired expression of MRP-2 in hyperbilirubinuria in rats (MRP-2 deficient condition) [137]. Both MRP-2 and MRP-3 may be altered in ethanol mediated liver inflammation, since MRP-3 expression has been shown to compensate for the decreased expression of MRP-2 in inflammatory conditions [137].

Another hepatic transporter known to participate in the regulation of intracellular cholesterol accumulation in hepatocytes is the ATP-binding cassette transporter A1 (ABCA1), a member of the ABC subfamily A of membrane transporters. Recent studies have suggested the association of hepatic steatosis with PUFA mediated decrease in ABCA1 protein expression, consequently leading to an increase in lipid storage in

hepatocytes [138-141]. Overexpression of ABCA1 resulted in decrease of cellular fatty acids and triglycerides, while their repression by ABCA1 siRNA increased both cellular fatty acids and triglycerides [138]. Rats with non-alcoholic steatohepatitis showed lower ABCA1 protein levels in liver cells as compared to normal rats [138]. Thus, we also investigated whether ABCA1 protein levels would be altered in liver of rats with alcoholic steatohepatitis (Figure 9).

In this thesis, we studied the PUFA mediated alteration in expression of efflux transporters (ABCA1, MRP-2 and MRP-3) in relation to disease severity in ALD. In addition to liver efflux transporters, hepatic uptake transporters, such as peroxisomal ABC transporters encoded by the ABCD genes are also thought to participate in the import of specific fatty acids in the peroxisomal matrix [144]. The hepatic expression of ABCD2 and ABCD3 are highly sensitive towards dietary PUFAs, indicating their possible involvement in PUFA metabolism [144].

Furthermore, fatty acid transport proteins (FATPs) or solute carrier family 27 (SLC-27) are integral membrane proteins that enhance the uptake of long-chain fatty acids into cells (Figure 9) [145]. Tumor-necrosis factor-α (TNF-α) has been shown to be a negative regulator of SLC-27 expression in the liver and adipocytes [146, 147]. This suggests a possible role of these uptake transporters in modulation of liver injury by means of regulating the hepatic PUFA content and the mechanisms exerted by these PUFAs thereafter.

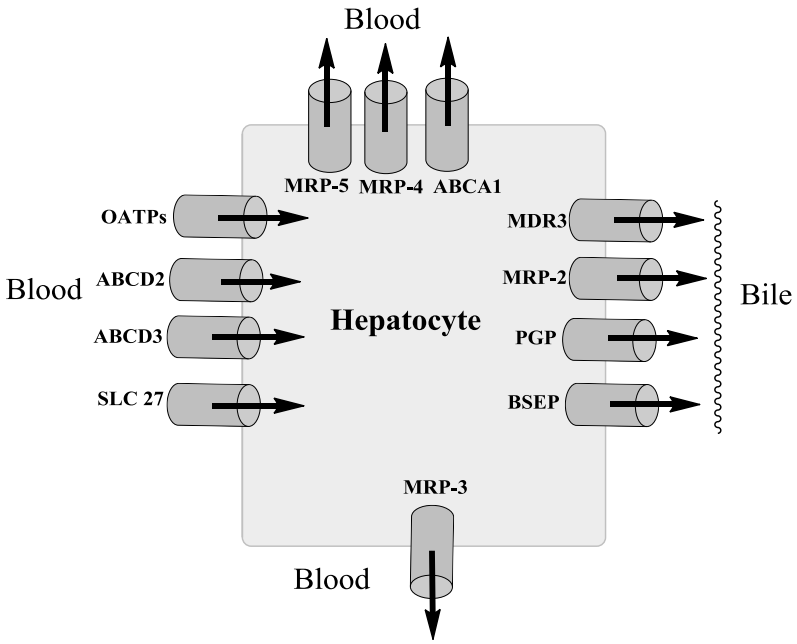


Figure 9. Hepatic membrane transporters involved in uptake of fatty acids and efflux of metabolites. Uptake transporters, ABCD2, ABCD3 and SLC-27 and efflux transporters, MRP-2, MRP-3 and ABCA1 involved in either uptake of fatty acids in liver or efflux of their metabolites, such as GSH conjugates, leukotrienes, etc out of liver [142, 143].

However, it still remains to be investigated how ethanol or diets rich in ω-3 and ω-6 PUFAs alter the expression of these uptake transporters and whether their expression correlates with disease severity in ALD.

In this thesis, we studied the alteration in expression of liver uptake (ABCD2, ABCD3, and SLC 27) and efflux transporters (MRP-2, MRP-3 and ABCA1) implicated in transport of fatty acids or their peroxidation metabolites. We found that ω-3 PUFA rich diets administered along with ethanol prevented the downregulation of hepatic efflux transporters (MRP-2 and ABCA1). In contrast, ω-6 PUFA rich diets administered with ethanol showed downregulation in protein expression of MRP-2 and ABCA1. This could result in accumulation of fatty acids and peroxidation metabolites in liver promoting lipid peroxidation and inflammation. However, no change in the gene expression of uptake

transporters was observed in any of the diets (ω-3 or ω-6 rich) administered with ethanol in comparison to their respective dextrose controls. These observations provide a good starting point for further studies investigating the role of MRP-2 and ABCA1 in alteration of lipid peroxidation and ethanol induced liver injury.

1.1.7. Formation of free radicals

Both acute and chronic ethanol administration leads to formation of radicals in the liver that are presumed to be the products of lipid peroxidation [14, 52, 148, 149]. However, no direct evidence regarding the structure or identity of these radicals has been found in the past because of unavailability of appropriate methodology. To detect short lived and highly reactive species, electron spin resonance (ESR) has been used with spin trapping technique in which unstable radicals react with spin traps to form relatively stable radical adducts [24, 29, 150-153]. Spin-trapping employs nitrone or nitroso compounds that react with the free radical to give a more-stable, ESR-observable free radical adduct [154]. The radical adduct can then be identified from the ESR spectral parameters, e.g. the hyperfine splitting constants [154].

In biological systems, spin trapping has been used to detect hydroxyl and superoxide radicals and other free radical metabolites of endogenous substances, xenobiotics, and lipid peroxidation products [24, 29, 152-155].

One of the most commonly used spin traps, the nitrone compound α-(4-pyridyl-1-oxide)-N-tertiary butyl nitrone (POBN), has been widely used to detect a variety of carbon-centered radicals in many spin trapping studies of lipid peroxidation in chemical, cellular and in vivo systems [154, 156, 157]. One of the major advantages of using POBN as a spin-trap agent is the long lifetime of hours or even days of the carbon-centered radical

adducts formed. However, the major limitation of using POBN for structural identification of free radicals is that POBN adducts show almost identical ESR spectra for many different carbon-centered radicals [154-159]. This is because hyperfine interactions with neighboring nuclei with non-zero spins, namely N, splits the signal into three and H further splits the signal into two, forming a total of six-line ESR spectra [158, 159] **(Figure 10)**.

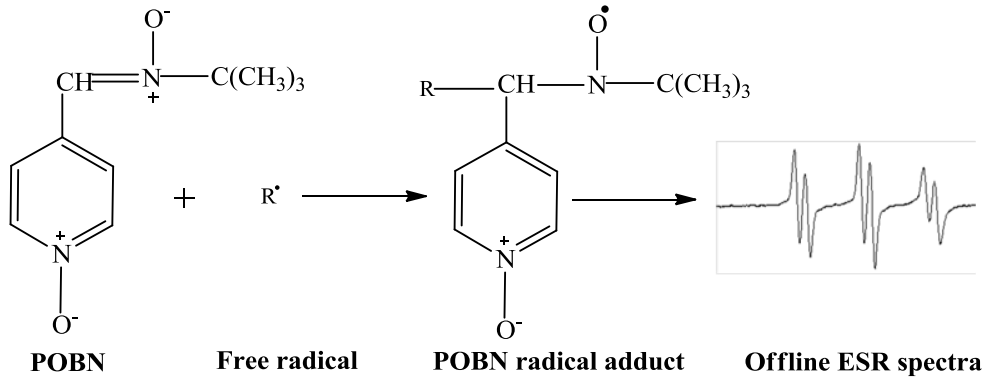


Figure 10. Spin-trapping of free radicals by POBN. Spin-trapping reaction with the characteristic six-line ESR spectra observed for almost all POBN radical adducts [154-159], making it impossible to identify the detailed chemical structure of individual radicals.

To overcome the difficulty in structural identification posed by using POBN as the spin-trap agent, the techniques of liquid chromatography (LC), electron spin resonance (ESR), and mass spectrometry (MS) have been used in combination **(Figure 11)**. This has resulted in a major breakthrough in radical identification since different radical adducts are first separated and sequentially eluted from the HPLC column according to their polarity, followed by ESR detection and characterization of molecular mass by MS (Figure 11). LC/ESR system enables the monitoring of POBN radical adducts via UV absorption, followed by ESR detection. The LC/MS system consists of an HPLC system and a Mass spectrometer (Figure 11) with chromatographic conditions identical to those used for online LC/ESR system.

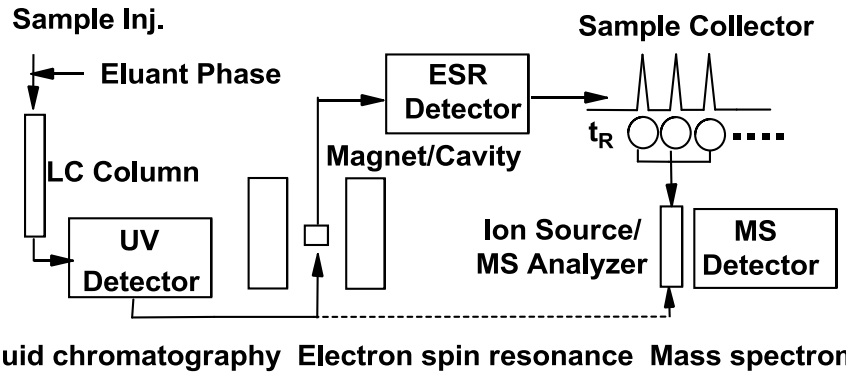


Figure 11. Radical identification using the combined techniques of LC/ESR and LC/MS. Radical adducts are first separated and sequentially eluted from the HPLC column, followed by ESR detection and characterization of molecular mass by MS [152, 156, 160-163, 174-178].

The total ion current chromatogram (TIC) obtained from the LC/MS of biological samples may be highly complex and show several compounds having similar retention times. In order to facilitate the identification of radical adducts in biological samples, both POBN and deuterated (D_9-POBN) were used in combination as spin trapping agents (called dual spin-trapping). LC/MS analysis of dual spin trapped bile samples from rats injected D_0-D_9 POBN mixture was used for m/z assignments and also exclude artifactual ions that might otherwise have been assigned to radical-related products. D_9-POBN spin trapping gave LC/MS profiles similar to those of POBN, except for slightly shorter retention times and the 9-da difference for each corresponding radical adduct [160-164]. With this protocol we tentatively characterized radicals generated in vivo by peroxidation of AA. Up to six-different radical adducts derived by AA-peroxidation were tentatively identified using bile from rats fed Saff-LDC diet. In addition, PUFA mediated alteration in their generation was also studied.

1.1.8. Animal models of ALD

Two frequently used animal models in ALD study are the lieber-di-carli liquid diet and the intragastric tubing feeding model. Tsukamoto and French developed continuous enteral–ethanol administration via intragastric infusion in which a catheter was implanted intragastrically, facilitating a continuous infusion of ethanol up to 16.5 g/kg/day [165, 166]. Thus, very high blood alcohol levels could be achieved using the Tsukamoto and French model. This model was also able to produce pathological changes that resembled human ALD, including micro vesicular and macro vesicular fat, meta-mitochondria, apoptosis, central lobular and pericellular fibrosis, portal fibrosis, bridging fibrosis, central necrosis, and infiltrating inflammatory leukocytes as well as lymphocytes [167-169]. However, the major limitation of this model included the complexity of the procedure involved and expensive maintenance of the animals along with high mortality rate of the animals used.

The voluntary oral ethanol feeding rat model first introduced in 1963 [170], involves oral administration of lieber di carli diet for 8 weeks along with isocaloric pair fed controls [171]. Lieber di carli diet includes 18% calories from protein, 12% from carbohydrates, 35% from fat and 36% energy derived from ethanol or from dextrose in case of controls. This model can replicate most of the biochemical changes, e.g. elevation in serum ALT/AST activities, increase in inflammatory cytokines, such as TNF-α observed in alcoholic liver disease [171].

The introduction of technique of feeding alcohol as part of the total liquid diet provided an extremely useful tool for the study of pathological disorders associated with alcoholism as it resulted in much greater daily ethanol intake of about 12-18 g/kg [172]. The liquid-diet model also facilitated the technique of pair-feeding which is essential to

control the nutritional variables like volume of the food intake. Another advantage of liquid diet technique is that it allows for relatively easy changes in dietary constituents [172], such as the type or percentage of fatty acids or alteration of the carbohydrate content of the diet. Such changes in the dietary constituents facilitate the monitoring of the effect of the targeted dietary constituents and associated pathways [172].

In the current study, we modified the PUFA content to create diets having different ω-6 to ω-3 ratios using 1) standard lieber di carli liquid diet, 2) safflower oil, rich in ω-6 LA and 3) fish oil, rich in ω-3 EPA and DHA. We studied the effect of these PUFAs (ω-3 and ω-6) on several aspects of ALD, such as modulation of lipid peroxidation, state of liver inflammation, and liver uptake and elimination mechanisms. The PUFA composition of the three different dietary regimens, the standard lieber di carli diet (LDC), safflower oil (Saff-LDC), and fish oil (Fish-LDC) diet is shown below **(Table 1)**.

Std. lieber-di carli diet	
Fatty Acid	%
Linoleic Acid	12
Linolenic Acid	0.08

ω-6:ω-3:150:1

Safflower oil	
Fatty Acid	%
Linoleic Acid	73
Linolenic Acid	0.19

ω-6:ω-3:384:1

Fish oil	
Fatty Acid	%
Linoleic Acid	2.2
Arachidonic acid	1.7
Eicosapentaenoic Acid	14
Docosahexaenoic Acid	7
Docosapentaenoic Acid	2
Linolenic acid	1.6

ω-3:ω-6:6.25:1

ω-6 PUFA
ω-3 PUFA

Table 1. PUFA composition of the three different diets. Safflower oil, rich in ω-6 LA and fish oil, rich in ω-3 EPA and DHA were used to modify the fat component of the standard lieber-di carli diet (LDC) to create diets having very high, low and moderately high ω-6 to ω-3 ratios, respectively.

1.2. Objective

Alcoholic fatty liver (hepatic steatosis) is a pathological condition that predisposes the liver to further injury (hepatitis and fibrosis) by several mechanisms, including free radical generation and lipid peroxidation [1-5]. The increased free radical production during alcohol mediated liver injury has been correlated with the degree of lipid peroxidation of PUFAs, e.g. ω-3 and ω-6 PUFAs [19, 52-62]. Several studies have shown that markers of lipid peroxidation and oxidative stress altered significantly when ω-3 or ω-6 PUFAs were co-administered with alcohol [52-62]. However, it is still not clear how PUFAs modulate lipid peroxidation and generation of reactive lipid metabolites associated with ethanol induced liver injury. There is also conflicting evidence regarding the type of PUFAs that exert beneficial or deleterious effects in ALD, which thus need to be further investigated [14, 19, 57, 67-71].

The objective of this research was to study the effect of ω-3 and ω-6 PUFA rich diets on modulation of lipid peroxidation, inflammatory and fibrotic factors, hepatic membrane transporters and the associated development of ethanol mediated fatty liver (steatosis) and inflammation. Thus, we investigated: (1) how ω-3 and ω-6 PUFAs affect the development of fatty liver or hepatic steatosis mediated by ethanol (and/or PUFAs), with associated changes in liver histopathology and function; (2) whether ω-3 and ω-6 PUFAs could differentially alter the generation of pro-inflammatory, pro-fibrotic factors and signal transduction pathways, e.g. NF-κB and modulate the state of liver inflammation; (3) how ω-3 and ω-6 PUFAs could alter lipid peroxidation mediated by the enzymes cycloxygenase (COX) and lipoxygenase (LOX), thus lead to the formation of different eicosanoids, the pro or anti-inflammatory products; (4) how ω-3 or ω-6 PUFAs alter the expression of liver

uptake and efflux transporters, and thus play crucial role in regulating the influx of fatty acid substrates and elimination of lipid metabolites from liver.

In addition, in order to provide a basis for further studies investigating the role of free-radicals in liver injury, we aimed to identify the AA-derived free radicals generated in vivo, along with the possible source (enzymatic and non-enzymatic) of their generation. To accomplish the objectives of this thesis, we designed and conducted the following studies described in forthcoming chapters, for which the methodology has been described in detail in Chapter 2.

1.3. Effect of ω-3 and ω-6 PUFA rich diets on ethanol induced liver injury

1.3.1. To determine the effect of ω-3 and ω-6 PUFA rich diets on ethanol induced liver pathology and function

In this study we determined the effect of ω-3 or ω-6 PUFAs on ethanol induced liver pathology and function, using three different diets fed with ethanol (ALD) and their respective controls fed isocaloric dextrose (Ctrl) for 8-weeks. We determined the effects of PUFAs on liver histology and clinical markers of liver function during their development (Chapter 3). The experimental design for this study (described in detail in Chapter 2) consisted of:

a) Feeding the three dietary regimen, 1) standard lieber di carli liquid diet (LDC), 2) safflower oil modified diet (Saff-LDC), rich in ω-6 LA and 3) fish oil modified diet (Fish-LDC), rich in ω-3s, e.g. EPA and DHA administered with ethanol (ALD) or isocaloric dextrose (Ctrl) for 8-weeks.

b) Measurement of animal weights in all groups prior to the start of feeding and every week on regular basis for 8-weeks. This also included monitoring the animals for any signs of sickness or stress related symptoms such as uneasiness, loss of appetite, etc.

c) Blood samples were collected every week via tail vein, serum was separated and used for biochemical measurements of clinical markers of liver injury like alanine amino transferase (ALT) and aspartate amino transaminase (AST) activity.

d) Bile and liver were collected after 8-weeks of feeding period for further analysis for measurement of free radicals and eicosanoids.

e) At the time of end point of experiment, small pieces from each liver (different lobes) were collected, formalin fixed and stained with hematoxylin and eosin (H & E) and used for light microscopy. Histological assessment of development of fatty liver and inflammation were carried out by a pathologist on a scale of 0-3, who had no prior knowledge of the dietary groups.

1.3.2. To determine the effect of ω-3 and ω-6 PUFA rich diets on ethanol induced liver inflammation and fibrosis

We determined the alteration of pro- and anti-inflammatory and pro-fibrotic factors in progression of inflammation and fibrosis from the three different modified dietary regimen, 1) LDC, 2) Saff-LDC and 3) Fish-LDC, administered with ethanol (ALD) or isocaloric dextrose (control) diet for 8-weeks. We evaluated the alteration of expression of pro and anti-inflammatory cytokines, transcription factors and pro-fibrotic factors, in relation to disease severity in ALD (Chapter 4). The experimental design for this study (described in detail in Chapter 2) consisted of:

a) RT-PCR analysis for determination of gene expression of pro- and anti-inflammatory (IL-1, IL-4, IL-6, TNF-α, IL-10) pro-fibrotic markers (TGF-β, smooth muscle actin (SMA) and collagen) and transcription factors (NF-κB and PPAR-α).

b) ELISA for determination of protein expression of TNF-α in rat livers

c) Western blot analysis for determination of activation of transcription factor NF-κB measured by determination of protein level of the phosphorylated NF-κB (p65) subunit.

1.3.3. To determine the alteration in AA peroxidation by ω-3 and ω-6 PUFA rich diets and the effect on ethanol induced liver inflammation

We studied the effect of dietary ω-3 and ω-6 PUFAs on lipid peroxidation, mediated by the enzymes COX or LOX and their relationship with ethanol mediated liver inflammation (Chapter 5). Ethanol has been shown to affect the metabolism of dietary fatty acids by liver which may lead to alteration in PUFA concentrations in the liver and alter lipid peroxidation [58, 66, 173]. Since PUFAs act as substrates for many lipid peroxidation enzymes, such as COX and LOX, we determined the concentrations of free AA, LA, EPA and DHA in the livers of rats fed different diets. The concentration of AA was measured since it is the precursor of several bioactive metabolites having pro-inflammatory effects, formed via its peroxidation mediated both by enzymatic as well as non-enzymatic peroxidation. The concentration of LA, EPA and DHA were also measured since they are the major components of the dietary supplements used to modify the fat component of the LDC diet. LA is the major PUFA constituent of safflower oil, and EPA and DHA are the major PUFA constituents of fish oil.

We also evaluated the alteration of hepatic COX and LOX expression by the different PUFA diets administered in ALD and Ctrl diets for 8-weeks. We then assessed the

generation of end products of COX and LOX mediated peroxidation of AA, such as eicosanoids–prostaglandins (PGs), hydroxyeicosatetraenoic acid (HETEs), and AA-derived free radicals (Chapter 5). The experimental design for this study (described in detail in Chapter 2) consisted of:

a) Real-time RT-PCR and western blot analysis for determination of gene and protein expression of lipid peroxidation enzymes COX and LOX, and oxidation enzyme CYP2E1.

b) LC/MS for determination of free fatty acid concentration of arachidonic acid (AA), linoleic acid (LA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) in rat livers.

c) LC/MS for quantification of eicosanoids–prostaglandins (PGE_2, $PGF_{2\alpha}$) and hydroxyeicosatetraenoic acids (HETEs) formed from COX and LOX mediated peroxidation, respectively, in rat livers.

d) Offline ESR analysis for measurement of AA-derived free radical generation in liver and bile using in vivo spin trapping.

e) LC/ESR and LC/MS analysis for characterization of AA-derived free radicals generated in vivo using dual spin trapping.

1.3.4. To determine the effect of ω-3 and ω-6 PUFA rich diets on ethanol induced alteration in hepatic membrane transporters

We studied the effect of ethanol and/or dietary PUFAs in alteration of several liver uptake and efflux transporters for their possible role in the uptake and elimination of fatty acids and their metabolites (Chapter 6). The experimental design for this study (described in detail in Chapter 2) consisted of:

a) RT-PCR for determination of gene expression of liver uptake transporters: SLC 27 A5, ABCD2, ABCD3 and efflux transporters: MRP-2, ABCA1, MRP-3.

b) Western blot analysis for determination of protein expression of efflux transporters MRP-2 and ABCA1 in rat liver.

1.4. Significance

Our results suggest that Fish-LDC diet (high ω-3 PUFA) prevented, while LDC and Saff-LDC diets (moderate and high ω-6 PUFA, respectively) promoted the development of ethanol induced fatty liver and inflammation. Fish-LDC diet reduced AA-peroxidation by lowering hepatic AA concentration as well as decreasing the expression of peroxidation enzymes, COX-2 and 5-LOX. This resulted in lower generation of pro-inflammatory, AA-derived metabolites such as PGs (Series-2), HETEs and free radicals. Due to high EPA and DHA concentration in rats fed Fish-LDC diet, there was increase in generation of anti-inflammatory, EPA and DHA-derived PGs (Series-3). Further, by preventing activation of NF-κB and reducing the induction of pro-inflammatory cytokine TNF-α, Fish-LDC diet significantly reduced liver inflammation. In addition, rats fed Fish-LDC diet showed high expression of liver efflux transporters, MRP-2 and ABCA1. Since MRP-2 is involved in elimination of peroxidation metabolites and GSH conjugates, its upregulation prevented the hepatic accumulation of reactive metabolites and thus reduced inflammation. Upregulation of ABCA1 may also be involved in elimination of fatty acids and triglycerides, consequently leading to reduced lipid peroxidation of fatty acids and accumulation of fat in rats fed Fish-LDC diet.

In contrast, ω-6 PUFA rich diets (LDC-ALD and Saff-LDC ALD) increased AA-peroxidation by increasing hepatic AA concentration and expression of COX-2 and 5-LOX. This resulted in high generation of pro-inflammatory, AA-derived metabolites, such as PGs (Series-2), HETEs and free radicals, increasing inflammation in these rats. Further,

by activating NF-κB and inducing TNF-α, these diets promoted ethanol mediated inflammation. Rats fed LDC-ALD and Saff-LDC ALD diets showed downregulation of MRP-2 and ABCA1, resulting in accumulation of peroxidation metabolites and fatty acids in liver, which may also promote fatty liver and inflammation in these rats.

The information gained from this study provides new insights into the effects of ω-3 and ω-6 PUFA rich diets in alteration of ethanol and/or PUFA mediated hepatic steatosis and inflammation. We found that AA-peroxidation may play critical role in development of ALD from its association with pro-inflammatory metabolite generation. To our knowledge, this is the first study to provide a basis to further explore the role of hepatic membrane transporters in modulation of lipid peroxidation and liver inflammation in ALD.

CHAPTER 2. MATERIALS AND METHODS

2.1. Animals and dietary regimen

The experimental design consisted of six groups of male Sprague dawley rats (200-220 g) (Charles River, Hollister, CA). Group I was the ALD group fed by ethanol containing diet (standard lieber di carli, LDC) [170, 172] (Bioserve, Frenchtown, NJ) for eight weeks orally (LDC ALD, n=20), and its control (Group II) was pair fed isocaloric dextrose containing diet (LDC Ctrl, n=20). Group III was fed with ethanol containing LDC diet modified by PUFAs using safflower oil, rich in ω-6 (Welch, Holme & Clark Co, Inc, Newark, NJ), (Saff-LDC ALD, n=20), control of Group III (Group IV) was fed with the Saff-LDC diet with isocaloric dextrose (Saff-LDC Ctrl, n=20). Group V was fed with ethanol containing LDC diet with modified PUFA using fish oil, rich in ω-3 (Omega Protein Inc., Houston, TX) (Fish-LDC ALD, n=20), and the pair fed control (Group VI) was fed with Fish-LDC diet with isocaloric dextrose (Fish-LDC Ctrl, n=20).

Experimental rats in each group were also sub-divided for acute treatment with AA and spin trap POBN to enable the detection of free-radicals. Rats with intra-peritoneal injections of these compounds were divided as follows: (i) no acute treatment (control, n=6), (ii) acute AA (0.5 g/kg, n=6), (iii) acute AA (0.5 g/kg) plus spin trap agent POBN (α-[4-pyridyl-1-oxide]-N-tert-butyl nitrone) (0.75 g/kg, n=6). In the last group, D_9-POBN was administered as spin-trap in one or two rats. The experimental design has been pictorially explained in the following figure **(Figure 12)**.

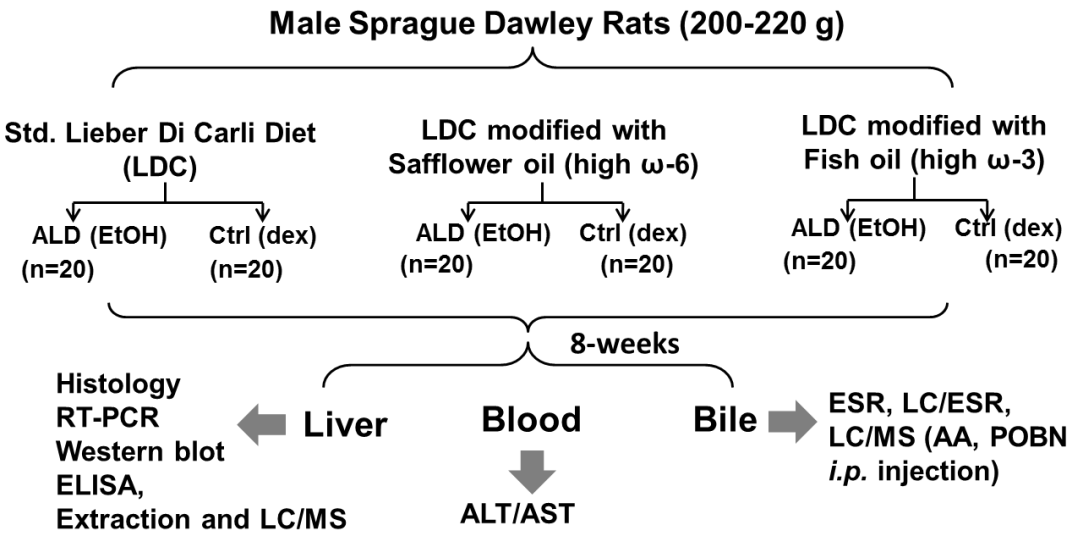


Figure 12. Experimental design to study the effect of ω-3 and ω-6 PUFA rich diets on ALD. Rats were fed three different diets ± ethanol for 8-weeks, bile, liver and blood were collected and subjected to histology and pathology, detection for free radicals, alteration in mRNA and protein expression of inflammatory factors, lipid peroxidation enzymes and hepatic membrane transporters.

In each dietary regimen, 36% of total calories were derived from ethanol (ALD) or dextrose (Ctrl), 35% of total calories were derived from fatty acids, 18% from proteins and 11% derived from carbohydrate sources in the diet. All liquid diets were prepared fresh every day. Oils used for PUFA modification were stored under nitrogen to prevent oxidation. Rats were maintained in a pathogen free animal facility at 21°C under a standard 12 hour light/12 hour dark cycle. The animal care and euthanasia procedures were in accordance with the guidelines given by IACUC (Protocol A0944) and NIH. Animals were weighed before starting the diets and subsequently every week.

Animals in each dietary group were pair fed (±ethanol): 1) rats in the ethanol (ALD) group were fed with the liquid diet on the first day, 2) their volume consumption was noted after about 24 hours to allow same volume of the liquid diet (consumed by the EtOH or ALD rats) to be then administered to the control (Ctrl) rats. The technique of pair-feeding is

essential to control the nutritional variables like volume of the food intake which in turn determine the blood alcohol and PUFA concentration in the different dietary groups. Pair feeding helped control the variability arising due to differential dietary intake of both ethanol and PUFAs. Since our study involved the determination of effect of PUFAs on severity of ethanol induced liver injury, pair feeding helped ensure uniform caloric intake of both ethanol and PUFAs.

2.2. Preparation of the liquid diets

The three different diets used in this study administered with (ALD) or without ethanol (Ctrl): 1) standard lieber di carli diet (LDC), 2) LDC modified using safflower oil (rich in ω-6 PUFA, Saff-LDC) and 3) LDC modified using fish oil (rich in ω-3 PUFAs, Fish-LDC).

2.2.1. Standard lieber di carli diet (LDC)

1) Preparation of control LDC diet (LDC Ctrl, 4.0 L):

Following the manufacturer's instructions, maltose dextrin (360 gm, corresponding to 36% of total calories) was weighed and volume q.s to 4.0 L (with 3.56 L of warm water). The solution was stirred until the maltose dextrin dissolved. One bag of dry mix (Bio-Serv, product F1697SP) was then mixed with the maltose dextrin solution and stirred until the mixture became homogenous.

2) Preparation of ethanol modified LDC diet (LDC ALD, 4.0 L):

Following the manufacturer's instructions, one bag of dry mix (Bio-Serv, product F1697SP) was mixed with 268 mL of 95% ethanol (corresponding to 36% of total calories), volume q.s to 4.0 L (with 3.56 L of warm water added) and stirred until the liquid mixture became homogenous.

2.2.2. Safflower oil modified LDC diet (Saff-LDC)

1) Preparation of Safflower oil control diet (Saff-LDC Ctrl, 4.0 L):

Following the manufacturer's instructions, maltose dextrin (360 gm) was weighed and dissolved in 3.56 L of warm water. One bag of dry mix (Bio-Serv, product F5712SP, liquid diet without added fatty acids) was then mixed into the maltose dextrin solution and stirred until the liquid mixture became homogenous. Following the manufacturer's instructions, fatty acid sources 39.6 gm/L, amounting to 172.22 mL oil safflower oil (specific gravity ~0.92) for 4.0 L was added and stirred until the mixture became homogenous.

2) Preparation of Safflower oil ALD diet (Saff-LDC ALD, 4.0 L):

One bag of dry mix (Bio-Serv, product F5712SP) was mixed with 3.56 L water and stirred until the liquid mixture became homogenous. Ethanol (268 mL of 95% ethanol) was then added to the solution. 172.22 mL safflower oil was then added and stirred until the mixture became homogenous.

2.2.3. Fish oil modified LDC diet (Fish-LDC)

1) Preparation of Fish oil control diet (Fish-LDC Ctrl, 4.0 L):

Maltose dextrin (360 gm) was weighed and dissolved in 3.56 L of warm water. One bag of dry mix (Bio-Serv, product F5712SP) was then mixed into the maltose dextrin solution and stirred until the liquid mixture became homogenous. Following the manufacturer's instructions, fatty acid sources 39.6 gm/L, amounting to 170.32 mL fish oil (specific gravity ~0.93) for 4.0 L was added and stirred until the mixture became homogenous.

2) Preparation of Fish oil modified ALD diet (Fish-LDC ALD, 4.0 L):

One bag of dry mix (Bio-Serv, product F5712SP) was mixed with 3.56 L water and stirred until the mixture became homogenous. Added 268 mL of 95% ethanol, 170.32 mL fish oil was then added and stirred until the mixture became homogenous.

2.3. Sample collection

Blood, bile, and liver tissue were collected from rats in each dietary group along with respective controls and stored for further analysis.

2.3.1. Collection of blood serum and plasma

During the feeding period, blood samples were collected from tail vein weekly (beginning from week-4). To collect serum, 100-200 μL of whole blood was collected in 1.5 mL centrifuge tubes and immediately centrifuged at 10,000 rpm for 15 min, serum was then collected by pipetting the clear supernatant in new tubes and stored at -20°C till further analyses. To collect plasma, 100-200 μL whole blood was collected in 1.5 mL centrifuge tubes containing about 20 μL of 1.0 M heparin (Sigma Aldrich, St. Louis, MO). The blood sample was then shaken vigorously and immediately centrifuged at 10,000 rpm for 15 minutes; plasma was then collected by pipetting the clear supernatant in new tubes and stored at -20°C till further analyses.

After the 8-weeks feeding period, blood samples were collected from the jugular vein (after the collection of bile for 3-4 hours). To collect serum, 500-1,000 μL whole blood was collected in 1.5 mL centrifuge tubes and immediately centrifuged at 10,000 rpm for 15 min; serum was then collected by pipetting the clear supernatant in new tubes, aliquoted into two-three tubes and stored at -80°C till further analyses. To collect plasma, 500-1000 μL whole blood was collected from the jugular vein in 1.5 mL centrifuge tubes

containing about 50 μL 1M heparin, the blood sample was then shaken vigorously and immediately centrifuged at 10,000 rpm for 15 min, plasma was then collected by pipetting out the clear supernatant in new tubes, aliquoted into two-three tubes and stored at -80°C till further analyses.

2.3.2. Collection of bile

Animals were fasted overnight with administration of water only. For surgery, all animals were weighed and anaesthetized using Nembutal (Pentobarbital, 0.9 μL/g). For bile duct canulation, a gentle cut was made to the muscle along the white line in the middle of abdomen, the bile duct was carefully separated with soft forceps and Q-tips. A gentle incision was made in bile duct using a fine iris needle, polyethylene tubing (i.d 0.28 mm) was inserted, bile flow was checked and if found normal, then the tubing and duct were carefully and gently fastened with a soft silk suture. To increase the detection sensitivity of the short-lived endogenously generated, AA-derived free radicals, AA (0.5 g/kg), and spin trap agent POBN (α-[4-pyridyl-1-oxide]-N-tert-butyl nitrone) (0.75 g/kg) (with D_9-POBN 1:1 mixture injected in one or two rats) were injected intraperitoneally. Both AA and POBN solutions were prepared in PBS and dissolved carefully. POBN solution was kept in water bath at 37ºC during the surgical procedure to prevent precipitation. Bile collection was started in tubes containing 50 mL mixture of metal chelators, 2, 2-dipyridyl (50 mM, DP) and bathocuprine-disulfonic acid disodium hydrate (50 mM, BC) to prevent ex vivo radical formation. A new collection tube was used every half hour for three hours. After collection of bile for three hours, bile volume collected every 30 min was recorded, the tube was transferred to dry ice immediately and stored at -80 °C for further analysis.

2.3.3. Collection of liver

Small pieces of liver from each lobe were cut, rinsed with PBS, and 1) fixed using neutralized buffered formalin 10% solution used for histology examination; 2) flash frozen using liquid nitrogen and stored -80 °C for later gene/protein analysis. All the remaining liver was collected, rinsed with PBS, frozen and stored at -80 °C for further analysis.

2.4. Sample analysis

Analysis of various specimens included histology and pathology examination of liver, hepatic gene and protein measurements, detection and structural identification of free radicals in bile and liver.

2.4.1. Histology and serology measurements

Formalin fixed liver samples were subjected to hematoxylin and eosin staining (H&E) and stained liver sections were analyzed using light microscopy. At least four different sections were examined per liver, images for microscopy were captured at a magnification of 40X (Olympus IX71 fluorescence microscope fitted with CCD camera). Histological assessment of development of fatty liver and inflammation was carried out on a scale of 0-3 by pathologist who had no prior knowledge of the dietary groups.

Activity of enzymes alanine amino transferase (ALT) and aspartate amino transaminase (AST) in serum were also tested as biochemical indicator of liver function. The activity of the enzymes was measured as the rate of oxidation of NADH to NAD, by measuring the resulting rate of decrease in absorbance and expressed in units per liter of serum. All kits used in this assay were obtained from Pointe Scientific Inc. (Brussels, Belgium). Reagents were reconstituted according to manufacturer's instructions: 1) 2.0 ml of reagent was pipetted into the cuvette and pre-warmed at 37°C for five minutes in the

spectrophotometer; 2) 0.05 ml of serum sample was transferred to this reagent, mixed and incubated at 37°C for one minute; 3) after one minute, absorbance was read and recorded at 340 nm. Readings were repeated every minute for the next five minutes; 4) mean absorbance difference/minute (Δ Abs/min) was calculated as the slope of the absorbance-time plot. The final values in IU/L were calculated by using the formula:

$$\text{ALT or AST (IU/L)} = \frac{(\delta \text{ Abs/min} \times 2.05 \times 1000)}{(6.22 \times 0.05 \times 1.0)} = \delta \text{ Abs/min} \times 6592$$

Where δ Abs/min = average absorbance change per minute; 1,000 = conversion of IU/mL to IU/L; 2.05 = total reaction volume (mL); 6.22 = millimolar absorptivity of NADH; 0.05 = sample volume (mL), 1.0 = light path in cm

2.4.2. Hepatic free fatty acid and eicosanoid determination

Liver tissue (~0.2 g) was homogenized in deionized water, free fatty acids and eicosanoids were extracted from the liver by methanol (3.0 mL, 15%), followed by acidification using 2 mM HCl to ~pH 3.0. These extracts were then subjected to solid phase extraction using C-18 SPE columns (Agilent, Santa Clara, CA) and eluted with 2.0 mL ethyl acetate. The eluants were then evaporated to dryness, reconstituted with ethanol (150 μL) and subjected to LC/MS analysis.

The LC/MS system consisted of an Agilent 1200 series HPLC system and an Agilent LC/MSD SL ion trap mass spectrometer. Suitable deuterated internal standards, such as AA-d_8 (200 ng), LA-d_4 (200 ng), PGE_2-d_4 (20 ng) and $PGF_{2\alpha}$-d_4 (20 ng) (Cayman Chemicals, Ann Arbor, MI) were used to quantify the levels of free fatty acids- arachidonic acid (AA), linoleic acid (LA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) and eicosanoids (PGs and HETEs) in liver extracts. HPLC separations were performed on a

C-18 column (Zorbax Eclipse-XDB, 4.6 × 75 mm, 3.5 μm) and guard column (Zorbax Eclipse-XDB, 4.6 × 12.5 mm, 5.0 μm) equilibrated with A (H_2O - 0.01% HOAc). 60.0 μL of reconstituted sample was injected into the HPLC system and eluted at a 0.8 mL/min flow rate with a gradient elution: (i) 0 to 12 min: 100% to 68% of solvent A, 0% to 32% of solvent B (ACN-0.01% HOAc); (ii) 12.0 to 28.0 min: 68% to 44% of A, 32% to 56% of B; (iii) 28.0 to 30.0 min: 14% of A and 86% of B; (iv) 30.0 to 44.0 min: 5% of A and 95% of B and (v) 44.0 to 50.0 min: 68% of A and 32% of B. Electrospray ionization in negative ion mode was used for all LC/MS measurements. Total ion current (TIC) chromatograms in full mass scan mode (m/z 50 to m/z 600) were performed to profile the fatty acids and eicosanoids in the liver samples. Other MS settings were capillary voltage, -4500 V; nebulizer press, 15 psi; dry gas flow rate, 5 L/min; dry temperature, 225°C; compound stability, 20%; and number of scans, 50.

2.4.3. Gene expression of lipid peroxidation enzymes and pro-inflammatory and pro-fibrotic factors

Real time RT-PCR analysis was used to evaluate mRNA levels of lipid peroxidation enzymes COX-2, 5-LOX, 15-LOX and CYP2E1, inflammatory and fibrotic markers such as TNF-α, interleukin (IL)-10, IL-4, IL-6, i-NOS, TGF-β, smooth muscle actin (SMA), collagen-1 and transcription factor PPAR-α. The total RNA from liver tissue was extracted using Qiagen RNeasy® Mini Kit (Qiagen, Valencia, CA, catalog number 74106). About 20 mg of tissue was extracted via steps involving tissue lysis, precipitation and RNA purification using the RNeasy mini columns following the manufacturer's instructions. The yield of RNA was then quantified by spectral absorption of light at 260 and 280 nm. Quantification was followed by genomic DNA elimination using 1.0 μg of the template

RNA and reverse transcription. The final step involved the real time PCR reaction using SYBR detection assay mixture as directed by the manufacturer. The forward and reverse primers were obtained from Qiagen (Quantitect primer assays) as described in **(Table 2)** below, aliquoted and stored according to manufacturer's instructions.

Gene	Quantitect primer assay
Beta–actin	QT00193473
COX-2	QT00192934
5-LOX	QT00184765
15-LOX	QT00181265
IL-4	QT01590316
IL-6	QT00193472
TNF-α	QT00178717
TGF-β	QT00187320
Smooth muscle actin	QT01615901
Collagen-1	QT02285619
PPAR-α	QT00176575
ABCC2	QT001869861
ABCD3	QT00178129
ABCD2	QT00185689
ABCC3	QT00191716
SLC 27 A5	QT00193242

Table 2. Quantitect primer assays used for RT-PCR. Quantitect primer (reverse and forward) assays used for mRNA determination using real-time RT-PCR, with their respective catalogue numbers for each primer assay.

Qiagen's Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, catalog number 205313) and Quantitect SYBR green RT-PCR kit (Qiagen, Valencia, CA, catalog number 204143) was used for reverse transcription and real-time polymerase chain reaction. The relative differences in expression among groups were expressed using cycle time (Ct) values after normalization with internal control, β-actin using the Eppendorf

realplex Mastercycler (Eppendorf, Westbury, NY). Thermal cycle protocol used for polymerase reaction is as described in table below **(Table 3)**. All experiments were performed in triplicates.

Cycle	Step	Temperature	Time	Description
1X	1	95 °C	15 min	Activation of DNA Polymerase
45X	2	95 °C	15 sec	Cycled template denaturation
	3	55 °C	25 sec	Annealing
	4	68 °C	30 sec	Extension

Table 3. The thermal cycler protocol. The thermal cycle protocol used for polymerase reaction showing the number of cycles required, the temperature used and the time required in each step.

2.4.4. Protein profiles of COX-2, 5-LOX, MRP-2, ABCA1 and NF-κB

Protein concentrations of lipid peroxidation enzymes, COX-2 and 5-LOX, hepatic membrane transporters, MRP-2 and ABCA1 and transcription factor, NF-κB were evaluated using western blot analysis.

2.4.4.1. Protein extraction and quantification

About 20 mg liver tissue was homogenized in 500 µL Tris buffer (pH 7.4) in presence of 100 mM phenylmethylsulphonyl fluoride (PMSF) and complete protease inhibitor cocktail (Roche Diagnostics, IN, USA). The homogenates were boiled at 95°C for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes.
Supernatants were separated and aliquoted into two-three tubes and stored at -20°C for further analysis.

Quantification of proteins was done by Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) using standard curves prepared with Bovine Serum Albumin (BSA, catalog number 23210, Thermo Scientific, Rockford, IL). Briefly, 5.0 µL of standard BSA solution

or sample solution were added to microplates and 25 μL of working Reagent A and 200 μL of Reagent B were then added into each well and mixed gently. After 15-min of incubation, absorbance was measured at 750 nm. Protein concentration was calculated using the standard curve obtained with BSA and adjusted before loading in the gel.

2.4.4.2. Western blot

Protein samples were adjusted to equal amounts (25 μg) by volume and then mixed with bromophenol blue-glycerol solution and loaded on the wells of a 10% poly-acrylamide resolving (pH 8.8) and 4% poly-acrylamide stacking gel (pH 6.8). Gels were then run at 120V (using a Mini-Trans blot cell by Biorad) until the samples reached the bottom of the glass plates. Proteins were then transferred onto PVDF membranes (Immunomobilon-P, catalog number IPVH00010), blocked with 5% non-fat milk for 1-2 hours, washed with tris buffer with tween-20 (0.1%), and then incubated overnight with primary antibodies against proteins of interest in buffer containing 1% non-fat milk and sodium azide (0.02-0.1%). For detection of activation of NF-κB, antibody was used against the phosphorylated p65 subunit of this transcription factor.

The immunoreactive bands were detected by a horseradish peroxidase (HRP)-conjugated secondary antibody. Protein signals were visualized using enhanced chemiluminescent substrate for detection of HRP on the immunoblots by exposure to autoradiographic films (Pierce ECL Western blotting Substrate, catalog number 32106, Thermo Scientific, Rockford, IL).

Quantification of immunoblots was done by densitometric analysis using Fluorchem FC8800 software in an Alpha Innotech imaging system. Details of antibody sources and concentrations of each primary antibody are listed, **Table 4**. All secondary

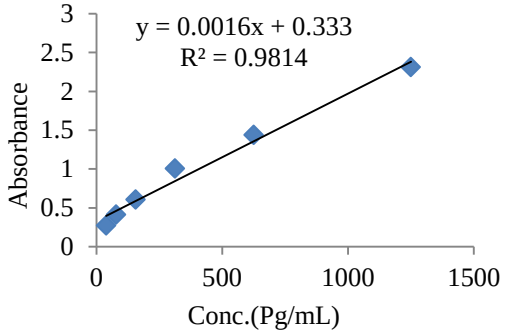
antibodies were used at a concentration of 0.05 µg/mL. All experiments were performed in triplicates.

Antibody	Source	Concentration (µg/mL)
5-LOX,	Santa Cruz Biotech, Inc., Santa Cruz, CA	0.5
COX-2,	Santa Cruz Biotech, Inc., Santa Cruz, CA	0.4
β-Actin,	Sigma Aldrich, St. Louis, MO	0.05
MRP-2,	Santa Cruz Biotech, Inc., Santa Cruz, CA	0.4
ABCA1,	Millipore, Temecula, CA	2.0
NF-kB (p65)	Cell Signaling Technology, Danvers, MA	1.0

Table 4. Primary antibodies with source and concentration for western blot. Please note that all secondary antibodies were used at a concentration of 0.05 µg/mL.

2.4.5. Quantification of TNF-α in liver

Quantification of hepatic TNF-α was done using sandwich ELISA. Protein were extracted and quantified as described in Sec 2.4.4.1. Extracted protein was then diluted to 25X and normalized to equal concentrations using the reagent diluent (PBS). Sandwich ELISA was performed and calculations done using the standard curve (minimum of six-point standard curve) prepared with TNF-α standard **(Figure 13)** according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, catalog number DY510).



Pg/mL	Absorbance
1250	2.3097
625	1.4378
312.5	1.0022
156.25	0.60275
78.125	0.41095
39.0625	0.2712

Figure 13. TNF-α standard curve plotted using standard TNF-α.

2.4.6. Offline ESR detection of free radical adducts

For offline ESR measurement of free radicals, bile samples obtained from rats injected AA (0. 5 g/kg, i.p) and spin trap POBN (0.75 g/kg, i.p) were directly transferred to the ESR flat cell for magnetic field scans.

For offline ESR measurement of free radicals in liver, tissues were extracted via a modified Folch extraction procedure. Briefly, ~0.5 g liver tissue was transferred to glass tubes containing 1 mL deionized water, 0.5 mL of 2, 2-dipyridyl (DP, 30 mM), 0.5 mL phenol (1.2 mM) and 3.0 mL mixture of chloroform and methanol (2:1). After homogenizing the tissue for 1 min, 9.0 mL mixture of chloroform and methanol (2:1) was added, shaken vigorously for 5 min, centrifuged at 12000 rpm and the lower organic layer dissolving the non-polar fat component was then collected, condensed and evaporated completely. The condensate was then reconstituted using 500 μL of mixture of chloroform and methanol (2:1) and immediately transferred to the ESR flat cell for magnetic field scans for offline ESR measurements. ESR spectra were obtained with a Bruker EMX spectrometer equipped with a super high Q cavity operating at 9.78 GHz at room temperature. Other ESR spectrometer settings were: magnetic field center, 3494.4 G; magnetic field scan, 70 G; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 1.0 G; receiver gain, 5.0×10^4; time constant, 0.655 s; and conversion time, 0.164 s.

2.4.7. Online LC/ESR detection of radical adducts

Bile samples (150 μL) were mixed with equal volume of 50% ACN in order to precipitate the proteins. The samples were then condensed for 20-30 min in a vaccufuge to completely remove the ACN and used for time scan detection using the on-line LC/ESR

system [52, 173]. The on-line LC/ESR system consisted of an Agilent 1200 series HPLC system and a Bruker EMX ESR system. The outlet of the Agilent UV detector was connected to a highly sensitive aquax ESR cell with red PEEK HPLC tubing (0.005 i.d.). The POBN radical adducts were monitored via UV absorption at 265 nm followed by ESR detection. There was a delay of about 9 s between the UV and the ESR detection in our on-line LC/ESR settings. LC separations were performed on a C-18 column (Zorbax Eclipse-XDB, 4.6 × 75 mm, 3.5 μm) and guard column (Zorbax Eclipse-XDB, 4.6 × 12.5 mm, 5.0 μm) equilibrated with A (H_2O - 0.1% HOAc). Sixty microliters of bile sample was injected into the HPLC system by autosampler, eluted at a 0.8 mL/min flow rate with gradient elution: (i) 0 to 40 min: 100% to 30% of solvent A, 0% to 70% of solvent B (ACN-0.1% HOAc); (ii) 40 to 45 min: 30% to 5% of A, 70% to 95% of B; (iii) 45 to 54 min: 5% of A and 95% of B; and (iv) 54 to 60 min: 5% to 100% of A and 95% to 0% of B. On-line ESR monitoring consisted of a time scan with the magnetic field fixed on the maximum of the middle line of the six-line spectrum of the POBN adduct as described elsewhere [174-176]. Other ESR settings were modulation frequency, 100 kHz; modulation amplitude, 3.0 G; microwave power, 20 mW; receiver gain, 4 × 105; and time constant, 2.6s. Ex vivo radical formation was prevented by addition of 50 mL mixture of chelating agents 2, 2-dipyridyl (50 mM, DP) and bathocuprine-disulfonic acid disodium hydrate (50 mM, BC) in the tubes used for bile collection.

2.4.8. LC/MS identification of radical adducts

Bile samples prepared as described in section 2.4.7 were subjected to LC/MS analysis for radical adduct identification. The LC/MS system consisted of an Agilent 1200 series HPLC system and an Agilent LC/MSD SL ion trap mass system. The outlet of the

UV detector in LC was connected to the MS system with red PEEK HPLC tubing as well. Chromatographic conditions were identical to those used for on-line LC/ESR. However, the LC flow rate (0.8 mL/min) into the MS inlet was adjusted to 40 μL/min via a splitter. There was a delay of ~35 s between the UV and the MS detection. Electrospray ionization in positive ion mode was used for all LC/MS measurements. Total ion current (TIC) chromatograms in full mass scan mode (m/z 50 to m/z 600) were performed to profile all products formed in vivo in the presence of POBN. Other MS settings were capillary voltage, -4500 V; nebulizer pressure, 20 psi; dry gas flow rate, 8 L/min; dry temperature, 60°C; compound stability, 20%; and number of scans, 50. Extracted ion current (EIC) chromatograms of ions of interest were projected from TIC to acquire MS chromatograms that could match with ESR chromatograms. EIC was performed to determine the number of isomers of given ions. Normally an isolation width of ± 0.5 Da was selected for EICs.

The LC/MS studies of bile samples from the rats injected with 1:1 mixture of D_0-D_9-POBN were also performed to confirm the structures of POBN adducts [161, 163, 174-178]. Ex vivo radical formation was prevented by addition of 50 mL mixture of chelating agents, 2, 2-dipyridyl (50 mM, DP) and bathocuprine-disulfonic acid disodium hydrate (50 mM, BC) in the tubes used for bile collection.

2.5. Statistical analysis

Mean and standard errors of individual values were calculated. For comparison of two groups (ALD and pair-fed control), Student's T–test was used to assess the significance of the difference. For other comparisons, analysis of variance (ANOVA) was performed by one-way ANOVA- Dunnett's Multiple Comparison Test. A p-value of <0.05 was considered to be statistically significant.

CHAPTER 3. EFFECT OF ω-3 AND ω-6 PUFA RICH DIETS ON ETHANOL INDUCED LIVER PATHOLOGY AND FUNCTION

3.1. Introduction

Alcoholic fatty liver (hepatic steatosis) is a pathological condition that predisposes the liver to further injury (hepatitis and fibrosis). Several studies have investigated the effects of PUFAs on the development of hepatic steatosis [15, 58-61, 65]. In an animal model of non-alcoholic steatohepatitis, the ω-3 fatty acid therapy was also found to be associated with significant decrease in hepatic steatosis [180]. There was also a study that showed that EPA-ethyl ester prevented the progression of hepatic fibrosis in a methionine- and choline-deficient diet induced model of non-alcoholic steatohepatitis with reduced ROS levels and serum oxidative stress [181]. Some studies reported that co-administration of arachidonic acid (AA) attenuated fat accumulation induced by conjugated linoleic acid in mouse liver [182]. In models of alcohol induced liver injury, conflicting evidence exists regarding which type of PUFAs (ω-3 or ω-6) exert beneficial or detrimental effect in development of fatty liver. Some investigators reported that rats fed fish oil (rich in ω-3 PUFAs) developed more severe ethanol induced liver injury than rats co-fed corn oil (rich in ω-6 PUFAs) and ethanol [14]. Ingestion of fish oil on the day prior to ethanol treatment was also found to reduce ethanol induced fatty liver by 73% in mice with acute ethanol induced fatty liver [67]. Thus, which type of PUFAs, e.g. ω-3 or ω-6, could exert beneficial or deleterious effect in alteration of ethanol induced liver injury is still not clear.

The objective of this study was to examine the effect of administration of ω-3 and ω-6 PUFA rich diets on ethanol induced hepatic steatosis and alteration of liver function. We hypothesized that dietary ω-3 and ω-6 PUFAs can be involved in alteration of ethanol

induced fatty liver leading to differences in liver histopathology and function, arising mainly from their different lipid peroxidation reactions. To examine this hypothesis, we used three different modified dietary regimen: 1) standard lieber di carli liquid (LDC) diet, 2) safflower oil modified diet (Saff-LDC), rich in ω-6 LA and 3) fish oil modified diet (Fish-LDC), rich in ω-3 EPA and DHA. All three diets were administered either with ethanol or control, i.e isolcaloric dextrose, for 8-weeks and their effect on liver histology. ALT and AST activity and animal growth were also measured as markers of liver function. The experimental design and methodology have been described in Chapter-2.

3.2. Results and discussion

3.2.1. Weight gain profile of experimental rats

Weight gain by rats was measured and used as indicator of any variation in growth of animals caused due to variable dietary intake among the different dietary groups. To monitor the effect of liquid diets on animal growth and metabolism, animal weights were recorded before the beginning of the feeding period, as well as every week for 8-weeks. The technique of pair-feeding (described in Chapter-2) allowed the precise control of the volume of food intake between the ALD and control (fed isocaloric dextrose) groups, thereby allowing for an accurate comparison of pathological condition between the ALD and control groups from the three different diets.

No significant difference was observed in the weight gain profile of rats from any of the dietary groups **(Figure 14)**. No loss of weight on chronic administration of ethanol was observed and a steady and consistent gain in weight was observed in all the dietary groups. Animals in the ALD and control groups consumed almost equal volumes of the respective

liquid diets, resulting in uniform growth among the different dietary groups, e.g. equal weight gain rates from our feeding design (Figure 14).

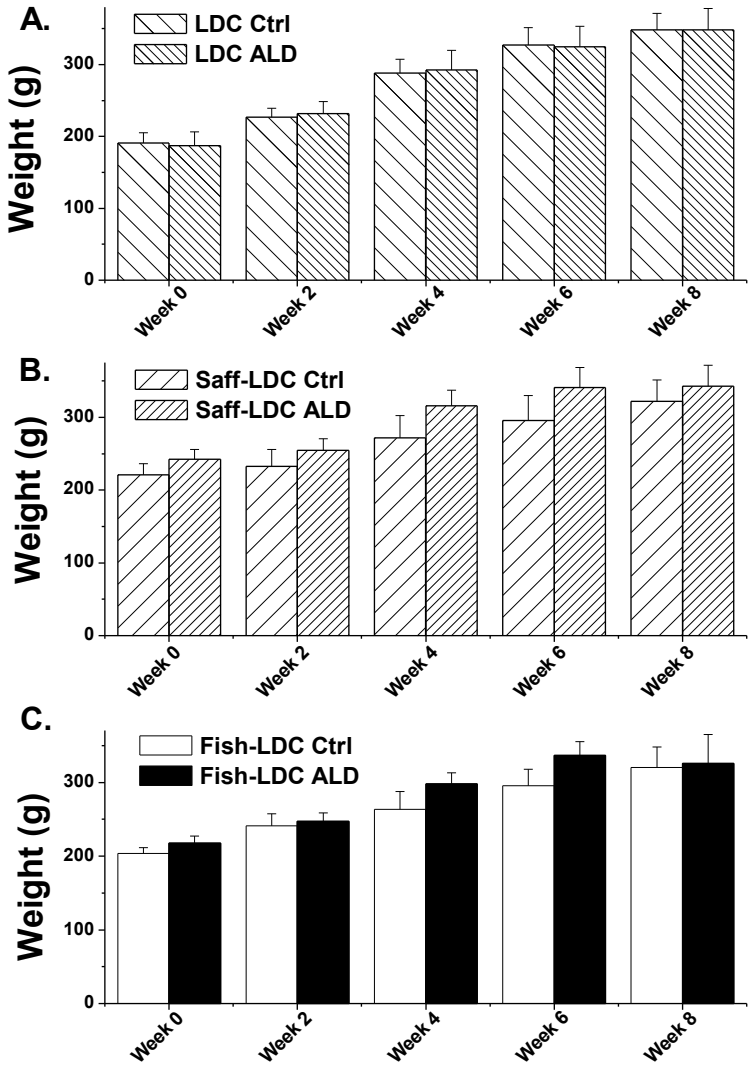


Figure 14. Animal growth measured by gain in body weight. Weight gain profile in rats fed: (A) Standard lieber di carli rats with ethanol (LDC ALD) and dextrose (LDC Ctrl), (B) Safflower oil modified diet with ethanol (Saff-LDC ALD) and dextrose (Saff-LDC Ctrl), and (C) Fish oil modified diet with ethanol (Fish-LDC ALD) and dextrose (Fish-LDC Ctrl). No significant difference was observed in the weight gain profile of rats from any of the dietary groups.

This result indicated no variability in growth of animals among the different dietary groups, both with ethanol and isocaloric dextrose control.

3.2.2. Liver serology studies

Clinical markers of altered activity of liver function-alanine amino transferase (ALT) **(Figure 15)** and aspartate amino transaminase (AST) **(Figure 16)** were determined weekly and at the end of the 8-week feeding period. Significant elevation in ALT activity (normal ALT value observed under our experimental condition ~ 40 IU/L) was observed at week-8 in LDC-ALD group in comparison to those in LDC-Ctrl group (Figure 15). Interestingly, significant elevation in ALT activity was observed at week-5 till week-8 in Saff LDC-ALD rats, in comparison to Saff LDC-Ctrl rats (Figure 15). However, no elevation in serum ALT activity was observed in Fish LDC-ALD rats at week-8 in comparison to Fish LDC-Ctrl rats (Figure 15). Early onset of liver function failure at week-5 (data not shown) was observed in rats fed Saff LDC-ALD diet having much higher ω-6 vs ω-3 ratio (384:1). In contrast, fish oil modified diet with very low ratio of ω-6 vs ω-3 (0.16:1), prevented the ethanol mediated development of liver injury. This data suggested that standard and particularly safflower oil modified diet rich in ω-6 PUFAs might promote the ethanol mediated progression of liver injury (marked by elevation in serum ALT).

Consistent to results of ALT activity, significant elevation in serum AST activity was observed at week-8 in rats fed LDC-ALD and Saff LDC-ALD diets, in comparison to their respective controls fed dextrose (Figure 16). No elevation in serum AST activity (normal AST value observed under our experimental condition ~ 200 IU/L) was observed in rats fed ethanol with fish oil modified diet (Fish LDC-ALD), in comparison to its control fed dextrose (Figure 16).

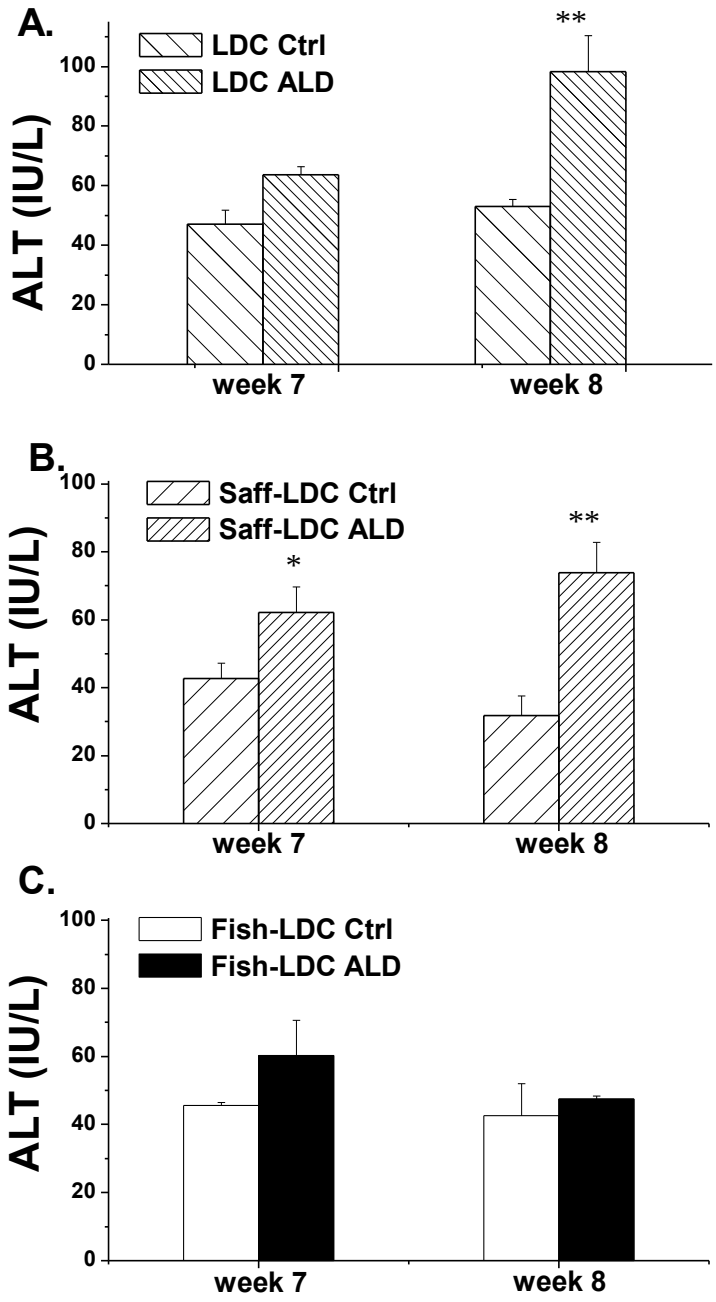


Figure 15. Serum ALT activity in different dietary groups. Clinical marker of altered liver function, alanine amino transferase (ALT) in serum samples determined at week 7 and week 8. (A) ALT activity determined in LDC Ctrl, LDC ALD, (B) Saff-LDC Ctrl, Saff-LDC ALD, and (C) Fish-LDC Ctrl, Fish-LDC ALD, groups, respectively. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, ** $p < 0.01$ compared to respective dietary controls.

This data further reinforced that standard and safflower oil modified diets with much higher ω-6 vs ω-3 ratio may promote the ethanol mediated progression of liver injury. In contrast, fish oil modified diet with very low ratio of ω-6 to ω-3 may prevent the ethanol mediated development of liver injury.

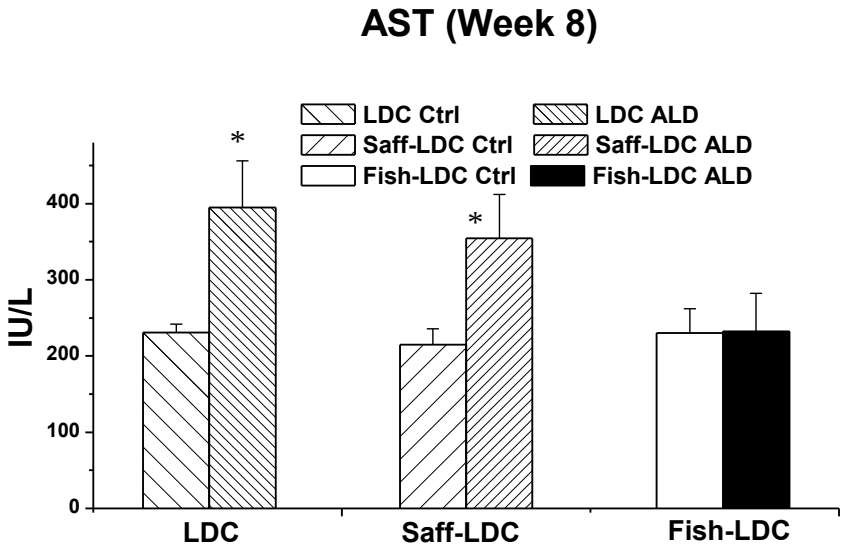


Figure 16. Serum AST activity in different dietary groups. Clinical marker of altered liver function, aspartate amino transaminase (AST) activity in serum samples determined at week 8 in LDC Ctrl, LDC ALD, Saff-LDC Ctrl, Saff-LDC ALD, Fish-LDC Ctrl, Fish-LDC ALD, groups, respectively. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, compared to respective dietary controls.

3.2.3. Liver histology using H & E staining

Liver histology was performed using H&E staining of liver sections obtained from the different dietary groups and evaluated for the presence of fat, necrosis and inflammation **(Figure 17)**.

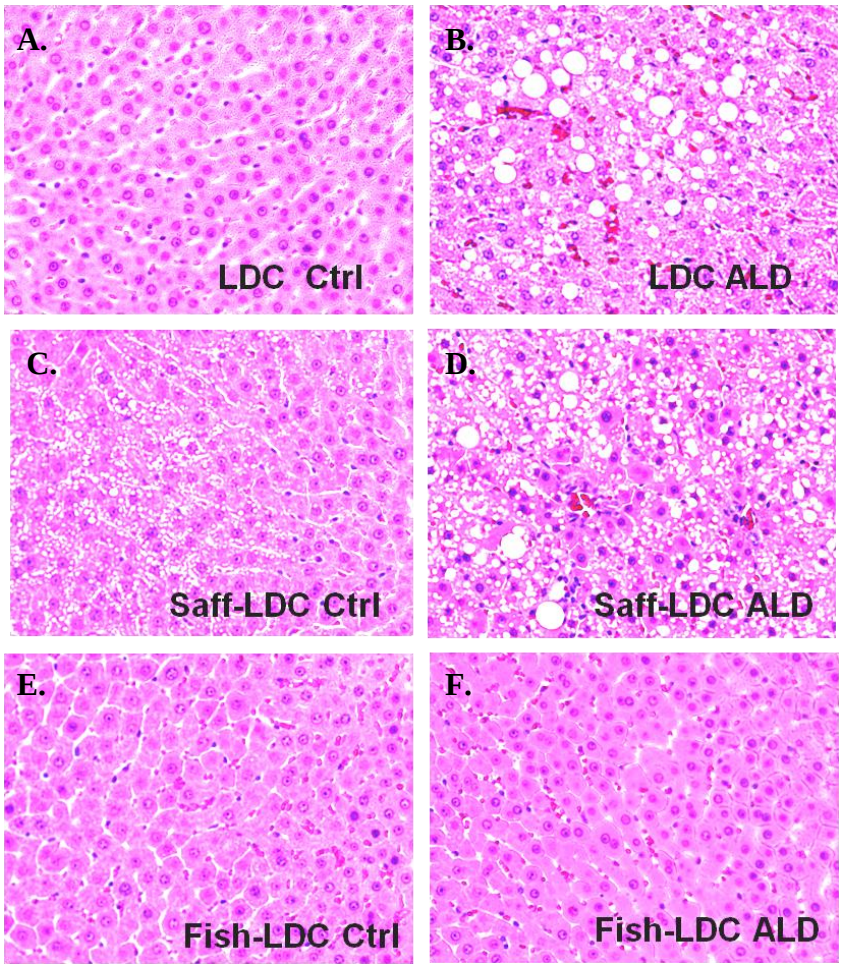


Figure 17. Rat liver histology in different dietary groups. H&E staining of rat liver sections, 40X showing fatty liver, inflammation and necrosis in (A) LDC Ctrl, (B) LDC ALD, (C) Saff-LDC Ctrl, (D) Saff-LDC ALD, (E) Fish-LDC Ctrl, and (F) Fish-LDC ALD. Values are representative from n=6-10 rats in each group.

Pathological assessment of liver histology was carried out on a scale of 0-3 by a pathologist who had no prior knowledge of the dietary groups **(Table 5)**. Livers of rats fed LDC-ALD diet showed mild to moderate fatty degeneration and limited dot necrosis. In comparison to LDC-ALD, LDC-Ctrl rats showed normal liver tissue structure and no fatty degeneration in the liver cells. In livers of rats fed Saff LDC-ALD diet, moderate to severe fatty degeneration, as well as focal (or dot) necrosis could be seen.

Inflammation was also observed in livers of rats fed LDC-ALD (inflammation score: 0.83) and Saff LDC-ALD diet (inflammation score: 1.29) (Table 5). In contrast, livers of rats fed Fish LDC diet were found to be free of any signs of fatty degeneration, necrosis and inflammation irrespective of feeding ethanol or control (Table 5). Interestingly, even in livers of rats fed Saff LDC Ctrl diet, mild fatty degeneration and inflammation was observed, signifying the initiation of steatosis in these rats even without ethanol. This data suggested that fish oil could prevent the ethanol mediated fatty liver degeneration and inflammation, while diets rich in ω-6 fatty acids, e.g. Saff LDC diet promoted the ethanol mediated fatty liver degeneration and significantly increased inflammation (Table 5). Since alcoholic fatty liver (steatosis) predisposes the liver to further injury (hepatitis and fibrosis), this data (Figure 15-17, Table 5) indicated that fish oil rich in ω-3 PUFAs could protect the liver from ethanol mediated hepatitis and fibrosis by preventing the onset of steatosis. In contrast, safflower oil rich in ω-6 PUFAs promoted the onset of ethanol mediated steatosis which could render the liver more susceptible to further injury by hepatitis and fibrosis.

Groups	**Average scores ± SEM**	
	Grade of fatty degeneration	**Inflammation**
LDC Ctrl	0.00 ± 0.00	0.00 ± 0.00
LDC ALD	*2.25 ± 0.33	0.83 ± 0.30
Saff-LDC Ctrl	0.63 ± 0.24	0.56 ± 0.13
Saff-LDC ALD	*2.71 ± 0.10	1.29 ± 0.15
Fish-LDC Ctrl	0.00 ± 0.00	0.00 ± 0.00
Fish-LDC ALD	0.00 ± 0.00	0.00 ± 0.00

Table 5. Liver pathology scores showing grades of fatty degeneration and inflammation. Pathology scores in LDC Ctrl, LDC ALD, Saff-LDC Ctrl, Saff-LDC ALD, Fish-LDC Ctrl and Fish-LDC ALD rats. Values are mean ± SEM from n=6 rats in each group, *$p < 0.05$, compared to respective dietary controls.

3.3. Discussion and conclusion

Both LDC and Saff-LDC diets promoted the ethanol mediated progression of liver injury as observed by elevation of serum ALT and AST activity (Figure 15, 16). In addition, rats fed Saff-LDC ALD diet developed the earliest onset of liver function failure (at week-5), perhaps due to its highest ω-6 to ω-3 ratio (384:1). Among all the three dietary groups (± ethanol), fish oil modified diet with the lowest ω-6 to ω-3 ratio (0.16:1) was found to maintain normal serum levels of ALT and AST activities which indicated that fish oil could prevent the ethanol mediated degeneration of liver function (Figure 15, 16).

Liver histology examination (Figure 17) and pathological assessment (Table 5), indicated high degree of fat deposition and inflammation in livers of rats fed ethanol along with diets rich in ω-6 fatty acids, such as LDC ALD and Saff-LDC ALD diets (Table 5). In contrast, livers of rats fed Fish-LDC ALD diet showed no fatty liver degeneration and inflammation. This data suggested that fish oil could prevent the ethanol mediated fatty liver and inflammation, while safflower oil promoted the ethanol mediated development of fatty liver and significantly increased inflammation (Table 5). Among all the three dietary control groups, only the liver of rats fed Saff-LDC Ctrl diet showed fat deposition and inflammation (Table 5) which signifies the development of non-alcoholic steatohepatitis in these rats, while all other control groups were found to be free of these pathological features.

Overall, our data indicated that fish oil, with the lowest ω-6 to ω-3 ratio may prevent the development of ethanol induced fatty liver, inflammation and liver function failure. In contrast, safflower oil with the highest ω-6 to ω-3 ratio acted like a ‘double hit’ when co-administered with ethanol, causing the maximum fatty degeneration and liver

inflammation among all groups. Even in the absence of ethanol, safflower oil diet could alone trigger the onset of fat deposition and liver inflammation. Since fish oil (rich in ω-3) was shown to prevent the onset of steatosis, it could potentially prevent the further progression of liver injury to hepatitis and fibrosis [2-11]. In contrast, safflower oil rich in ω-6 PUFAs could increase the susceptibiblity of liver to further injury by hepatitis and fibrosis, perhaps by promoting the onset of ethanol mediated steatosis.

CHAPTER 4. EFFECT OF ω-3 AND ω-6 PUFA RICH DIETS ON ETHANOL INDUCED LIVER INFLAMMATION AND FIBROSIS

4.1. Introduction

Oxidative stress induced cellular responses have been shown to play an important role in innate immune cell activation by ethanol [88]. Clinical studies have demonstrated that patients with ethanol induced liver injury show increased levels of inflammatory cytokines, such as IL-1, IL-6 and TNF-α, while immunoregulatory cytokines like IL-10, help regulate the immune response by reducing the production of inflammatory cytokines [81-83] (Figure 7). Transcription factor NF-κB has been shown to be an important component of the TNF-α proliferative pathway through TNF-receptor 1 (Figure 8).

In addition to ethanol, dietary ω-3 and ω-6 PUFAs have also been shown to affect the secretion of cytokines in several inflammatory pathological conditions [56, 70, 71]. For example, when administered to healthy volunteers, ω-3 PUFAs have been shown to decrease bacterial endotoxin induced production of the pro-inflammatory cytokines, IL-1 and TNF-α from peripheral blood lymphomonocytes [92-94]. In contrast, fats rich in ω-6 PUFAs were shown to enhance IL-1 production and tissue responsiveness to cytokines [100]. ω-3 PUFAs have also been shown to modulate the activation of NF-κB [92, 94-98]. Some reports have indicated that linoleic acid (ω-6 PUFA) activated NF-κB and stimulated TNF-α production in porcine peripheral blood mononuclear cells [99]. Although some studies have investigated the role of dietary PUFAs in alteration of inflammatory cytokines in several pathological conditions, their effect on cytokine modulation in the liver in ethanol mediated hepatic steatosis is still not clear.

In addition to the enhanced pro-inflammatory cytokine production in ALD, ethanol can also induce the release of pro-fibrogenic factors, such as transforming growth factor-β (TGF-β) and smooth muscle actin (SMA) from hepatic stellate cells (HSCs), up-regulates the gene expression of collagen I and enhances type I collagen protein production by HSCs [113, 114]. Reactive oxygen species, e.g. hydrogen peroxide, superoxide anion and pro-inflammatory cytokines have been implicated in abnormal activation of hepatocytes and HSC and induction of fibrosis [78]. However, it is not yet clear how the hepatic expression of these pro-fibrotic factors (TGF-β, collagen I and SMA) is altered by dietary ω-3 and ω-6 PUFAs and its correlation with progression of steatosis to fibrosis in ALD. Based on the observations from Chapter-3, diets rich in ω-6 PUFAs were found to perhaps promote the progression of ethanol mediated fatty liver and inflammation, while diet rich in ω-3 PUFAs prevented the development of ethanol mediated fatty liver and also significantly reduced inflammation (Figure15-17, Table-5). We studied whether Fish-LDC diet would downregulate the expression of pro-inflammatory and pro-fibrotic factors, thereby reducing ethanol mediated liver inflammation and preventing the progression of steatosis to fibrosis. We also investigated whether LDC and Saff-LDC diets (with moderate and high ω-6 content) would result in upregulation of pro-inflammatory and pro-fibrotic factors in the liver and thus promote ethanol mediated liver inflammation and susceptibility to fibrosis.

4.2. Results and discussion

To determine the effect of ethanol and/or dietary PUFAs on liver inflammation and fibrosis, we measured the alteration in gene and protein expression of several inflammatory and fibrotic factors, e.g. TNF-α and TGF-β.

4.2.1. Expression of inflammatory and fibrotic factors

mRNA levels of pro-inflammatory (TNF-α, i-NOS, IL-1, IL-4, IL-6), pro-fibrotic (TGF-β, SMA, collagen-1), and anti-inflammatory (IL-10) factors were determined in livers of rats fed LDC, Saff-LDC and Fish-LDC diets. mRNA expression of pro-inflammatory cytokine TNF-α was found to be significantly upregulated by ethanol in rats fed LDC and Saff-LDC diets **(Figure 18)**. However, decrease in TNF-α mRNA was observed in rats fed ethanol with Fish-LDC diet relative to Fish-LDC Ctrl diet (Figure 18).

Gene expression of anti-inflammatory cytokine, IL-10 was found to be significantly upregulated in rats fed Fish-LDC ALD diet in comparison to rats fed Fish-LDC Ctrl diet. This could be one of the possible preventive mechanisms by which fish oil could prevent the ethanol mediated increase in liver inflammation. In contrast, no change in IL-10 expression was observed in LDC ALD and Saff-LDC ALD groups, in comparison to their respective dextrose controls. Gene expression of pro-inflammatory factor, i-NOS, was found to be upregulated in rats fed ethanol in all the three dietary regimens, in comparison to their respective dextrose controls (Figure 18). No significant alteration in the gene expression of other pro-inflammatory cytokines (IL-1, IL-4, IL-6), was observed between ethanol and dextrose control groups in all the three dietary regimens (Figure 18).

Out of the several pro-fibrotic factors tested, only smooth muscle actin (SMA) was found to be increased in rats fed LDC-ALD diet in comparison to LDC-Ctrl diet, indicating the ethanol mediated activation of hepatic stellate cells in these rats. TGF-β and collagen-1 did not show any elevation in rats fed ethanol with LDC or Saff-LDC diet. In contrast, in rats fed Fish-LDC diet, a decrease in gene expression of ethanol induced TGF-β was observed (Figure 18) which could lead to lower protein expression of TGF-β.

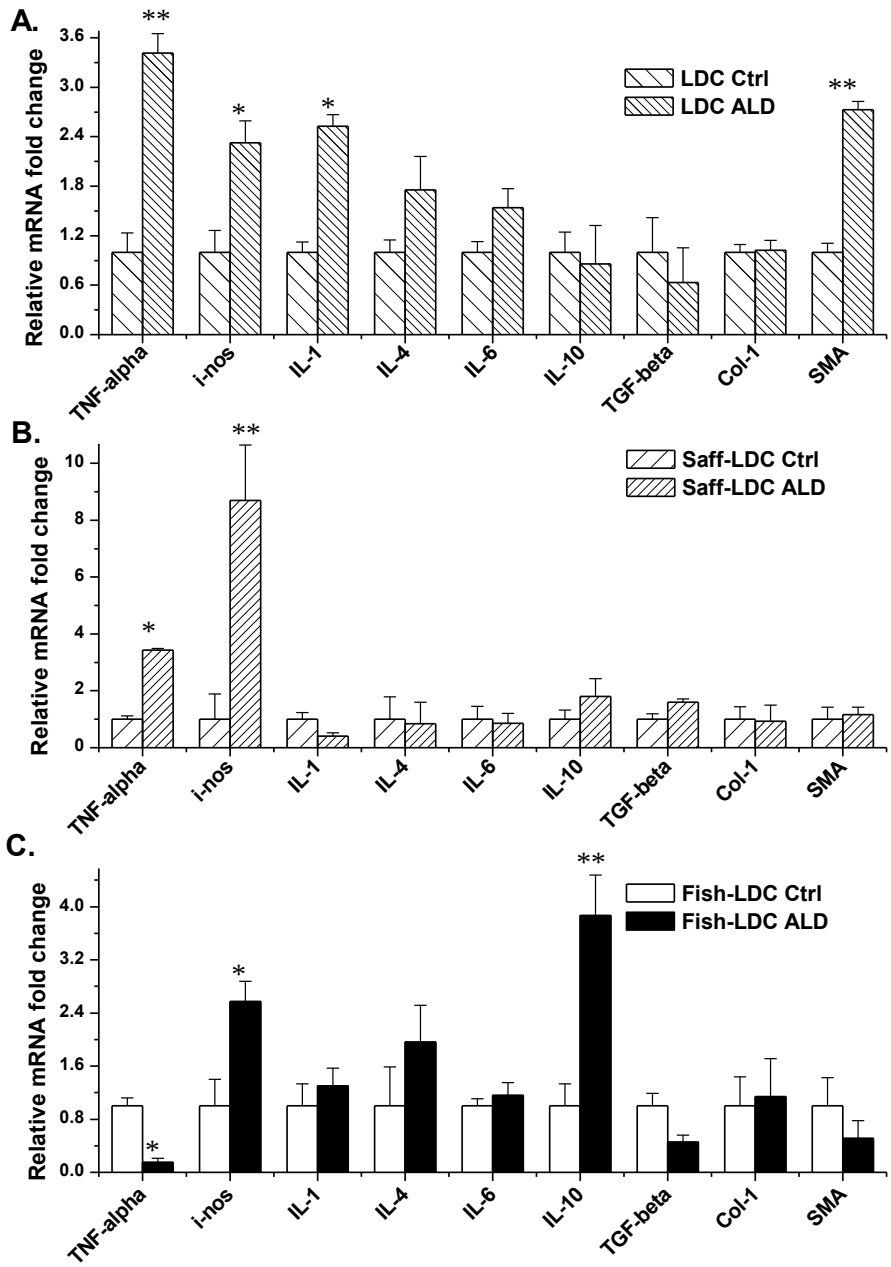


Figure 18. mRNA of inflammatory and fibrotic factors relative to respective control diets. Relative mRNA of pro-inflammatory (TNF-α, i-NOS, IL-1, IL-4, IL-6), anti-inflammatory (IL-10) and pro-fibrotic (TGF-β, SMA, collagen-1) factors in rats fed: (A) LDC-ALD and LDC-Ctrl diets, (B) Saff-LDC ALD and Saff-LDC Ctrl diets, and (C) Fish-LDC ALD and Fish-LDC Ctrl diets. β-actin was used as the internal-standard. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, **$p < 0.01$ compared to respective dextrose controls.

mRNA expression of TNF-α was found to be significantly upregulated in rats fed Saff-LDC ALD diet in comparison to LDC ALD diet **(Figure 19)**. This result indicated that addition of safflower oil in diet containing ethanol could further increase the expression of hepatic TNF-α. Thus, both safflower oil and ethanol acted as potential stimulants of inflammation in rats fed Saff-LDC ALD diet. No significant alteration in the mRNA expression of other inflammatory factors was observed in rats fed Saff-LDC ALD diet with respect to LDC ALD diet. On the other hand, downregulation of TNF-α and i-NOS was observed by addition of fish oil in rats fed Fish-LDC ALD diet with respect to LDC ALD diet (Figure 19). In addition, anti-inflammatory cytokine, IL-10 was found to be significantly upregulated in rats fed Fish-LDC ALD diet in comparison to LDC ALD. Thus, downregulation of pro-inflammatory factors, TNF-α, i-NOS and increase in anti-inflammatory IL-10 could be the possible mechanisms by which fish oil prevented ethanol mediated liver inflammation. No significant alteration in the gene expression of other pro-inflammatory cytokines (IL-1, IL-4, IL-6) was observed between Fish-LDC ALD diet in comparison to LDC ALD diet (Figure 19).

No significant change in gene expression of any of the pro-fibrotic factors tested was observed in rats fed Saff-LDC-ALD diet in comparison to LDC-ALD, indicating that addition of safflower oil did not further induce fibrosis in these rats during the 8-week of feeding. In contrast, rats fed Fish-LDC ALD diet showed decrease in gene expression of TGF-β with respect to LDC ALD fed rats (Figure 19) which suggested that fish oil could possibly prevent the onset of ethanol mediated fibrosis via downregulation of TGF-β in liver.

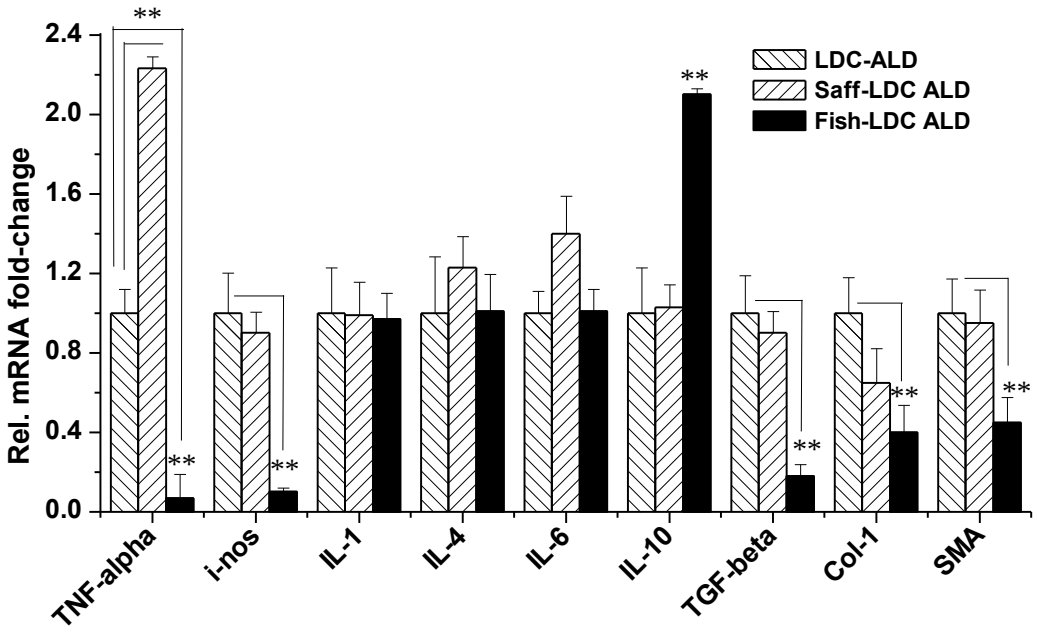


Figure 19. mRNA of inflammatory and fibrotic factors relative to LDC-ALD diet. Relative mRNA of pro-inflammatory (TNF-α, i-NOS, IL-1, IL-4, IL-6), anti-inflammatory (IL-10) and pro-fibrotic (TGF-β, SMA, collagen-1) factors in rats fed Saff-LDC ALD and Fish-LDC ALD with respect to LDC-ALD. β-actin was used as the internal-standard. Values are mean ± SEM from n=4-6 rats in each group, *p < 0.05, **p < 0.01 compared to LDC-ALD.

Protein expression of TNF-α was found to be significantly upregulated by ethanol in rats fed LDC and Saff-LDC diets with respect to their controls **(Figure 20)**. Addition of safflower oil in diet containing ethanol did not further increase the protein expression of TNF-α. In contrast, addition of fish oil in diet significantly downregulated TNF-α in rats fed Fish LDC ALD diet with respect to LDC ALD diet (Figure 19, 20). In addition, rats fed Saff-LDC Ctrl diet showed higher expression of TNF-α than both LDC-Ctrl and Fish-LDC Ctrl fed rats. This result indicated that safflower oil (high ω-6 vs ω-3) could cause an increase in TNF-α even in the absence of ethanol in diet, thus resulting in liver inflammation and development of NASH in these rats (Figure 20).

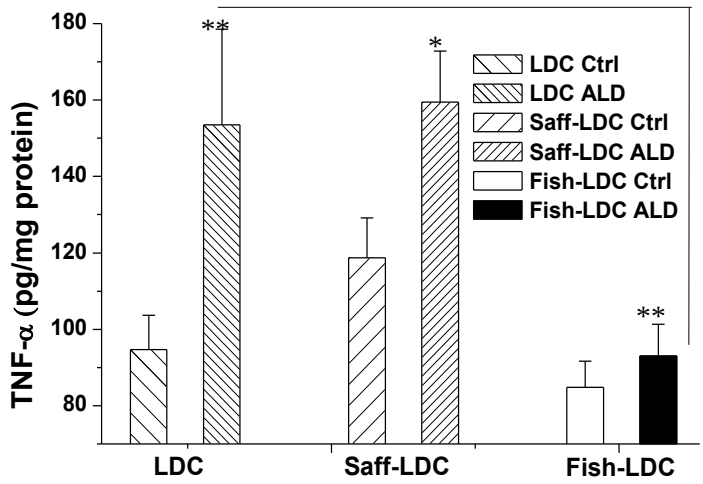


Figure 20. Protein expression of TNF-α. TNF-α measured in the liver using ELISA in rats fed LDC-ALD and LDC-Ctrl, Saff-LDC ALD and Saff-LDC Ctrl, Fish-LDC ALD and Fish-LDC Ctrl diets. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, **$p < 0.01$, compared to respective dietary controls and compared to LDC-ALD.

4.2.2. Activation of NF-ΚB in liver

Activation of NF-κB in the liver was measured by the expression of its phosphorylated NF-κB p65 subunit. Activated form of NF-κB was observed in rats fed LDC-ALD diet (rich in ω-6 PUFA) **(Figure 21)**, suggesting that ethanol caused the activation of this transcription factor possibly involved in the secretion of TNF-α. Interestingly, activated form of NF-κB was observed both in the livers of Saff-LDC ALD and Saff-LDC Ctrl rats. The highest expression of NF-κB was observed in livers of rats fed Saff-LDC ALD (Figure 21), which suggested that safflower oil co-administered with ethanol caused the maximum activation of NF-κB. In contrast, very low expression of activated phosphorylated form of NF-κB was observed in rats fed Fish-LDC ALD diet, and NF-κB was not even detected in rats fed Fish-LDC Ctrl diet (Figure 21). This data suggested that while fish oil (Fish-LDC diet) could significantly reduce the ethanol mediated activation of NF-κB and its associated induction of TNF-α, LDC diet failed to

prevent and Saff-LDC diet further increased the ethanol mediated activation of NF-κB and its associated induction of TNF-α.

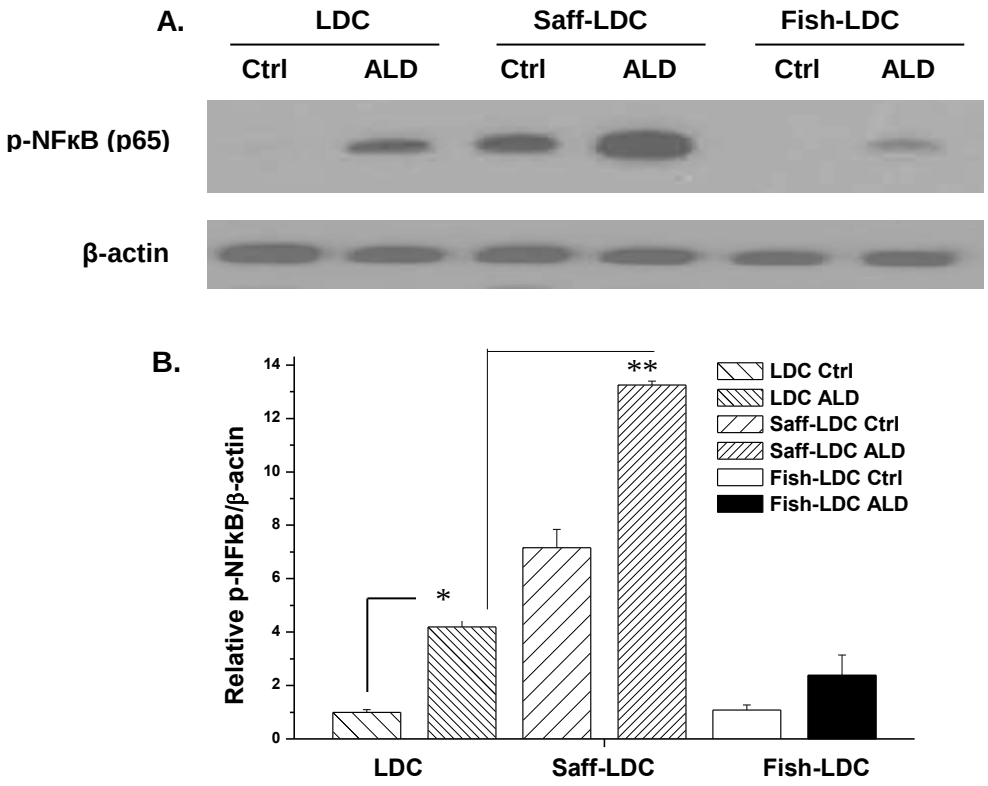


Figure 21. Western blot showing the expression of activated form of NF-κB. Effect of PUFAs on activation of NF-κB measured by the phosphorylation of the NF-κB p-65 subunit which precedes the activation of this transcription factor and its translocation into the nucleus resulting in transcription of TNF-α. (A) Western blot analysis of pNF-κB, and (B) Histogram representing mean ± SEM of the densitometric scans from four separate experiments, normalized by comparison with β-actin and expressed as relative to LDC Ctrl value, *p < 0.05, **p < 0.01, compared to respective dietary controls, *p < 0.05, **p < 0.01, compared to LDC-ALD.

Data from Figure 19-21 and Table-5 indicated that diet containing both ethanol and safflower oil, i.e. Saff-LDC ALD diet acted like a 'double hit' with both ethanol and ω-6 PUFA as stimulants, potentiating the activation of NF-κB causing the maximum induction of TNF-α which resulted in highest scores for inflammation. In contrast, fish oil diet was found to prevent the activation of NF-κB, which possibly contributed in the downregulation

of TNF-α gene expression and prevented upregulation in its protein expression (Figure 19-20), thus exerting anti-inflammatory effect (Table-5). On comparing the protein expression of pNF-κB among the control groups of the three different dietary regimens, rats fed Saff-LDC diet showed significantly higher expression of pNF-κB than both LDC and Fish-LDC fed rats. This result indicated that safflower oil could activate NF-κB even in the absence of ethanol in the diet (Figure 21), resulting in increased transcription of TNF-α and consequently increased liver inflammation in rats fed Saff-LDC diet.

4.3. Discussion and conclusion

Downregulation of TNF-α and i-NOS mRNA was observed in rats fed Fish-LDC ALD diet with respect to LDC ALD diet (Figure 19). Increase in mRNA of anti-inflammatory cytokine, IL-10 was also observed in rats fed Fish-LDC ALD diet in comparison to rats fed LDC ALD diet. This data suggested that downregulation of pro-inflammatory TNF-α, i-NOS and increase in anti-inflammatory, IL-10 could be the possible preventive mechanisms by which fish oil prevented ethanol mediated liver inflammation (Figure 19). In contrast mRNA expression of TNF-α was found to be significantly upregulated in rats fed Saff-LDC ALD diet in comparison to rats fed LDC ALD diet (Figure 19), suggesting that safflower oil in diet containing ethanol could further increase the expression of hepatic TNF-α.

Protein expression of TNF-α was found to be significantly upregulated by ethanol in rats fed LDC and Saff-LDC diets with respect to their controls (Figure 20). Safflower oil did not further increase TNF-α expression in diet containing ethanol, while fish oil significantly downregulated TNF-α in diet containing ethanol (Figs. 19, 20). In addition, rats fed Saff-LDC Ctrl diet showed higher expression of TNF-α than both LDC-Ctrl and

Fish-LDC Ctrl fed rats. Thus, safflower oil (high ω-6 vs ω-3) could increase TNF-α even in absence of ethanol in diet, resulting in liver inflammation and initiation of NASH in these rats (Figure 20).

No significant change in gene expression of any of the pro-fibrotic factors tested was observed in rats fed Saff-LDC-ALD diet in comparison to LDC-ALD diet, indicating safflower oil did not induce fibrosis in these rats during the 8-week feeding period tested in this study. In contrast, Fish-LDC ALD diet decreased gene expression of TGF-β with respect to LDC ALD diet (Figure 19), which indicated that fish oil could possibly prevent the onset of ethanol mediated liver fibrosis via downregulation of TGF-β. However, no significant change in SMA or collagen 1 gene expression was observed by fish oil in diet containing ethanol. This study provides a starting point for further investigation of downregulation of TGF-β as a possible mechanism in prevention of ethanol mediated fibrosis by fish oil or other ω-3 rich diets.

Activation of NF-κB was observed when ethanol was administered with LDC diet, indicating that ethanol could potentially activate this transcription factor. Since NF-κB has been implicated in the induction of TNF-α (Figure 8), activation of NF-κB may thus be one of the possible mechanisms of the ethanol mediated increase in TNF-α in rats fed LDC-ALD diet compared to rats fed LDC-Ctrl diet. Rats fed Saff-LDC ALD diet showed the highest activation of NF-κB among all dietary groups; also with significantly higher activation compared to LDC-ALD group. This result re-emphasized that Saff-LDC ALD diet could act like a ‘double hit’ with both ethanol and ω-6 PUFA acting as stimulants further potentiating the ethanol mediated activation of NF-κB. This high activation of NF-κB resulted in the high induction of TNF-α in this group (Figs. 19, 20) consistent to the

highest scores for inflammation (Table-5). In contrast, significant decrease in activation of NF-κB was observed in Fish-LDC ALD group (Figure 21) compared to LDC-ALD. This result indicated that fish oil could prevent the ethanol mediated liver inflammation by preventing activation of NF-κB, leading to lower expression of TNF-α (consistent to the pathology examination scores for inflammation, Table-5).

Interestingly, rats fed Saff-LDC Ctrl diet also showed significantly higher expression of phosphorylated (activated) NF-κB than both LDC Ctrl and Fish-LDC Ctrl fed rats. This result re-emphasized that safflower oil could activate NF-κB even in absence of ethanol in diet (Figure 21), initiating the development of NASH by increasing the transcription of TNF-α, consequently increasing inflammation.

CHAPTER 5. ALTERATION IN AA PEROXIDATION BY ω-3 AND ω-6 PUFAs AND ITS EFFECT ON ETHANOL INDUCED LIVER INFLAMMATION

5.1. Introduction

The effects of PUFAs in ethanol mediated liver injury are thought to be mediated through increased oxidative stress by lipid peroxidation [15, 58-61, 65], including the reaction catalyzed by enzymes lipoxygenase (LOX) and cycloxygenase (COX) [35, 39, 41, 43]. COX catalyzes the synthesis of PGG_2 from arachidonic acid (AA) which is further converted to PGH_2. PGH_2 is subsequently converted into a series of PGs and TXA_2 via COX [35] (Figure 4). COX-2, the inducible isoform of COX can be induced by lipid peroxidation and inflammatory stimuli such as cytokines and endotoxins [37, 39, 183, 184]. Reactive oxygen intermediates and lipid hydroperoxides have also been shown to regulate the expression of COX-2 [39, 183, 184].

Out of the various isoforms of LOX (5-, 15-, 12 and 8-LOX), 5-LOX has previously been implicated in the progression of chronic liver diseases [41-46]. 5-LOX catalyzes the oxygenation of AA to produce 5-hydroxeicosatetraenoic acid (5-HETE) followed by synthesis of leukotrienes which display a variety of pro-inflammatory actions [41, 45, 46] (Figure 4). CYP450 2E1, an enzyme from the CYP450 family of microsomal enzymes, has also been implicated in ethanol induced oxidation and generation of reactive oxygen species, e.g. acetaldehyde, superoxide etc. The alteration in generation of eicosanoids (e.g. PGs and HETEs) from COX and LOX mediated peroxidation of AA has been associated with ethanol mediated liver injury [36-46]. However, the generation of PGs and HETEs from dietary ω-3 or ω-6 PUFAs in ALD has not been studied. In this thesis, we studied the alteration in generation of PGs and HETEs formed by peroxidation of AA, the fatty acid

precursor of several bioactive lipid metabolites having pro-inflammatory activities. We investigated how ω-3 or ω-6 PUFAs as substrates for COX and LOX affect the peroxidation of AA and result in alteration of AA mediated inflammatory factors in ALD (Figure 5).

In addition to generation of eicosanoids, e.g. PGs and HETEs, both acute and chronic ethanol administration also lead to formation of free radicals in the liver [14, 52, 147, 148]. In vivo lipid peroxidation is a very complex free radical event in which different types of PUFA-derived free radicals may be generated via a number of peroxidation pathways (Figure 1). However, they have not been structurally identified before mainly due to the lack of appropriate methodology. In this thesis, we studied the generation of AA-derived free-radicals mediated by several enzymatic and non-enzymatic lipid peroxidation pathways. We tentatively identified the structures of these radicals along with their possible source of generation in vivo. In addition, we studied whether dietary ω-3 or ω-6 PUFAs affect the generation of AA-derived free-radicals in ALD and how these reactive lipid species exert pro- or anti-inflammatory activity, thus contributing to either progression or retardation of liver inflammation and injury.

Electron spin resonance (ESR) with spin trapping technique was used to detect free radicals derived from AA. During spin trapping, the short-lived radicals react with spin traps such as POBN to form relatively stable radical adducts (Figure10) [149, 150]. We utilized the combined technique of liquid chromatography (LC) and ESR in combination with mass spectrometry (MS), wherein radical adducts are first separated and sequentially eluted from the HPLC column, followed by ESR detection and characterization of

molecular mass by MS [29, 152, 159-163] (Figure11). The details of the experimental methodology, techniques and reagents have been described in Chapter-2.

In addition to alteration of lipid peroxidation, the amount of dietary fat intake has been shown to be the principal determinant of the degree of fatty infiltration in animals and humans ingesting alcohol [53, 54, 58]. Long term ethanol consumption has been shown to inhibit the enzymes involved in beta-oxidation of fatty acids [119-123]. Ethanol has also been shown to increase the rate of fatty acid and triglyceride synthesis in liver by inducing the expression of key enzymes of lipid synthesis, resulting in fat accumulation and mitochondrial dysfunction [119-123]. This increase in free fatty acid concentration in the liver may in turn alter the rate and extent of lipid peroxidation.

Peroxisome proliferator activated receptor-alpha (PPAR-α), a member of the nuclear hormone receptor superfamily is the key transcriptional regulator of many genes involved in fatty acid oxidation system in liver including acyl-coA oxidase, liver fatty acid-binding protein and lipoprotein lipase [124]. PPAR-α is activated upon binding with fatty acids, hence contributes to control their intracellular concentration in the liver [125]. Several studies have investigated the role of PPAR-α in the pathogenesis of alcoholic fatty liver [125-127]. Fatty acid levels are increased in the liver during the metabolism of ethanol, therefore the proteins transcribed by PPAR-α were expected to be induced by alcohol consumption. However, some studies reported that the mRNA level of PPAR-α was decreased by ethanol feeding, leading to increased concentration of free fatty acids in the liver [126, 127]. This may in turn, alter the rate and extent of lipid peroxidation, since these free fatty acids act as substrates for lipid peroxidation.

Thus, alteration in the expression of PPAR-α may not only alter the rate of lipid peroxidation but also affect the type of PUFAs available as substrates to undergo lipid peroxidation. In this thesis, we studied the alteration in PPAR-α expression after administration of diets rich in ω-3 and ω-6 PUFAs. We also examined the correlation of PPAR-α expression with lipid peroxidation and the severity of ethanol induced fatty liver or steatosis.

5.2. Results and discussion

5.2.1. Hepatic free fatty acid profile

To determine the basal substrate (PUFA) levels for hepatic lipid peroxidation, ω-6 (AA and LA) and ω-3 (EPA and DHA) PUFA concentrations were measured in liver **(Figure 24)**. Concentration of PUFAs after acute administration of AA were also measured **(Figure 25)**. Concentration of LA, EPA and DHA were also measured since they were the major fatty acid constituents of Saff-LDC and Fish-LDC diets, e.g. LA being the major PUFA constituent of safflower oil, while EPA and DHA being the major PUFA constituents of fish oil. Ethanol has also been shown to affect the metabolism of dietary fatty acids in liver, leading to alteration in PUFA concentrations in the liver which may affect the rate and extent of lipid peroxidation.

LC/MS was used for determination of free-fatty acid concentrations in the extracted liver samples (Figure 24, 25). Details of the experimental methods, techniques and reagents have been described in Chapter-2. Extracted ion chromatograms (EICs) of the fatty acids and internal standards, LA-d_4 and AA-d_8 (200 ng) **(Figure 22, 23)** were used for quantification of the endogenous PUFAs.

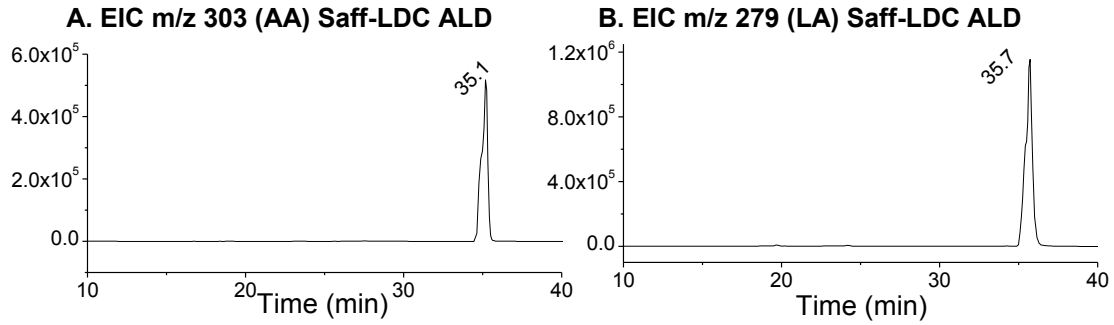


Figure 22. LC/MS analysis for determination of hepatic LA and AA concentration. EICs are representatives selected from the Saff-LDC ALD group, n=4-6 rats in each group. (A) EIC m/z 303, AA, and (B) EIC m/z 279, LA. AA-d_8 (20 ng/µL), t_R 38.1 min (m/z 311) and LA-d_4 (20 ng/µL), t_R 34.5 min (m/z 283) were used as internal standards added prior to liver extraction.

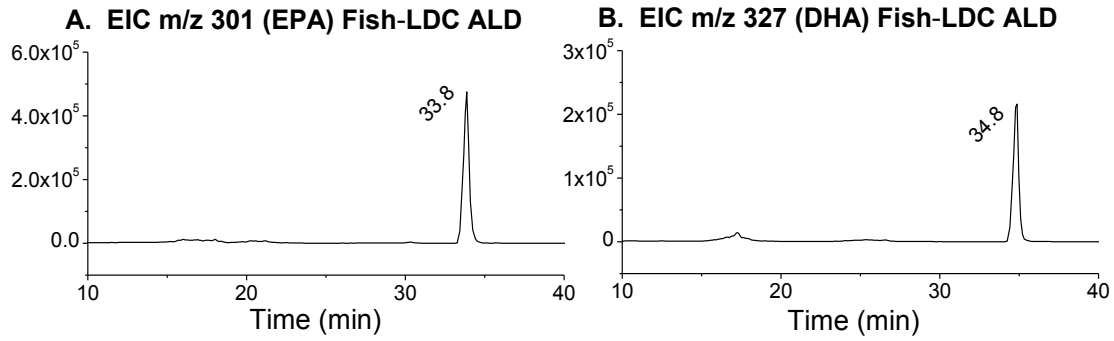


Figure 23. LC/MS analysis for determination of hepatic EPA and DHA concentration. EICs are representatives selected from the Fish-LDC ALD group, n=4-6 rats in each group. (A) EIC m/z 301, EPA, and (B) EIC m/z 327, DHA. EIC are representative from n=4-6 rats in each group. LA-d_4 (20 ng/µL), t_R 34.5 min (m/z 283) was used as internal standard added prior to liver extraction.

Significant elevation in basal hepatic AA concentration was observed in rats fed LDC-ALD and Saff-LDC ALD diet in comparison to their respective control diets (Figure 24). Saff-LDC ALD diet resulted in much higher hepatic AA concentration than the LDC-ALD diet possibly via increasing the uptake of AA by hepatic membrane transporters. No significant difference in the basal free AA concentration was observed in the LDC-Ctrl and Saff-LDC Ctrl fed rats.

No significant increase in the basal free AA concentration was observed in the livers of rats fed Fish-LDC ALD diet in comparison to its control (Figure 24). AA concentration was also found to be much lower in Fish-LDC ALD rats in comparison to LDC-ALD rats possibly via decreased uptake of AA by hepatic membrane transporters. Arachidonate levels in Fish-LDC Ctrl fed rats were significantly lower than both LDC-Ctrl and Saff-LDC Ctrl fed rats (Figure 24). This result suggested that fish oil in diet could reduce the free AA concentration in the liver, possibly resulting in lower generation of AA-derived pro-inflammatory metabolites, thus reducing liver inflammation.

No significant alteration in the levels of free LA was observed in any dietary group fed ethanol relative to their respective controls (Figure 24). The data suggested that the metabolism of LA may not be altered by ethanol consumption. As expected, the levels of free EPA and DHA were found to be much higher in Fish-LDC groups (both ethanol and control) than in LDC and Saff-LDC groups (Figure 24).

EPA and DHA are substrates for COX and LOX mediated peroxidation and generate PGE_3 and resolvins, anti-inflammatory metabolites [57, 68, 69, 71] (Figure 5). Increase in free EPA and DHA concentration with the resulting increase in their peroxidation may lead to increase in formation of anti-inflammatory products, which thereby reduced liver inflammation (Chapter-3, Table-5). Hepatic concentration of free AA was also measured after acute treatment with AA. Comparison of free AA concentration between the ethanol and control group after acute AA treatment indicated that ethanol can alter the metabolism and uptake of AA in the liver **(Figure 25)**.

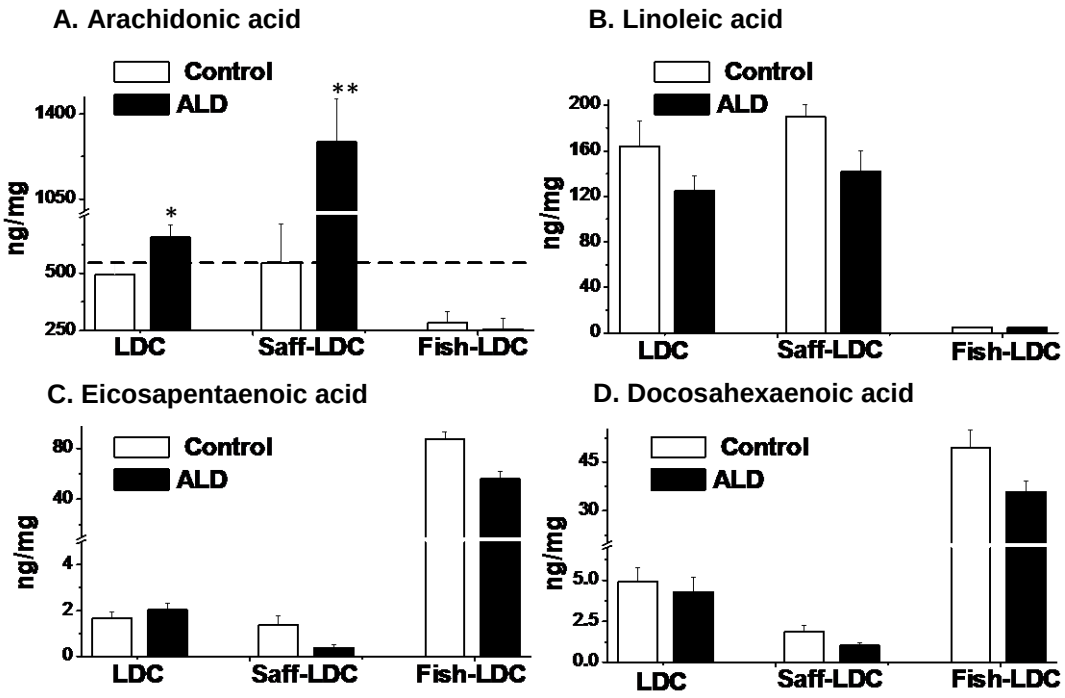


Figure 24. LC/MS determination of basal concentration of AA, LA, EPA and DHA in liver. Rats were fed LDC, Saff-LDC and Fish-LDC, ALD and Ctrl diets. (A) Measurement of AA, (B) Measurement of LA, (C) Measurement of EPA, and (D) Measurement of DHA. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, ** $p < 0.01$ compared to respective dietary controls.

Significant elevation of hepatic AA was observed in LDC-ALD and Saff-LDC ALD fed rats with respect to their controls, showing that ethanol could cause the alteration of hepatic AA uptake and metabolism, resulting in accumulation of free AA in liver of these rats (Figure 25). This free AA could act as substrate for lipid peroxidation and to increase generation of AA-derived pro-inflammatory metabolites in LDC-ALD and Saff-LDC ALD fed rats. No significant change in hepatic AA concentration was observed between LDC-ALD and Saff-LDC ALD fed rats treated with acute dose of AA.

However, lowest AA concentration was observed in Fish-LDC ALD rats in comparison to LDC-ALD and Saff-LDC ALD fed rats, suggesting fish oil could prevent the ethanol induced elevation in hepatic AA concentration. Data from Figs. 24 and 25 showed that fish oil could lower the hepatic AA concentration, both basal as well as after acute treatment, possibly by lowering the uptake of AA via hepatic membrane transporters. In addition, no change in free AA concentration was observed in livers of rats fed Fish-LDC ALD diet relative to Fish-LDC Ctrl diet.

Thus, unlike ω-6 based diets, fish oil diet could prevent hepatic inflammation by preventing the accumulation of free AA in liver (Figure 25). Lower hepatic AA concentration resulted in lower generation of AA-derived pro-inflammatory metabolites which thereby reduced liver inflammation in fish oil fed rats. In contrast, ω-6 based LDC and Saff-LDC diets with ethanol resulted in high hepatic AA uptake which led to increase in AA concentration, both basal as well as after acute treatment.

High AA concentration could lead to increased generation of AA-derived pro-inflammatory metabolites of peroxidation, thus elevated liver inflammation in rats fed LDC and Saff-LDC diets (Chapter-3, Table-5). Treatment with acute AA was also aimed to increase the sensitivity of detection of AA-derived spin trapped radical adducts formed by lipid peroxidation and spin trapping (Figs. 36, 37).

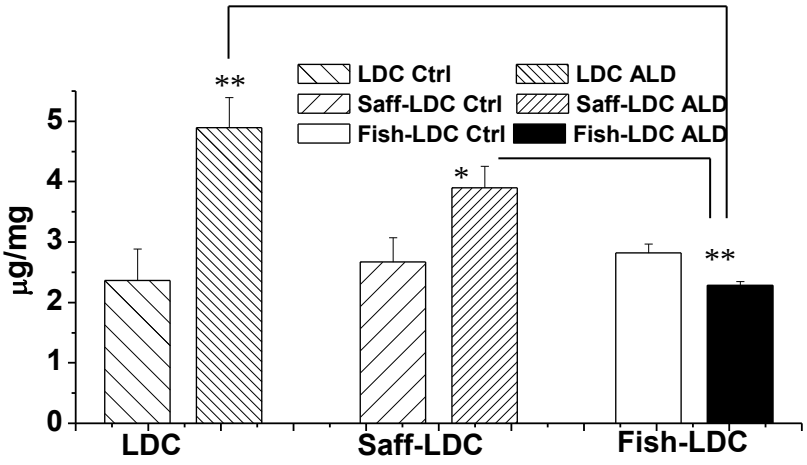


Figure 25. LC/MS determination of AA concentration in liver after acute treatment with AA. Acute dose of AA was injected i.p, in rats fed LDC Ctrl and LDC ALD diets, Saff-LDC Ctrl and Saff-LDC ALD diets, Fish-LDC Ctrl and Fish-LDC ALD diets. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, ** $p < 0.01$, compared to respective dietary controls, ** $p < 0.01$, compared to LDC-ALD.

5.2.2. Gene expression of COX, LOX and CYP450 2E1

mRNA level of lipid peroxidation enzymes COX-1, COX-2, 5-LOX, 15-LOX and oxidation enzyme CYP2E1 was determined using reverse transcriptase real time-polymerase chain reaction.

mRNA expression of COX-2 and 5-LOX were found to be significantly elevated in livers of LDC-ALD and Saff-LDC ALD fed rats with respect to their controls **(Figure 26)**. However, no significant elevation in mRNA of COX-2 and 5-LOX was observed in livers of rats fed Fish-LDC ALD relative to its control (Figure 26). No significant change in the mRNA levels of COX-1 and 15-LOX was observed in the different dietary groups fed ALD or Ctrl diets (Figure 26). mRNA levels of CYP450 2E1 were found to be upregulated in rats fed LDC-ALD diet compared to rats fed LDC-Ctrl diet.

However, no significant alteration in mRNA level of CYP450 2E1 was observed in livers of rats fed Saff-LDC ALD and Fish-LDC ALD diets compared to their respective dextrose controls (Figure 26).

mRNA expression of COX-2 and 5-LOX in livers of rats fed Saff-LDC ALD diet did not show any difference compared to LDC ALD diet, indicating no change in gene expression of these lipid peroxidation enzymes between the two dietary regimens **(Figure 26, 27)**. In contrast, relative mRNA of COX-2 and 5-LOX in livers of Fish-LDC ALD fed rats showed significant downregulation compared to rats fed with LDC ALD. Data from Figs. 26 and 27 suggested that fish oil could decrease COX-2 and 5-LOX gene expression in liver, overcoming the ethanol induced increase in their expression.

Thus, ω-3 PUFA rich Fish-LDC diet may prevent the ethanol mediated upregulation of COX-2 and 5-LOX protein via decreasing their mRNA expression and consequently decreasing the rate of lipid peroxidation. In contrast, ω-6 PUFA rich Saff-LDC and LDC diets promoted the ethanol mediated upregulation of COX-2 and 5-LOX protein expression via possibly increasing their mRNA which resulted in increased rate of lipid peroxidation. COX-2 catalyzes the synthesis of PGs (series-2) from AA which exert pro-inflammatory effects, while PGs (series-3) are generated from EPA/DHA which show anti-inflammatory activity (Figure 4) [35-39, 183, 184]. 5-LOX catalyzes the oxygenation of AA to produce 5-hydroxeicosatetraenoic acid (5-HETE) followed by synthesis of leukotrienes which also display a variety of pro-inflammatory actions [41, 45, 46] (Figure 4).

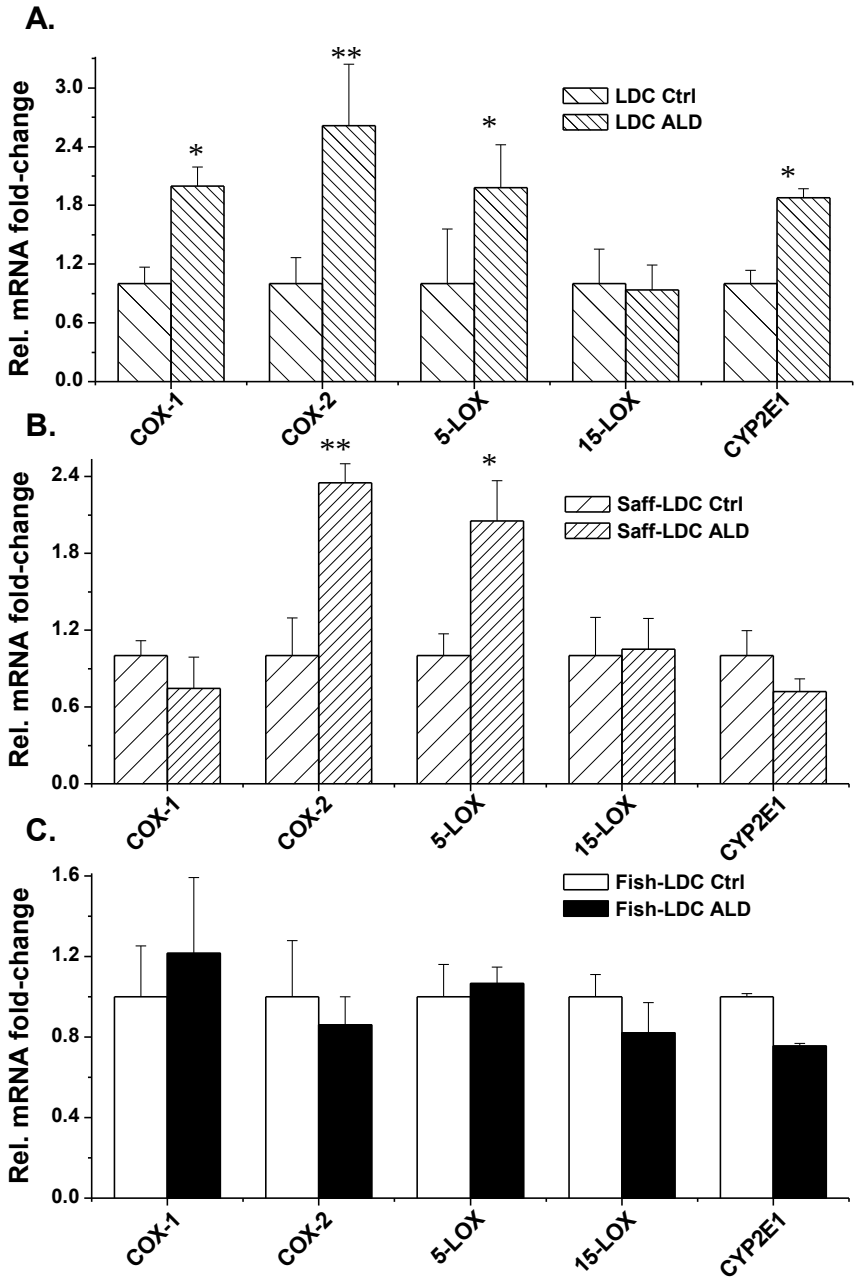


Figure 26. Effect of diet on mRNA of lipid peroxidation and oxidation enzymes. mRNA levels of COX-1, COX-2, 5-LOX, 15-LOX and CYP450 2E1 in livers of rats fed: (A) LDC Ctrl and LDC ALD, (B) Saff-LDC Ctrl and Saff-LDC ALD diets, and (C) Fish-LDC Ctrl and Fish-LDC diets. β-actin served as the house keeping gene. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, ** $p < 0.01$ compared to respective dietary controls.

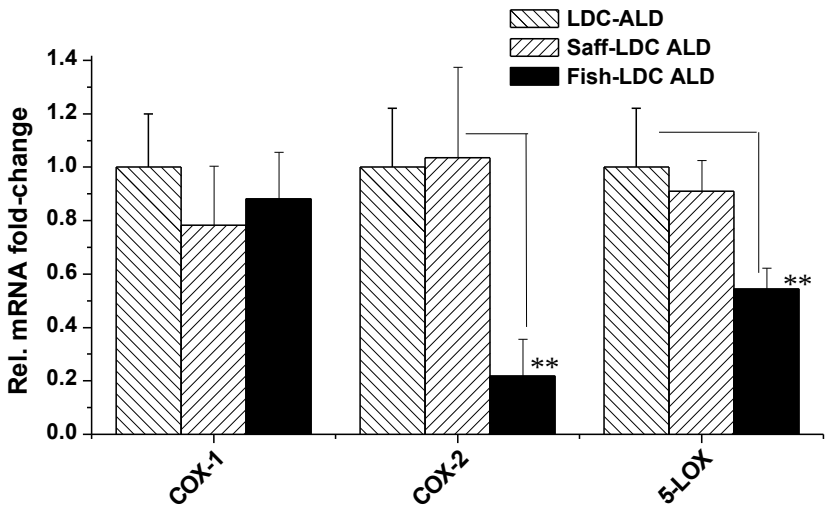


Figure 27. Effect of Fish-LDC and Saff-LDC diet on mRNA of COX-1, COX-2 and 5-LOX. Relative mRNA fold change of COX-1, COX-2 and 5-LOX in Saff-LDC ALD and Fish-LDC ALD with respect to LDC-ALD. β-actin served as the house keeping gene. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, ** $p < 0.01$ compared to LDC-ALD.

5.2.3. Protein level of COX-2 and 5-LOX

Consistent with gene expression, the protein expression of COX-2 and 5-LOX were found to be elevated in livers of rats fed ethanol with LDC and Saff-LDC diets relative to their dextrose controls **(Figs. 28, 29)**. In contrast, downregulation in protein levels of COX-2 and 5-LOX was observed in livers of rats fed Fish-LDC diet relative to its dextrose control (Figs. 28, 29). Protein expression of COX-2 in liver of rats fed Saff-LDC ALD diet was also found to be elevated with respect to LDC ALD, indicating that Saff-LDC ALD diet increased the expression of COX-2 more than ethanol alone in rats fed LDC ALD diet (Figure 28). In contrast, protein expression of COX-2 in liver of rats fed Fish-LDC ALD diet was found to be significantly downregulated with respect to LDC ALD diet (Figure 28), which suggested that fish oil could overcome the ethanol induced increase in COX-2 expression.

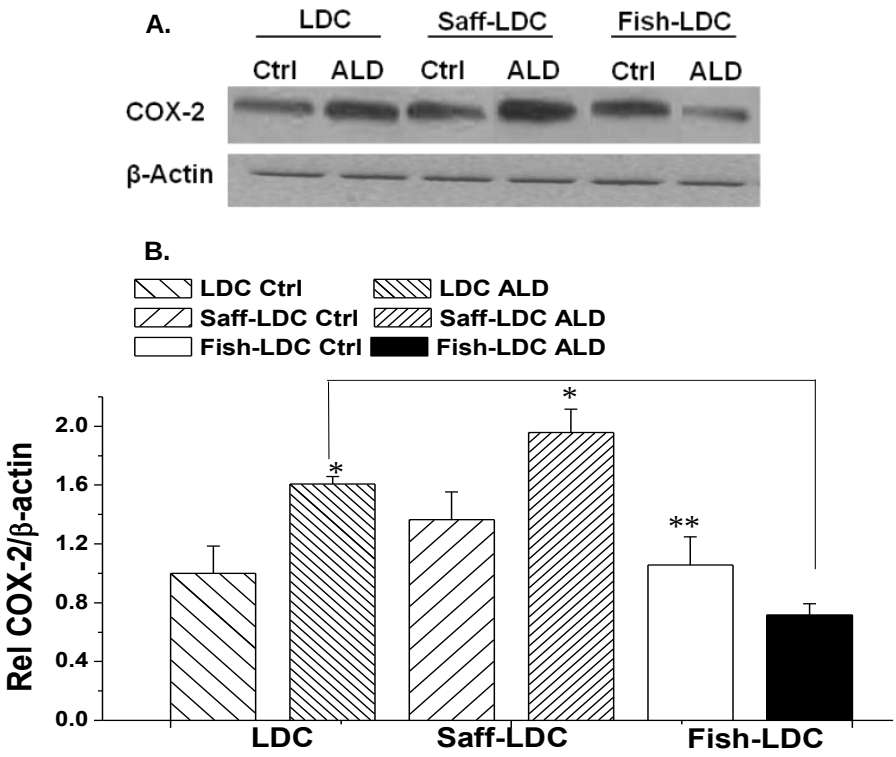


Figure 28. Protein expression of COX-2. COX-2 expression in LDC Ctrl and LDC ALD, Saff-LDC Ctrl and Saff-LDC ALD, Fish-LDC Ctrl and Fish-LDC ALD dietary groups. (A) Western blot analysis of COX-2, and (B) The histogram representing mean ± SEM of the densitometric scans from four separate experiments, normalized by comparison with β-actin and expressed as relative to LDC Ctrl value, *$p < 0.05$, compared to respective dietary controls, ** $p < 0.01$, compared to LDC-ALD group.

Protein expression of 5-LOX in liver of rats fed Saff-LDC ALD diet was found to be significantly elevated in comparison to LDC ALD fed rats, which showed that Saff-LDC ALD diet increased 5-LOX expression more than ethanol alone in rats fed LDC ALD diet (Figure 29). In contrast, rats fed Fish-LDC ALD diet showed significant downregulation of 5-LOX expression (Figure 29) compared to LDC ALD and Saff-LDC ALD diet, which indicated that ω-3 PUFA in rats fed Fish-LDC ALD diet could overcome the ethanol induced increase in 5-LOX expression in liver.

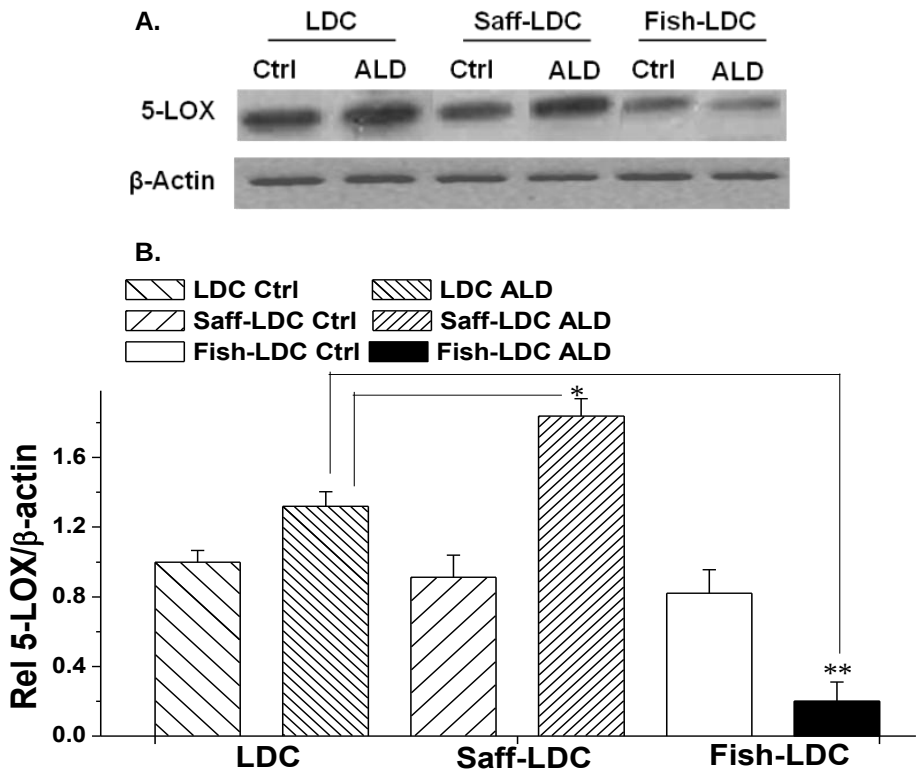


Figure 29. Protein expression of 5-LOX. 5-LOX expression in LDC Ctrl and LDC ALD, Saff-LDC Ctrl and Saff-LDC ALD, Fish-LDC Ctrl and Fish-LDC ALD dietary groups. (A) Western blot of 5-LOX, and (B) Histogram representing mean ± SEM of the densitometric scans from four separate experiments, normalized by comparison with β-actin and expressed as relative to LDC Ctrl value, *$p < 0.05$, **$p < 0.01$, compared to respective dietary controls, *$p < 0.05$, ** $p < 0.01$, compared to LDC-ALD group.

Our results (Figs. 27-29) indicate that fish oil could prevent the ethanol mediated induction of lipid peroxidation enzymes COX-2 and 5-LOX, resulting in decrease in lipid peroxidation mediated by these enzymes. In contrast, safflower oil (high in ω-6 PUFA), further promoted the ethanol mediated upregulation of COX-2 and 5-LOX protein expression, thus increasing lipid peroxidation. Increase in AA-peroxidation by COX-2 and 5-LOX could possibly contribute to increased generation of pro-inflammatory metabolites in rats fed Saff-LDC ALD diet and led to severe inflammation in liver of these rats (Chapter-2, Table-5). In contrast, decrease in COX-2 and 5-LOX mediated AA-

peroxidation in rats fed Fish-LDC ALD diet, possibly contributed to decrease in generation of pro-inflammatory metabolites.

5.2.4. Gene expression of transcription factor PPAR-α

Saff-LDC ALD diet was found to significantly downregulate PPAR-α gene expression compared to Saff-LDC Ctrl diet **(Figure 30)**. However, no significant alteration in the PPAR-α gene expression was observed in rats fed LDC ALD and Fish-LDC ALD diets, in comparison to their respective controls (Figure 30). Decrease in PPAR-α gene possibly contributed to dysregulation of fatty acid oxidation and accumulation of fatty acids and triglycerides, resulting in development of fatty liver in Saff-LDC ALD fed rats. Increase in fatty acid concentration in their liver may further contribute to increase in lipid peroxidation which led to generation of pro-inflammatory lipid species. This study provides a new research direction to evaluate the alteration in expression of enzymes transcribed by PPAR-α, such as liver fatty acid-binding protein (L-FABP), lipoprotein lipase (LPL) and acyl-coA oxidase (ACOX) in ethanol induced liver injury.

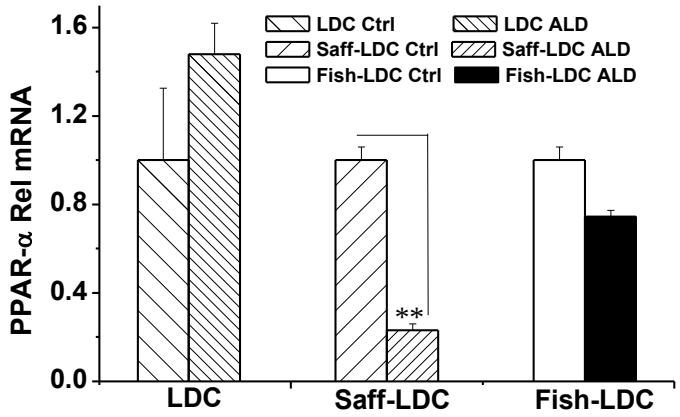


Figure 30. Relative mRNA expression of PPAR-α. PPAR-α is involved in fatty acid metabolism including beta-oxidation pathways in liver. Figure shows the relative mRNA expression of PPAR-α in rats fed LDC-Ctrl, LDC-ALD, Saff-LDC Ctrl, Saff-LDC ALD, Fish-LDC Ctrl and Fish-LDC ALD diets. Values are mean ± SEM from n=4-6 rats in each group, ** $p < 0.01$, compared to respective dietary controls.

5.2.5. Hepatic eicosanoid (PGs and HETEs) profile

LC/MS was used to quantify the generation of bio-active eicosanoids from COX catalyzed AA peroxidation (PGE_2 and $PGF_{2\alpha}$) and LOX catalyzed AA peroxidation (5-, 8-, 12-, 15-HETEs). Extracted ion current (EICs) chromatograms of the PGs and HETEs and the internal standards, PGE_2-d_4 and $PGF_{2\alpha}$-d_4 **(Figure 31, 32)** were used for quantification of the endogenous metabolites.

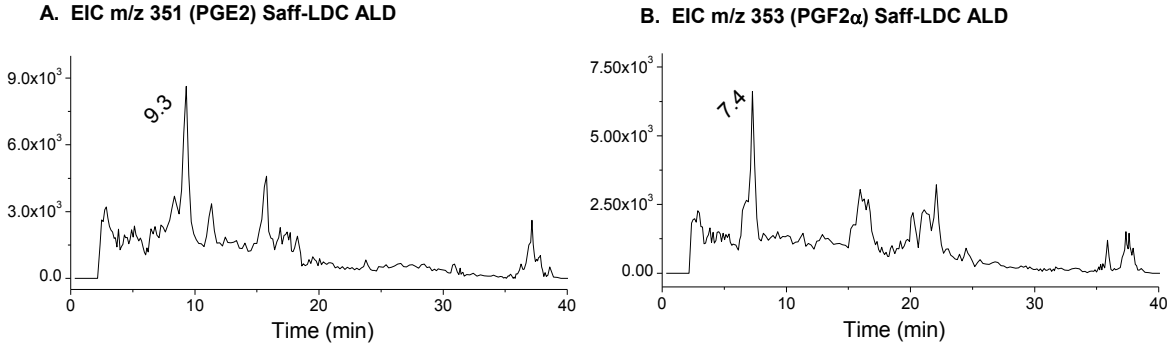


Figure 31. LC/MS analysis for determination of hepatic PGE_2 and $PGF_{2\alpha}$ concentration. EICs are representatives selected from the Saff-LDC ALD group, n=4-6 rats in each group. (A) EIC m/z 351, PGE_2, and (B) EIC m/z 353, $PGF_{2\alpha}$. PGE_2-d_4 (2 ng/μL), t_R 9.1 min (m/z 355) and $PGF_{2\alpha}$-d_4 (2 ng/μL), t_R 7.3 min (m/z 357) were used as internal standards added prior to liver extraction.

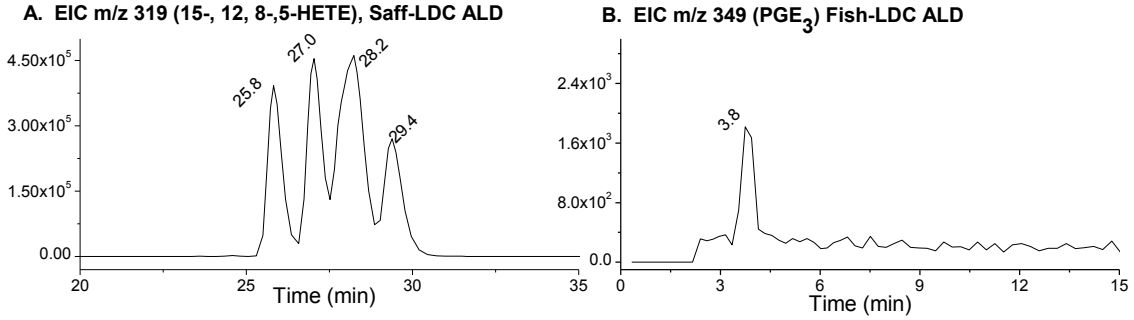


Figure 32. LC/MS analysis for determination of hepatic HETE and PGE_3 concentration. (A) EIC m/z 319, 15-, 12-, 8- and 5-HETE, representative selected from the Saff-LDC ALD group injected with acute AA, and (B) EIC m/z 349, PGE_3, representative selected from the Fish-LDC ALD group. EIC are representative from n=4-6 rats in each group. PGE_2-d_4 (2 ng/μL), t_R 9.1 min (m/z 355) was used as internal standard added prior to liver extraction.

No significant increase in the basal level of COX-AA metabolites (PGs) was observed between rats fed ethanol and dextrose in any of the dietary groups (Figure 33, 34). High concentration of PGE_2 and $PGF_{2\alpha}$ was observed in rats fed LDC-ALD and Saff-LDC ALD diet followed with acute dose of AA relative to their dextrose controls **(Figure 33)**. This result was consistent to the increase in free AA concentration along with COX and LOX expression in liver after treatment with acute AA.

In contrast, no elevation in levels of PGE_2 and $PGF_{2\alpha}$ was observed in rats fed Fish-LDC ALD diet compared to its dextrose control even after acute treatment with AA (Figure 33). Overall, PGE_2 and $PGF_{2\alpha}$ levels were found to be much lower in Fish-LDC fed rats (both ethanol and control) compared to LDC and Saff-LDC fed rats. Thus, fish oil could lower the generation of AA-derived pro-inflammatory PGs through competing for COX-2 and 5-LOX mediated peroxidation, consequently reducing inflammation.

Besides lower generation of pro-inflammatory PGs (series-2) and HETEs, PGE_3, a member of the anti-inflammatory PG-3 series of eicosanoids was only detected in rats fed Fish-LDC diet (Figure 33). PGE_3 has been shown to be further metabolized into resolvins which possess strong anti-inflammatory activity in addition to some immunoregulatory activities [57, 68, 71]. Thus, fish oil not only resulted in lower generation of AA-derived pro-inflammatory PGs, but also increased the generation of EPA and DHA-derived anti-inflammatory PG-3 series of eicosanoids which may prevent liver inflammation.

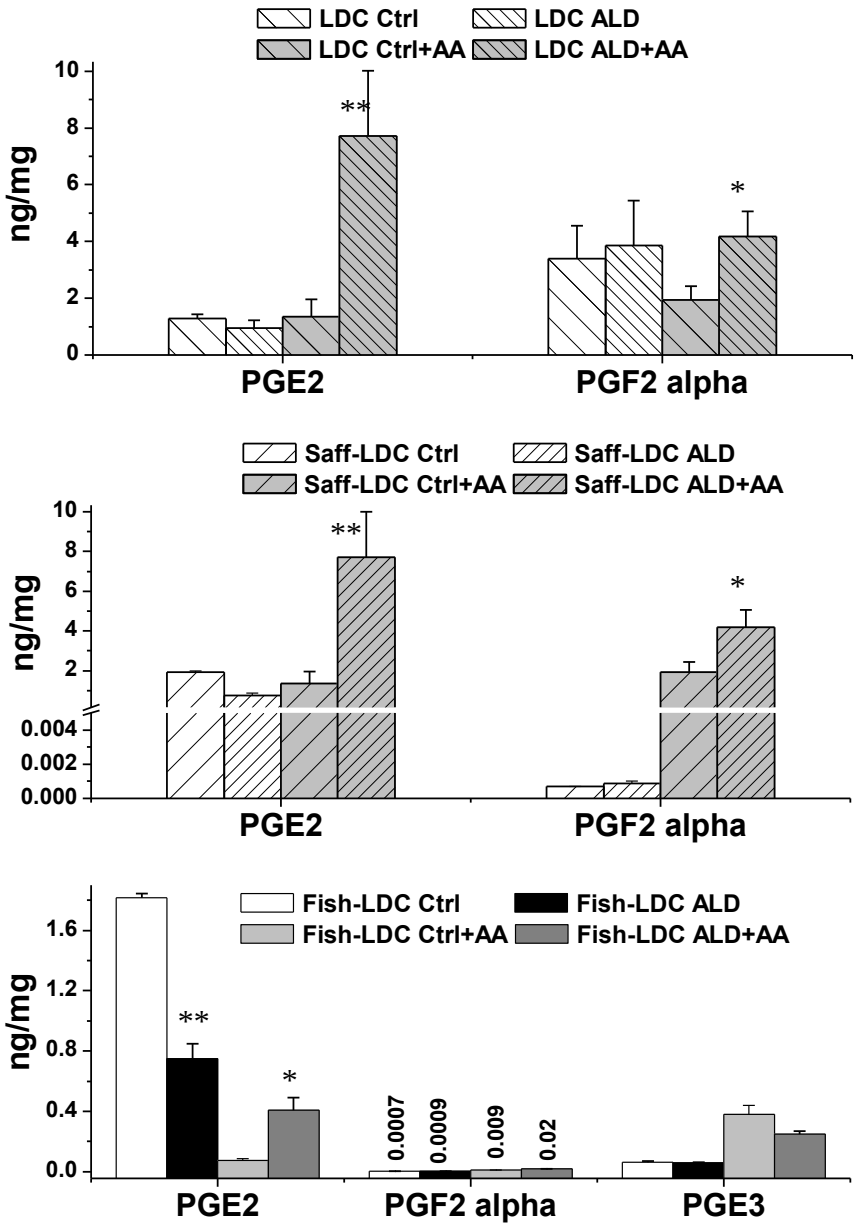


Figure 33. Determination of PGE_2, $PGF_{2\alpha}$ and PGE_3 generated via the COX-AA peroxidation. Concentration of PGs measured in liver of rats with or without acute treatment with AA (injected i.p) from: (A) LDC Ctrl and LDC ALD diets, (B) Saff-LDC Ctrl and Saff-LDC ALD diets, and (C) Fish-LDC Ctrl and Fish-LDC diets. Values are mean ±SEM from n=4-6 rats in each group, *$p < 0.05$, **$p < 0.01$ compared to respective dietary controls.

Significant decrease in the basal level of 15-, 12-, 8-, 5-HETEs (LOX-AA metabolites) was observed between rats fed Fish-LDC ALD diet compared to Fish-LDC Ctrl, while no change was observed between rats fed LDC-ALD and Saff-LDC ALD diets with respect to their dextrose control **(Figure 34)**.

Significant increase in concentration of 15-, 12-, 8-, 5-HETEs in rats fed Saff-LDC ALD diet and 5-HETE in LDC-ALD rats was also observed in rats treated with acute AA relative to their dextrose controls. Increase in concentration of 5-HETE was consistent to the increase in concentration of free AA and 5-LOX expression in liver after treatment with an acute dose of AA in these rats. Increase in 15-, 12-, 8-HETEs may be attributed to the possible increase in activities of these enzymes. After acute treatment with AA, no elevation in HETEs was observed in rats fed Fish-LDC ALD relative to its control diet (Figure 34).

In addition, overall HETE level was found to be lower in Fish-LDC fed rats compared to Saff-LDC and LDC groups, indicating fish oil could lower the generation of HETEs, pro-inflammatory products. By competing for LOX mediated peroxidation and decreasing AA concentration by lowering its uptake via hepatic membrane transporters, fish oil could limit the generation of AA-derived pro-inflammatory HETEs (15-, 12-, 8-HETEs), thus reduced inflammation (Figure 34, Table-5).

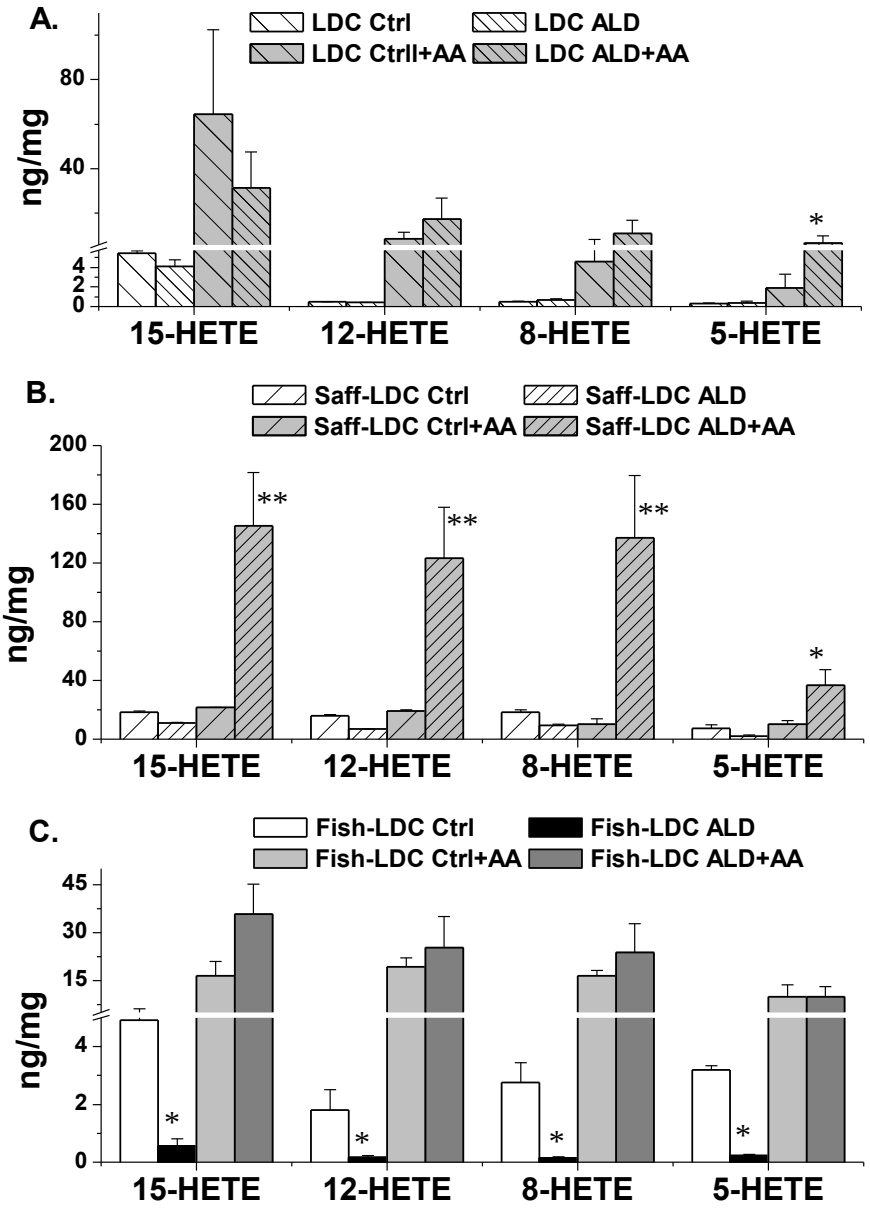


Figure 34. Determination of 15-, 12-, 8-, 5-HETEs generated via the LOX-AA peroxidation. Concentration of HETEs measured in liver of rats with or without acute treatment with AA (injected i.p) from: (A) LDC Ctrl and LDC ALD diets, (B) Saff-LDC Ctrl and Saff-LDC ALD diets, and (C) Fish-LDC Ctrl and Fish-LDC ALD diets. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, **$p < 0.01$ compared to respective dietary controls.

5.2.6. Measurement of free radicals

5.2.6.1. Offline ESR

Six-line spectra was observed when offline ESR was recorded using liver (a_H = 3.04 and a_N= 14.97) and bile (a_H = 2.77 and a_N =14.96) samples collected from rats after acute administration of AA (0.5 g/kg, i.p) and spin trap POBN (0.75 g/kg, i.p) **(Figs. 35, 36)**. Signal intensity (for both liver and bile) was below detection limit in samples collected without acute AA treatment (data not shown). Livers were extracted via a modified folch extraction procedure, while bile was directly transferred to the ESR flat cell and subjected to offline ESR analysis (Materials and Methods, Chapter-2).

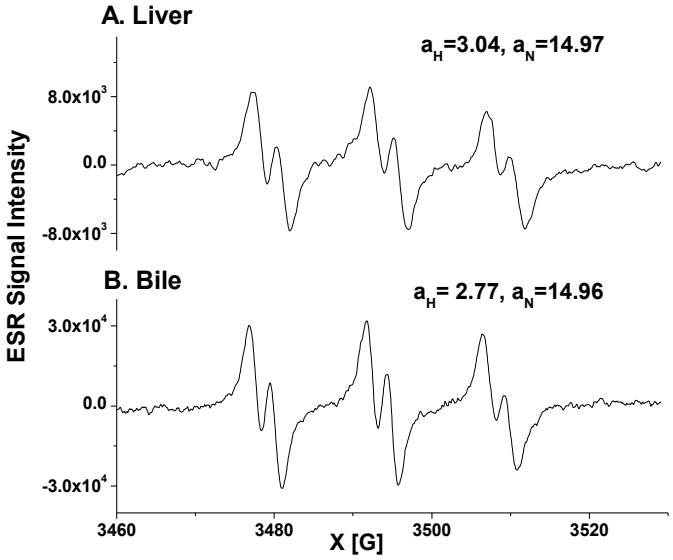


Figure 35. Offline ESR spectra determined using liver and bile. Representative spectra showing offline ESR obtained from: (A) liver, and (B) bile samples from Saff-LDC ALD group. Six-line spectra was observed when offline ESR was recorded using liver (a_H = 3.04 and a_N= 14.97) and bile (a_H = 2.77 and a_N =14.96) collected from rats after acute administration of AA and spin trap POBN.

Offline ESR signal intensity in bile of rats fed LDC and Saff-LDC diets was found to be much higher than the ESR signal intensity in rats fed Fish-LDC diet. This data indicated much lower free radical generation in rats fed Fish-LDC diet in comparison to

rats fed LDC and Saff-LDC diets, possibly due to the lower COX-2 and 5-LOX expression, as well as lower uptake of AA in liver **(Figure 36)**.

Significant elevation in signal intensity was observed in bile of rats fed Saff-LDC ALD diet, while no change in signal intensity was observed in bile of rats fed Fish-LDC ALD diet with respect to their control rats. Interestingly, signal intensity was found to be lower in bile of rats fed LDC-ALD diet in comparison to LDC-Ctrl diet (Figure 36). This observation was later attributed to the lower expression of hepatic efflux transporter MRP-2 and its potential role in the efflux of radical adducts and their possible phase-II conjugates (for example cysteine conjugates), respectively (Chapter-6).

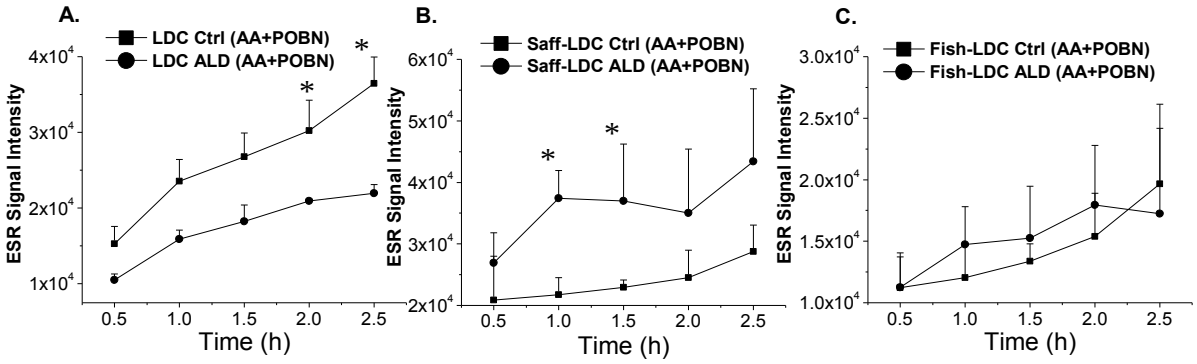


Figure 36. Offline ESR signal intensity determined using bile. Samples were collected from rats injected AA and POBN i.p in groups: (A) LDC Ctrl and LDC ALD, (B) Saff-LDC Ctrl and Saff-LDC ALD, and (C) Fish-LDC Ctrl and Fish-LDC ALD, respectively. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$ compared to respective dietary controls.

Using liver samples, however, significant elevation in ESR signal intensity was observed in rats fed LDC-ALD diet, compared to its dextrose control. In contrast, decrease in ESR signal intensity was observed in livers of rats fed modified diets with ethanol (both Saff-LDC ALD and Fish-LDC ALD), in comparison to their respective dextrose controls **(Figure 37)**. For rats fed Fish-LDC diet, this observation was attributed to the lower

expression of COX-2 and 5-LOX in Fish-LDC ALD rats relative to Fish-LDC Ctrl rats, leading to lower AA-derived radical generation.

For rats fed Saff-LDC diet, this observation was credited to the differential regulation of hepatic membrane transporters like MRP-2, MRP-3 and ABCA1 (Chapter-6) and possibly several other transporters involved in efflux of fatty acids and their metabolites, respectively.

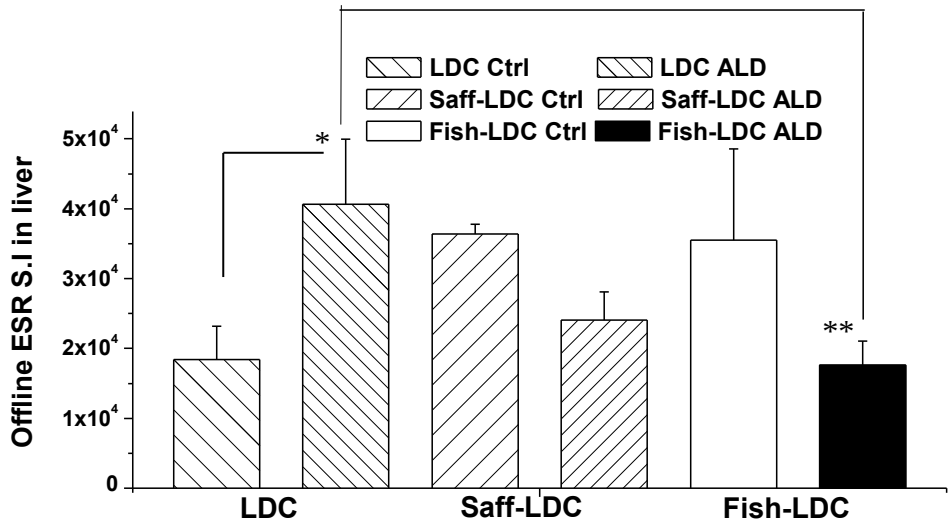


Figure 37. Offline ESR signal intensity determined using liver. Samples were obtained from rats acutely injected AA and POBN i.p in LDC Ctrl, LDC ALD, Saff-LDC Ctrl, Saff-LDC ALD, Fish-LDC Ctrl and Fish-LDC ALD groups. Values are mean ±SEM from n=4-6 rats in each group. *$p < 0.05$ compared to respective dietary control, ** $p < 0.01$, compared to LDC-ALD group.

5.2.6.2. Online LC/ESR

Using online LC/ESR a total of seven different types of AA-derived radicals were observed in bile of rats fed LDC-ALD diet (LC/ESR conditions described in Chapter-2, Sec 2.4.7). Only four of these peaks were observed in bile collected from rats fed LDC-Ctrl diet **(Figure 38)**.

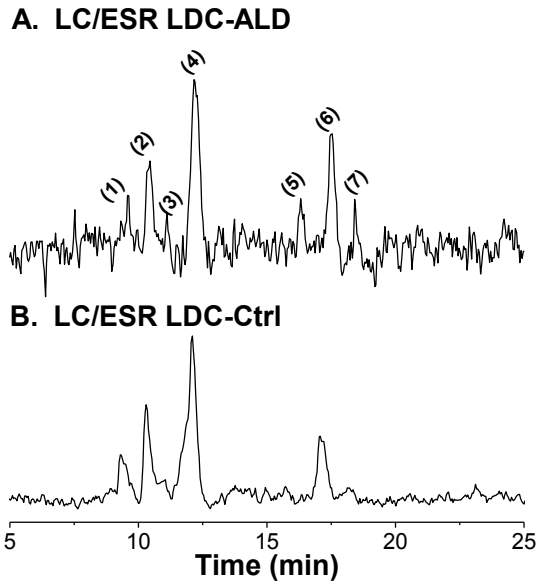


Figure 38. Online LC/ESR chromatogram from LDC fed rats. Chromatogram obtained from rats injected acute dose of AA and POBN, i.p, in (A) LDC ALD, and (B) LDC Ctrl groups, respectively. Chromatogram are representative from n=4-6 bile samples from each group. LC/ESR conditions used have been previously described in Chapter-2.

A total of six different types of AA-derived radicals were observed using LC/ESR in bile of rats fed Saff-LDC diet. Elevation in signal intensity was observed for some of these adducts in bile collected from Saff-LDC ALD rats relative to samples from rats fed Saff-LDC Ctrl diet **(Figure 39)**.

However, only four of these peaks (LC/ESR peak 1, 2, 3, 4) were observed in bile collected from rats fed Fish-LDC diet, LC/ESR peak 5 and 6 were undetectable in Fish-LDC group (Figure 39). In addition, Fish-LDC diet resulted in lower COX and LOX mediated free radical generation in comparison to both LDC and Saff-LDC diets.

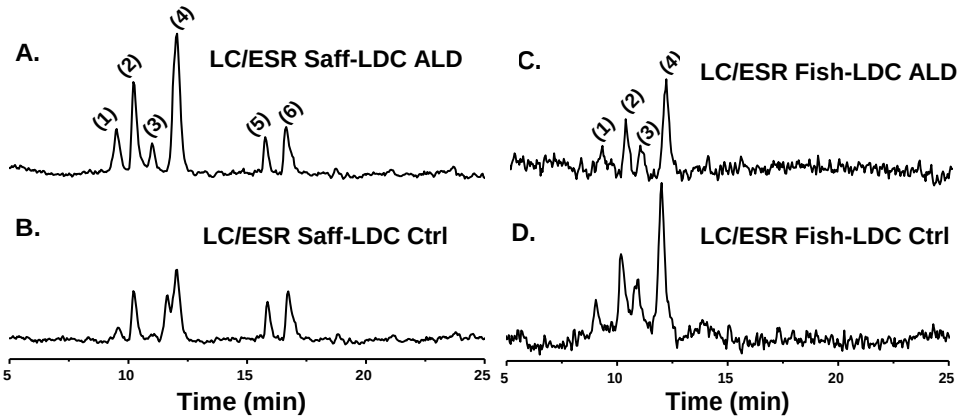


Figure 39. Online LC/ESR chromatogram from Saff-LDC and Fish-LDC fed rats. LC/ESR was recorded using bile samples obtained from rats injected AA and POBN, i.p from: (A) Saff-LDC ALD, (B) Saff-LDC Ctrl, (C) Fish-LDC ALD, and (D) Fish-LDC Ctrl groups, respectively. Spectra are representative from n=4-6 bile samples from each group.

5.2.6.3. Online LC/MS

LC/MS analysis of dual spin trapped bile samples from rats injected D_0-D_9 POBN mixture was used for m/z assignments and also exclude artifactual ions that might otherwise have been assigned to radical-related products. D_9-POBN spin trapping gave LC/MS profiles similar to those of POBN, except for slightly shorter retention times and the 9-da difference for each corresponding radical adduct [160-164].

Molecular ions (m/z) of POBN radical adducts were tentatively identified as 387, 250 (isomer 1), 548, 250 (isomer 2), 350 and 311 with retention times 9.3 min, 10.1 min, 11.0 min, 12.1 min, 15.7 and 16.7 min, corresponding to LC/ESR peaks 1, 2, 3, 4, 5 and 6, respectively, in bile of rats fed Saff-LDC diet (Figure 39).

LC/ESR peak 1 (m/z 387) (Figure 40) was identified to be the possible cysteine conjugate of POBN/$^{\bullet}C_5H_{11}$ (m/z 266) **(Figure 40)** [163].

POBN/$^{\bullet}C_5H_{11}$ (m/z 266) has previously been identified to be formed via β-scission of the PGF_2-type alkoxyl radical formed during COX mediated AA peroxidation (Figure 43) [163].

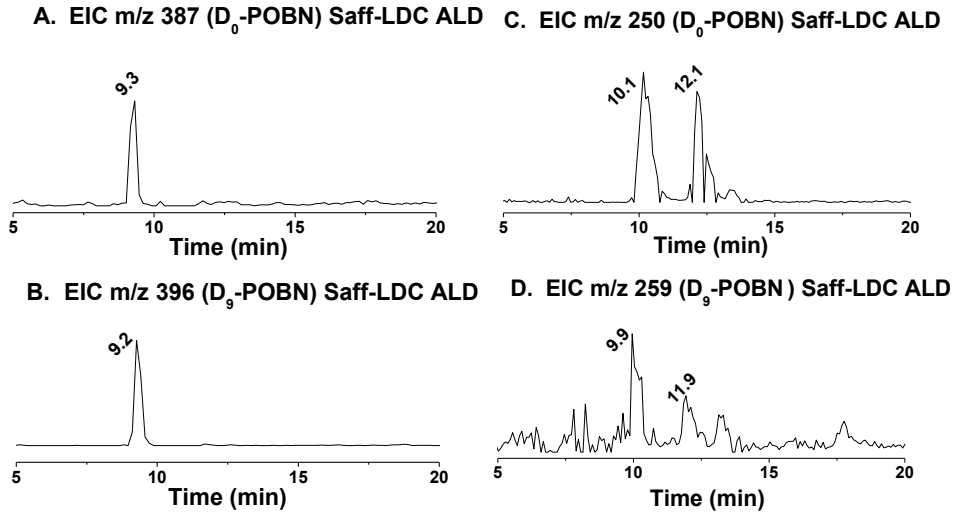


Figure 40. LC/MS EICs of radical adducts corresponding to LC/ESR peak 1, 2, 4. (A) LC/ESR Peak 1 (m/z 387), (B) corresponding D_9-POBN ion of peak 1 (m/z 396), (C) LC/ESR Peak 2, 4 (m/z 250, isomer 1 and 2), and (D) corresponding D_9-POBN ion of peak 2, 4 (m/z 259), determined using dual-spin trapped (D_0-D_9, 1:1 mixture) bile sample collected from Saff-LDC ALD rats. Spectra are representative from n=4-6 bile samples from each group.

LC/MS/MS spectra of LC/ESR Peak 1 (m/z 387) was confirmed by the LC/MS/MS of its corresponding D_9-POBN ion (m/z 396). Fragment ions with m/z 211 and 220 having 9 da difference between the POBN and the corresponding D_9-POBN adduct were observed **(Figure 41)**.

LC/ESR peaks 2 and 4, POBN/$^{\bullet}C_3H_3O$ (m/z 250) (Figure 39) were identified to be the two isomers of radical adducts eluting at t_R 10.2 min and 12.1 min, respectively. These adducts were proposed to be the MDA-derived radical adducts, possibly arising from 5- or 15-LOX mediated peroxidation or auto-oxidation of AA (Figure 2, 40, 44) [163].

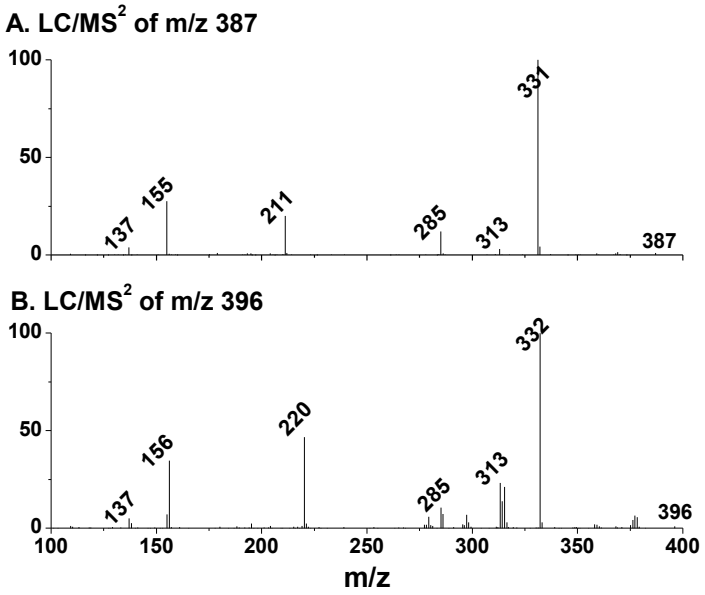


Figure 41. LC/MS/MS of m/z 387 and 396 corresponding to LC/ESR peak 1. LC/MS/MS analysis of dual spin trapped bile samples from rats injected D_0-D_9 POBN were used for confirmation of radical structure by identifying ion pairs showing a 9 da difference. (A) LC/ESR peak 1 (m/z 387), and (B) corresponding D_9-POBN ion (m/z 396) showing ion fragments with m/z 211 and 220, respectively.

Radical adduct corresponding to LC/ESR peak 3 was characterized as POBN/$^{\bullet}C_{20}H_{33}O_5$ (m/z 548), the PGF_2-type radical adduct, originating from the PGF_2-type alkoxyl radical **(Figure 42, 43)** formed during the COX mediated AA peroxidation [163].

Radical adduct corresponding to LC/ESR peak 6, m/z 311 was tentatively identified to be the methyl conjugate (Phase II conjugate) of radical adduct, POBN/$^{\bullet}C_6H_{13}O$ (m/z 296), an exclusive radical derived from fragmentation (β-scission) of PGF_2-type alkoxyl radical formed during COX mediated AA peroxidation [163] (Figure 42, 43).

Radical adduct corresponding to LC/ESR peak 5, POBN/$^{\bullet}C_9H_{15}O_2$ (m/z 350) was proposed to be derived from hydroxynonenal (HNE)-type radical, possibly arising from the auto-oxidation of AA (Figure 3, 42, 45).

The proposed HNE-type (m/z 350, LC/ESR peak 5) and methyl conjugate of COX/AA derived radical adducts (m/z 311, LC/ESR peak 6) were not observed in rat bile obtained from Fish-LDC fed rats. Fish oil lowered the uptake of AA in liver via hepatic transporters and also reduced AA-peroxidation, particularly via COX-2 and 5-LOX. This could result in generation of some radical adducts, such as the possible HNE-type and the COX/AA derived radicals below detection limit in rats fed Fish-LDC diet.

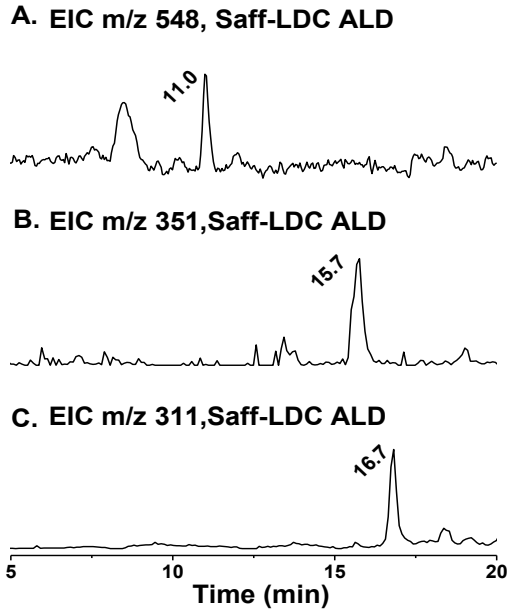


Figure 42. EICs of radical adducts corresponding to LC/ESR peaks 3, 5, 6. (A) LC/ESR peak 3 (m/z 548), the PGF_2-type radical adduct originating from the PGF_2-type alkoxyl radical, (B) LC/ESR peak 5 (m/z 350), radical adduct proposed to be derived from HNE-type radical, and (C) LC/ESR peak 6 (m/z 311), methyl conjugate of radical adduct m/z 296 derived from fragmentation of PGF_2-type alkoxyl radical observed in bile samples collected from Saff-LDC ALD rats, treated with acute AA and spin trap POBN. EICs are representative from n=4-6 bile samples from each group.

Enzymatic pathways involved in AA-derived radical generation included the COX-2 and 5-LOX mediated, while non-enzymatic pathway included the auto-oxidation mechanisms.

The possible pathways of generation of the six-different type of spin-trapped radical adducts derived from AA have been shown in the schemes below **(Figure 43-45)**.

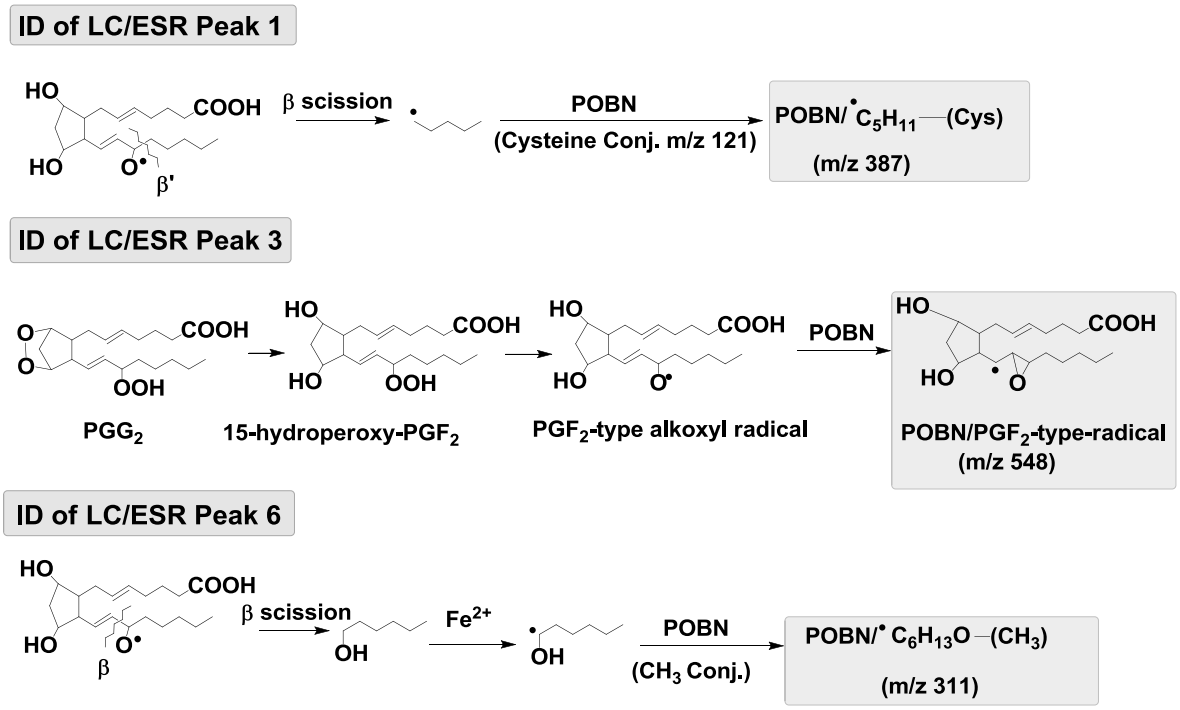


Figure 43. Possible mechanism of generation of radical adducts, LC/ESR peak 1, 3, 6. Radical adducts corresponding to LC/ESR peak 1, 3 and 6 identified as m/z 387, m/z 548 and m/z 311 could be generated from the COX-AA peroxidation and subsequent Phase-II conjugation with cysteine (LC/ESR peak 1) or methyl (LC/ESR peak 3), respectively.

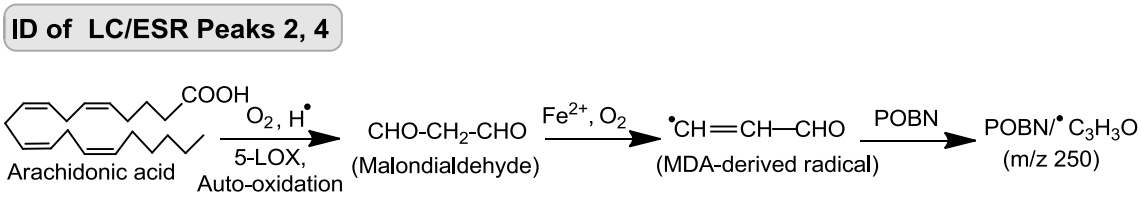


Figure 44. Possible mechanism of generation of radical adducts, LC/ESR peak 2, 4. Radical adducts corresponding to LC/ESR peak 2 and 4 with m/z 250 could possibly be derived from MDA, generated from the 5-LOX mediated AA peroxidation in vivo (Figure 2).

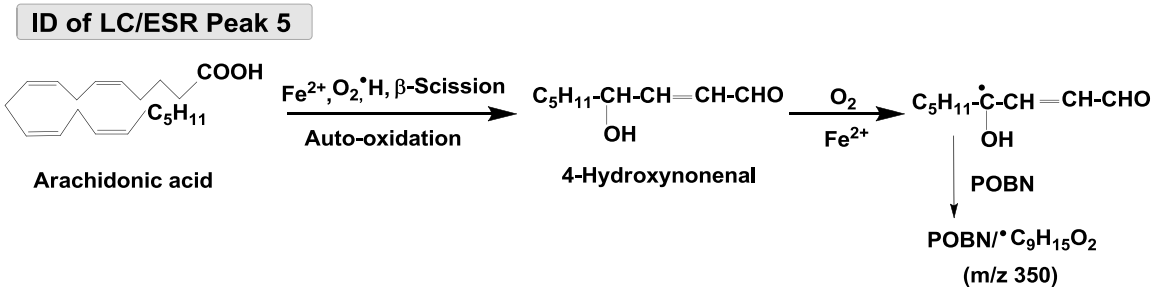


Figure 45. Possible mechanism of generation of radical adduct, LC/ESR peak 5. Radical adduct corresponding to LC/ESR peak 5 with m/z 350 tentatively identified as 4-hydroxynonenal derived radical adduct could be generated endogenously possibly by the auto-oxidation of AA (Figure 3).

LC/ESR peak 1 (m/z 387) was identified to be possibly generated by conjugation of cysteine with POBN/$^{\bullet}C_5H_{11}$ (m/z 266), radical formed via β-scission of the PGF_2-type alkoxyl radical during COX or LOX mediated AA peroxidation (Figure 40, 43, Table 6) [163].

LC/ESR peaks 2 and 4 (m/z 250) identified to be the two isomers of radical adduct, POBN/$^{\bullet}C_3H_3O$ (m/z 250) (Figure 40) were proposed to be possibly derived from MDA formed during 5-LOX mediated AA peroxidation or auto-oxidation of AA (Figure 44). LC/ESR peak 3, characterized as POBN/$^{\bullet}C_{20}H_{33}O_5$ (m/z 548) was assigned to be the PGF_2-type radical adduct originating from the PGF_2-type alkoxyl radical formed during the COX mediated AA peroxidation (Figure 42, 43, Table 6) [163].

Radical adduct corresponding to LC/ESR peak 5, POBN/$^{\bullet}C_9H_{15}O_2$ (m/z 350) was proposed to be derived from hydroxynonenal (HNE)-type radical, possibly arising from the auto-oxidation or COX/LOX mediated peroxidation of AA (Figure 42, 45, Table 6).

Radical adduct corresponding to LC/ESR peak 6, m/z 311 was tentatively identified to be the methyl conjugate (Phase II conjugate) of radical adduct, POBN/$^{\bullet}C_6H_{13}O$ (m/z

296), an exclusive radical derived from fragmentation (β-scission) of PGF_2-type alkoxyl radical formed during COX mediated AA peroxidation [163] (Figure 42, 43, Table 6).

The proposed HNE-type (m/z 350, LC/ESR peak 5) and methyl conjugate of COX/AA derived radical adduct (m/z 311, LC/ESR peak 6) were not observed in bile obtained from Fish-LDC fed rats. This could be possibly because fish oil lowered the uptake of AA in liver via hepatic transporters and also reduced AA-peroxidation, particularly via COX-2 and 5-LOX. Summary of radical adduct identification with the possible source (enzymatic as well as non-enzymatic) of their endogenous generation have been shown in Table 6 below.

LC/ESR	LC/MS ID	Possible Source from AA peroxidation
1	POBN/$^{\bullet}C_5H_{11}$-Cys (m/z 387)	Cysteine (m/z 121) conjugate of POBN/$^{\bullet}C_5H_{11}$ (m/z 266) from COX
2	POBN/$^{\bullet}C_3H_3O$ (m/z 250), isomer 1	Possible MDA-derived radical from 5-LOX or auto-oxidation pathway
3	POBN/$^{\bullet}C_{20}H_{33}O_5$ (m/z 548)	PGF_2 type radical derived from COX
4	POBN/$^{\bullet}C_3H_3O$ (m/z 250), isomer 2	Possible MDA-derived radical from 5-LOX or auto-oxidation pathway
5	POBN/$^{\bullet}C_9H_{15}O_2$ (m/z 350)	Possible HNE-derived radical from auto-oxidation pathway
6	POBN/$^{\bullet}C_6H_{13}O$-CH_3 (m/z 311)	Methyl (m/z 15) conjugate of POBN/$^{\bullet}C_6H_{13}O$ (m/z 296) from COX

Table 6. Summary of radical adduct identification and possible source of generation.

Thus, radical adducts were identified using LC/MS of spin trapped and dual spin-trapped bile samples injected with AA and spin trap POBN or D_0-D_9 POBN, respectively.

Bile obtained from Saff-LDC ALD rats were used for identification. Radical adducts were identified as those having m/z 387, 250 (isomer 1), 548, 250 (isomer 2), 351 and 311 with LC/ESR retention times 9.3, 10.2, 11.0, 12.1, 15.7 and 16.7 min, respectively. Proposed pathways of their generation in vivo have been described in Figures 43-45.

5.3. Discussion and conclusions

In this thesis, we studied the relationship between free fatty acid concentration in liver and their metabolism via lipid peroxidation in relation to severity of liver injury. High basal arachidonate levels were observed in livers of rats fed LDC and Saff-LDC diets in comparison to Fish-LDC diet, wherein very low levels of free AA and high levels of EPA and DHA were detected (Figure 24, 25). Hepatic arachidonate levels correlated closely with severity of liver pathology (Figure 17, 24, 25, Table-5). Increased AA as substrate for peroxidation led to generation of the related inflammatory mediators, e.g. PGs, HETEs and AA-derived free radicals, which correspond to inflammation in liver of rats fed LDC-ALD and Saff-LDC ALD diets.

Possible mechanisms responsible for alteration of hepatic free AA-concentration may involve the role of hepatic membrane transporters as well as AA-oxidation mechanisms. Low AA and high EPA and DHA concentration were found in livers of Fish-LDC fed rats (Figure 24). Both EPA and DHA generate PGE_3 and resolvins as metabolites (which possess anti-inflammatory activity) from COX or LOX mediated peroxidation [57, 68, 69, 71] (Figure 5, 33). Thus, in rats fed Fish-LDC diet, increase in PGE_3 along with lower PGE_2 concentration may correspond to decreased liver inflammation.

Acute AA was injected intraperitoneally to determine the extent of alteration of liver metabolism after chronic ethanol and/or PUFA administration. Free AA concentration

in rats fed LDC-ALD and Saff-LDC-ALD diets followed by acute AA was found to be higher than their respective dextrose controls. In contrast, no change in the free AA levels were observed in rats fed Fish-LDC ALD diet, followed by acute AA treatment relative to its control (Figure 25). In addition, free AA levels were found to be much lower in Fish-LDC ALD rats than in LDC-ALD rats. The results (Figure 25) indicated that administration of fish oil, even with acute AA treatment could lead to decrease in the uptake of free AA via hepatic membrane transporters. Thus, ethanol induced liver injury may also involve the role of several hepatic membrane transporters.

Gene expression of PPAR-α, transcription factor involved in beta-oxidation of fatty acids was determined since beta-oxidation mechanisms can potentially alter the fatty acid concentration in liver. Lower PPAR-α expression could dysregulate fatty acid oxidation resulting in accumulation of fatty acids and triglycerides which possibly led to development of fatty liver in these rats. Saff-LDC ALD diet showed significant downregulation of PPAR-α gene expression relative to its control (Figure 30). Increase in fatty acid concentration in their liver may have contributed to the increased lipid peroxidation leading to generation of pro-inflammatory species. However, no significant alteration in the PPAR-α gene expression was observed in rats fed LDC-ALD and Fish-LDC ALD diets relative to their respective dextrose controls (Figure 30). This study provides a new research direction to further evaluate the alteration in expression of enzymes transcribed by PPAR-α, such as liver fatty acid-binding protein (L-FABP), lipoprotein lipase (LPL) and acyl-coA oxidase (ACOX) to fully understand the role of this transcription factor in PUFA mediated alteration of ALD.

Both gene and protein expression of lipid peroxidation enzymes COX-2 and 5-LOX were elevated in livers of rats fed LDC-ALD and Saff-LDC ALD diets with respect to their controls (Figure 26-29). Protein expression of COX-2 and 5-LOX in liver of rats fed Saff-LDC ALD diet was also found to be increased in comparison to LDC ALD fed rats. This indicated that ω-6 PUFA in diet further potentiated the ethanol induced increase in COX-2 and 5-LOX expression (Figure 27). No alteration in COX-2 and 5-LOX gene expression was observed between LDC ALD and Saff-LDC ALD fed rats which suggested the possible role of post-transcriptional modifications induced by safflower oil in their induction.

In contrast, both gene and protein expression of COX-2 and 5-LOX in liver of rats fed Fish-LDC ALD diet showed significant downregulation compared to rats fed LDC-ALD diet (Figure 27). This data indicated that addition of fish oil in ethanol containing diet could decrease AA-peroxidation by downregulation of COX-2 and 5-LOX.

Overall, increase in peroxidation of AA led to increased generation of PGs and HETEs and AA-derived free radicals in livers of rats fed Saff-LDC ALD diet, which may correspond to the combined effect of AA and ethanol mediated liver inflammation (Figure 26-29, Chapter-3, Table-5). In contrast, lower AA-peroxidation in livers of rats fed Fish-LDC ALD diet, led to decrease in generation of AA-derived PGs, HETEs and free radicals, thus decreasing AA and ethanol mediated liver inflammation (Chapter-3, Table-5). Further, Fish-LDC fed rats not only showed lower radical generation in bile than those fed Saff-LDC diet, but also some radical adducts, e.g. the proposed HNE-type (m/z 350, LC/ESR peak 5) and COX-AA derived radical adduct (m/z 311, LC/ESR peak 6) were not observed in bile from Fish-LDC diet (Figure 39). Fish oil in diet prevented the generation of radicals

derived from auto-oxidation and COX mediated pathways, possibly by lower COX-2 expression and reduced hepatic AA concentration, typically due to decrease in its uptake in liver.

Results from Chapter-3 and 4, including liver histopathology examination, liver function tests and gene/protein expression of inflammatory and fibrotic factors provided evidence of fatty liver and inflammation in rats fed Saff-LDC ALD and LDC-ALD diets. In contrast, livers of rats fed Fish-LDC ALD diet were found to be free of fatty liver and inflammation (Chapter-3, Figure 17, 18, Table 5). The rate and extent of lipid peroxidation assessed by the generation of reactive lipid species in livers strongly correlated with the results obtained from histopathology examination, thus clearly indicating the relationship of lipid peroxidation and liver inflammation. High AA-peroxidation generating PGs (series-2), HETEs and AA-derived radicals in rats fed Saff-LDC ALD and LDC-ALD diets (rich in ω-6 PUFAs) led to fatty liver and inflammation in these rats. In contrast, high EPA and DHA-peroxidation generating PGE_3 with low AA-peroxidation and reduced PG (series-2), HETEs and AA-derived radicals in rats fed Fish-LDC diet led to prevention of ethanol induced fatty liver and inflammation in these rats.

In comparison to rats fed with LDC-ALD diet, Saff-LDC ALD diet group also showed higher AA concentration and COX-2 and 5-LOX expression. This result showed that safflower oil could further elevate the ethanol induced increase in lipid peroxidation mediated by these enzymes, with both ethanol and ω-6 PUFA acting as stimulants of inflammation (Figs. 24, 27-29). In contrast, rats fed Fish-LDC ALD diet showed much lower AA concentration and expression of COX-2 and 5-LOX, compared to LDC-ALD diet. This data suggests that addition of fish oil could decrease AA-peroxidation and lower

the generation of AA-derived pro-inflammatory metabolites in ethanol induced liver inflammation (Figs. 24, 27-29). Thus, in this study we observed a relationship between the type of dietary PUFAs and severity of ethanol induced liver injury. Fish oil (rich in ω-3 PUFAs) was found to exert anti-inflammatory effect, while safflower oil (rich in ω-6 PUFAs) was shown to promote the ethanol induced liver steatosis and inflammation.

CHAPTER 6. EFFECT OF ω-3 AND ω-6 PUFA RICH DIETS ON ETHANOL INDUCED ALTERATION IN HEPATIC MEMBRANE TRANSPORTERS

6.1. Introduction

Multidrug resistant associated proteins (MRPs) are efflux transporters known to play a major role in hepato-biliary elimination of many structurally diverse xenobiotics, including glutathione (GSH) conjugates of 4-HNE [187] and endogenous molecules which are produced from lipid peroxidation (e.g. LTC_4, and PGs) [130]. Out of the several isoforms of MRP, MRP-1 and MRP-3 pump their substrates, e.g. glucuronide and GSH conjugates into the bloodstream rather than excreting them into the bile or urine [131, 132]. MRP-3 together with MRP-2 is also called the GS-X pump to transport GSH conjugates [133]. MRP-2 is mainly expressed on the canalicular membrane of hepatocytes and plays vital role in the defense against oxidative stress because of its role in export of GSH conjugates as well as leukotrienes [188]. Rat MRP-2 expression has been found to decrease in various cholestatic models and in non-alcoholic fatty liver disease. It has also been reported that MRP-3 overexpression compensates for the impaired expression of MRP-2 in MRP-2 deficient conditions, such as in hyperbilirubinuria in rats [134-136]. With their role in elimination of GSH conjugates, GSSG and metabolites e.g., LTC_4, both MRP-2 and MRP-3 may play a role in alteration of ethanol mediated liver injury (Figure 9) [136].

Another hepatic transporter known to participate in the regulation of intracellular fatty acid and cholesterol accumulation in hepatocytes is the ATP-binding cassette transporter A1 (ABCA1), a member of the ABC subfamily A of membrane transporters. Recent studies have suggested the association of hepatic steatosis with PUFA mediated decrease in ABCA1 protein expression leading to increased lipid storage in hepatocytes

[137-140]. Overexpression of ABCA1 resulted in decrease of cellular fatty acids and triglycerides, while ABCA1 gene silencing by siRNA increased both cellular fatty acids and triglycerides [137]. Rats with non-alcoholic steatohepatitis showed lower ABCA1 protein levels while the mRNA level was not altered in liver as compared to normal rats (Figure 9) [137]. With its role in elimination of cellular free fatty acids and triglycerides, ABCA1 may play a role in alteration of the rate of lipid peroxidation and degree of fatty liver, respectively. The alteration in the expression of efflux transporters, ABCA1, MRP-2 and MRP-3 in rats fed ethanol along with dietary ω-3 and ω-6 PUFAs and their relationship with liver injury has been studied in this thesis.

In addition to liver efflux transporters, hepatic uptake transporters, such as peroxisomal ABC transporters encoded by the ABCD genes, ABCD2 and ABCD3 are also thought to participate in the import of specific fatty acids in the peroxisomal matrix [143]. Furthermore, fatty acid transport proteins (FATPs) or solute carrier family 27 (Slc-27) are integral membrane proteins that enhance the uptake of long-chain fatty acids into cells (Figure9) [144]. These factors suggest a possible role of these uptake transporters in modulation of liver injury by means of regulating the hepatic PUFA content, thereby altering the rate of lipid peroxidation. We studied the alteration in gene expression of uptake transporters involved in influx of fatty acids from the blood stream into liver of rats administered ethanol and dietary ω-3 or ω-6 PUFAs and their correlation with disease severity in ALD.

Results from the previous chapters (Chapter 3 and 4) indicated that diets rich in ω-6 PUFAs when administered along with ethanol promote the progression of liver inflammation and injury, while diets rich in ω-3 PUFAs when administered with ethanol

prevent the progression of liver inflammation and injury. In this chapter, we studied whether ω-3 or ω-6 PUFAs differentially altered the expression of liver uptake and efflux transporters which may also play crucial role in modulation of lipid peroxidation and liver injury in ALD.

6.2. Results and discussion

6.2.1. mRNA expression of liver uptake and efflux transporters

mRNA levels of uptake (ABCD2, ABCD3 and SLC27 A5) and efflux (MRP-2 and MRP-3) transporters was determined using real-time reverse transcriptase reaction in livers of rats fed the LDC, Saff-LDC and Fish-LDC diets. mRNA of MRP-3 showed significant upregulation mediated by ethanol in all dietary groups **(Figure 46)**. However, mRNA levels of other hepatic transporters did not show any significant alteration in any of the diets with administration of ethanol with respect to their respective controls (Figure 46). Previous reports by other investigators also suggested similar findings. No significant change in mRNA levels of efflux transporters MRP-2 and ABCA1, and significant alteration in their protein expression was reported in liver inflammation or steatosis, suggestive of a possible role of post-transcriptional modifications in their expression [139, 142, 143, 189].

mRNA of uptake transporters, ABCD2 and ABCD3 was found to be upregulated in Saff-LDC ALD fed rats with respect to LDC-ALD fed rats, suggesting that addition of safflower oil (rich in ω-6) could increase the gene expression of these transporters. Gene expression of ABCD2 but not ABCD3 was found to be upregulated in Fish-LDC ALD fed rats with respect to LDC-ALD fed rats which suggested that addition of fish oil (rich in EPA and DHA) could increase ABCD2 gene expression. mRNA level of MRP-3 showed

significant upregulation mediated by safflower oil but not fish oil **(Figure 47)**. No significant change in mRNA levels of MRP-2 and SLC-27A5 was observed with either safflower oil or fish oil (Figure 47).

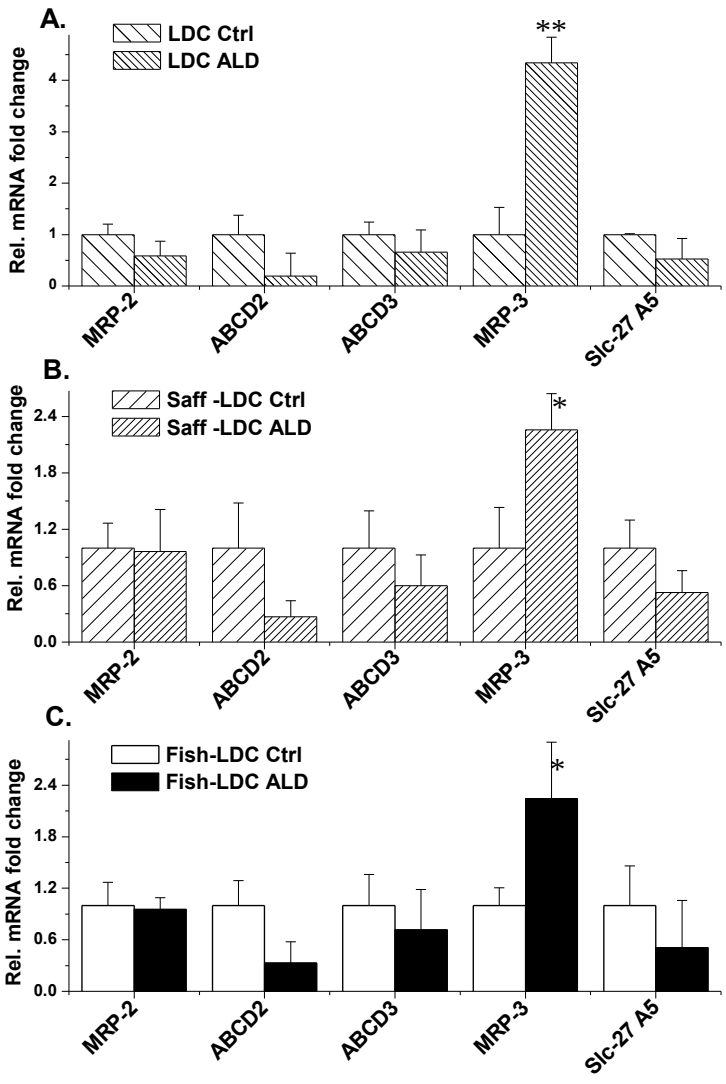


Figure 46. Ethanol mediated alteration in mRNA of hepatic transporters in rats fed different PUFA diets. RT-PCR was used to evaluate the fold change in mRNA of uptake (ABCD2, ABCD3 and SLC27 A5) and efflux (MRP-2 and MRP-3) transporters by ethanol in: (A) LDC ALD with respect to LDC Ctrl, (B) Saff-LDC ALD with respect to Saff-LDC Ctrl, and (C) Fish-LDC ALD with respect to Fish-LDC Ctrl. β-actin was used as the internal control. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, compared to respective dietary controls.

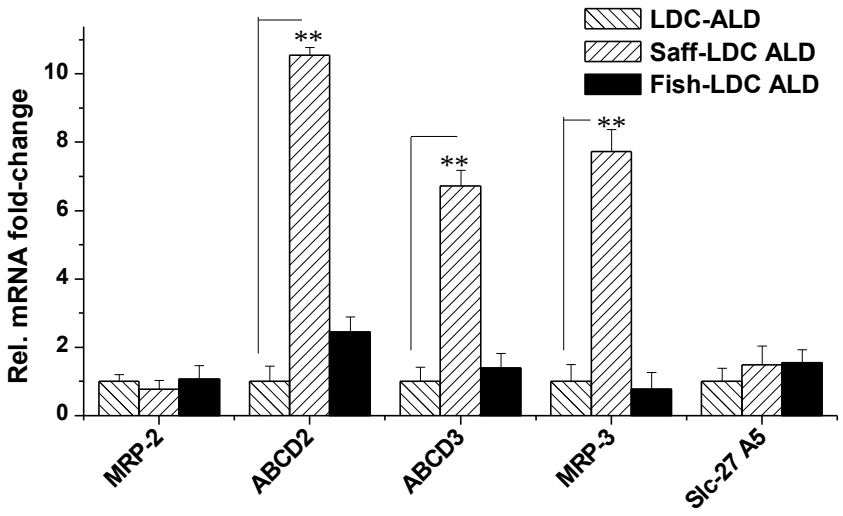


Figure 47. PUFA mediated alteration in mRNA of hepatic transporters. RT-PCR was used to evaluate mRNA fold change of uptake (ABCD2, ABCD3 and SLC27 A5) and efflux (MRP-2 and MRP-3) transporters by Saff-LDC ALD and Fish-LDC ALD diets with respect to LDC-ALD. β-actin was used as the internal control. Values are mean ± SEM from n=4-6 rats in each group, *$p < 0.05$, **$p < 0.01$, compared to LDC-ALD.

6.2.2. Protein expression of liver efflux transporters

We evaluated the protein expression of MRP-2 and ABCA1 using western blot analysis to confirm the change in expression of these liver efflux transporters. Previous reports by other investigators suggested no significant change in mRNA levels of MRP-2 and ABCA1, but significant alteration in the protein levels of these efflux transporters have been observed in steatosis. This implied the possible role of post-transcriptional modifications of these liver efflux transporters in inflammatory conditions [189].

Although change in the mRNA expression was not detected, protein expression of hepatic efflux transporter MRP-2 was found to be significantly downregulated in LDC-ALD and Saff-LDC ALD fed rats relative to their dextrose controls. The result suggested that ethanol may be involved in downregulation of MRP-2 protein **(Figure 48)**.

In contrast, upregulation of MRP-2 protein was observed in Fish-LDC ALD rats with respect to its control (Figure 48). MRP-2 protein expression was also found to be significantly downregulated in rats fed Saff-LDC ALD diet, in comparison to rats fed LDC ALD diet. This data indicated that safflower oil (with high ω-6 vs ω-3 ratio) when administered with ethanol could decrease MRP-2 protein expression more than that by ethanol alone.

However, MRP-2 protein was found to be significantly upregulated in rats fed Fish-LDC ALD diet compared to rats fed LDC ALD diet (Figure 48). This result demonstrated that fish oil not only prevented the downregulation of MRP-2 protein, but could also increase its expression in liver. Downregulation of MRP-2 protein by ethanol and safflower oil possibly led to decrease in elimination and accumulation of pro-inflammatory lipid peroxidation metabolites, e.g. eicosanoids, radical adducts and their conjugates (e.g. cysteine conjugates) in rats fed Saff-LDC ALD diet. This could result in increased liver inflammation in rats fed LDC-ALD and Saff-LDC ALD diets, consistent to results from liver histopatholgy studies (Chapter-3, Table-5).

In contrast, upregulation of MRP-2 protein possibly led to increased elimination of pro-inflammatory metabolites of lipid peroxidation and prevented ethanol induced liver inflammation in rats fed Fish-LDC ALD diet (Chapter-3, Table-5).

Protein expression of hepatic efflux transporter, ABCA1 was found to be significantly downregulated in LDC-ALD and Saff-LDC ALD fed rats relative to their dextrose controls. This result indicated that ethanol mediated the downregulation of ABCA1 protein in these rats **(Figure 49)**.

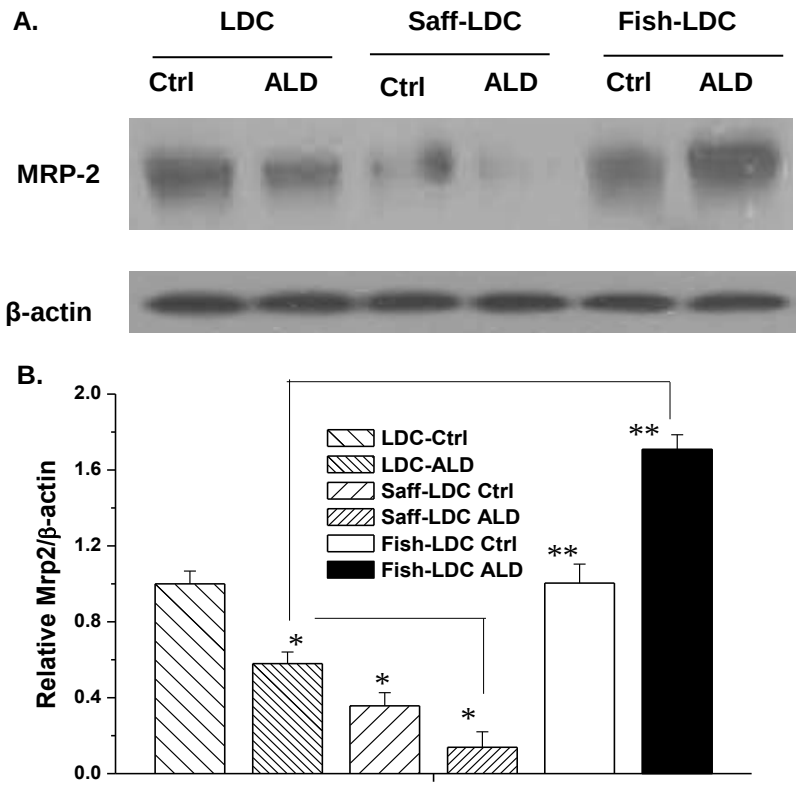


Figure 48. Protein expression of MRP-2 in rats fed different diets. (A) Western blot analysis of MRP-2, and (B) The histogram representing mean ± SEM of the densitometric scans from four separate experiments, normalized by comparison with β-actin and expressed as relative to LDC Ctrl value, *$p < 0.05$, compared to respective dietary controls, ** $p < 0.01$, compared to LDC-ALD group.

In contrast, upregulation of ABCA1 was observed in Fish-LDC ALD rats, with respect to its control (Figure 49). In addition, ABCA1 protein was found to be significantly downregulated in rats fed Saff-LDC ALD diet in comparison to rats fed LDC ALD diet. This suggested that safflower oil in diet could further decrease ABCA1 protein expression (Figure 49). In contrast, significant upregulation was observed in rats fed Fish-LDC ALD diet compared to rats fed LDC ALD diet (Figure 49). Thus, fish oil could not only overcome the ethanol mediated downregulation of ABCA1 protein, but also increased its hepatic expression.

Downregulation of ABCA1 by diet containing both ethanol and safflower oil could possibly led to decrease in elimination and thus accumulation of fatty acids and triglycerides. This could result in increased fatty liver (mediated by triglycerides) and lipid peroxidation (because of increase in free fatty acids). Increase in lipid peroxidation led to inflammation in liver of rats fed LDC-ALD and Saff-LDC ALD diets (Chapter-3, Table-5). In contrast, upregulation of ABCA1 in rats fed Fish-LDC ALD diet possibly led to increased elimination of fatty acids and triglycerides from liver, thus reduced ethanol induced fatty liver and inflammation (Chapter-3, Table-5).

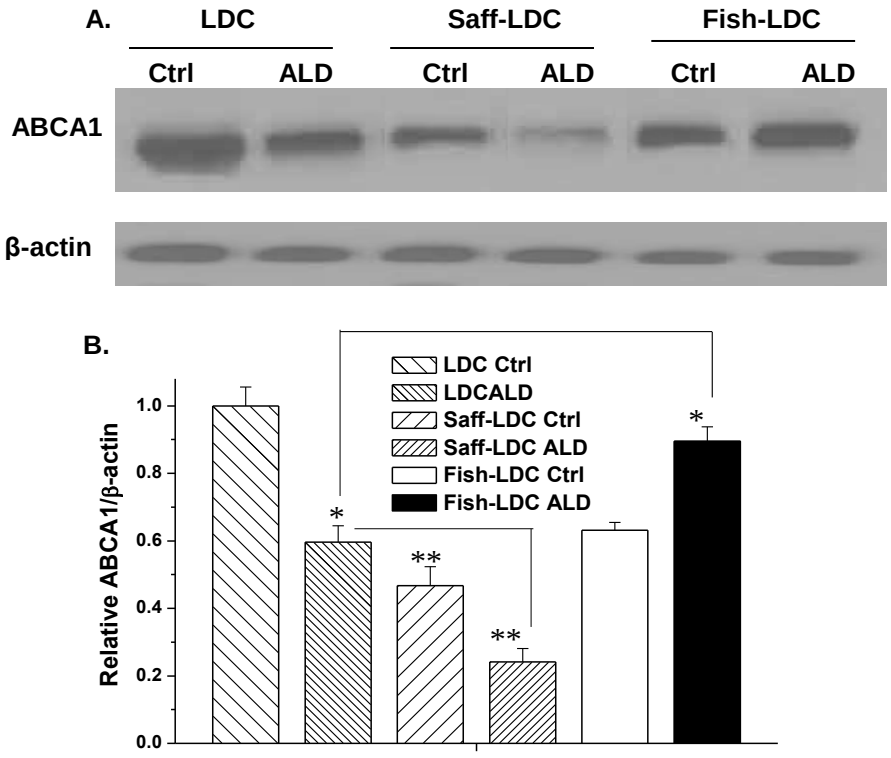


Figure 49. Protein expression of ABCA1 in rats fed different diets. (A) Western blot analysis of ABCA1, and (B) The histogram representing mean ± SEM of the densitometric scans from four separate experiments, normalized by comparison with β-actin and expressed as relative to LDC Ctrl value, *$p < 0.05$, **$p < 0.01$ compared to respective dietary controls, *$p < 0.05$ compared to LDC-ALD group.

6.3. Discussion and conclusion

Efflux transporters, MRP-2 and ABCA1 may play a role in alteration of ethanol mediated steatosis and liver inflammation. Ethanol mediated decrease in MRP-2 (Figure 48) possibly led to accumulation of pro-inflammatory lipid peroxidation metabolites, e.g. eicosanoids, radical adducts and conjugates (e.g. cysteine conjugates). This could result in increased inflammation in the liver of rats fed LDC-ALD and Saff-LDC ALD diets as observed in liver histopatholgy studies (Chapter-3, Table-5). In addition, Saff-LDC ALD diet significantly downregulated MRP-2 protein expression in comparison to rats fed LDC ALD diet. Our data suggests that both ethanol and safflower oil acted as stimulants to decrease MRP-2 expression. Thus, due to its high ω-6 content, Saff-LDC ALD diet showed the maximum decrease in MRP-2 protein expression. This decrease in MRP-2 protein by ethanol and safflower oil could possibly lead to decrease in elimination and thus, accumulation of pro-inflammatory lipid peroxidation metabolites. This could result in increased ethanol and/or PUFA induced liver inflammation in rats fed LDC-ALD and Saff-LDC ALD diets (Chapter-3, Table-5). In contrast, Fish-LDC ALD diet significantly upregulated MRP-2 protein expression in comparison to rats fed LDC ALD diet. This result suggests that fish oil not only prevented the ethanol mediated downregulation of MRP-2 protein in liver, but also increased its hepatic expression which may be one of the mechanisms by which it exerts anti-inflammatory activity. This upregulation of MRP-2 by fish oil possibly led to increased elimination of pro-inflammatory metabolites of lipid peroxidation which resulted in reduced liver inflammation in rats fed Fish-LDC ALD diet (Chapter-3, Table-5).

Hepatic efflux transporter, ABCA1 was also found to be downregulated by ethanol since LDC-ALD and Saff-LDC ALD rats showed lower protein expression relative to their dextrose controls (Figure 49). However, ethanol mediated downregulation of ABCA1 was prevented by the addition of fish oil in diet, since Fish-LDC ALD showed even higher protein expression relative to its dextrose control (Figure 49). Saff-LDC ALD diet also significantly downregulated ABCA1 protein expression in comparison to rats fed LDC ALD diet. This data suggested that safflower oil combined with ethanol led to a greater decrease in ABCA1 protein expression than caused by ethanol alone. In contrast, Fish-LDC ALD diet significantly upregulated ABCA1 protein expression in comparison to rats fed LDC ALD diet. This result suggested that fish oil not only prevented the ethanol mediated downregulation, but could also increase the expression of ABCA1 in ethanol induced inflammatory condition in liver. Downregulation of ABCA1 by both ethanol and safflower oil could possibly lead to decrease in elimination and thus accumulation of fatty acids and triglycerides in liver of rats fed LDC-ALD and Saff-LDC ALD diets. This could result in increased ethanol induced fatty liver (mediated by triglycerides) and lipid peroxidation of free fatty acids resulting in inflammation in liver of these rats (Chapter-3, Table-5). In contrast, upregulation of ABCA1 protein in rats fed Fish-LDC ALD diet possibly led to increased elimination of fatty acids and triglycerides from the liver, resulting in reduced ethanol induced fatty liver and inflammation (Chapter-3, Table-5).

CHAPTER 7. DISCUSSION AND CONCLUSIONS, CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

7.1. Discussion and conclusions

Alcoholic fatty liver (steatosis) is a pathological condition that predisposes the liver to further injury (hepatitis and fibrosis) by several mechanisms, including free radical generation and lipid peroxidation [16-19]. Increased free radical production during alcohol mediated liver injury has been correlated with lipid peroxidation of PUFAs, such as ω-3 and ω-6 PUFAs [16-19]. Several studies have shown that markers of lipid peroxidation altered significantly when diets supplemented with ω-3 or ω-6 PUFAs were administered along with alcohol [55]. However, how these PUFAs modulate lipid peroxidation and the role of lipid peroxidation metabolites in ALD is poorly understood. Although several studies have shown that ω-3 PUFAs possess anti-inflammatory activity [190], conflicting evidence also reported that rats fed fish oil (a rich source of ω-3 PUFAs), along with ethanol, could develop more severe liver injury than rats fed corn oil (a rich source of ω-6 PUFAs) [55]. Conflicting results from different studies arise mainly due to differences in feeding techniques, animal models, composition and purity of the fatty acid supplements used, biological parameters measured and the different analytical methods.

It is still not clear which type of dietary PUFAs, i.e. ω-3 or ω-6, may play beneficial role in preventing steatosis in ALD. Thus, the objective of this research was to study the effect of ω-3 and ω-6 PUFA rich diets on ethanol induced fatty liver (steatosis) and inflammation. In the current study, we modified the PUFA content to create diets having different ω-6 to ω-3 ratios using 1) standard lieber di carli liquid diet, 2) safflower oil, rich in ω-6 LA and 3) fish oil, rich in ω-3 EPA and DHA. We studied the effect of these PUFAs

(ω-3 and ω-6) on several aspects of ALD, including modulation of lipid peroxidation, alteration of hepatic membrane transporters and their effect on development of fatty liver and inflammation. Using the combined technique of LC/ESR/MS with spin-trapping, we tentatively identified several AA-derived free radicals generated in vivo with the possible source of their generation, and also studied the ethanol and/or PUFA induced modulation in radical generation.

Liver histology and pathological assessment indicated high degree of fat deposition and inflammation in livers of rats fed LDC ALD and Saff-LDC ALD diets, namely the ω-6 based diets. In contrast, livers of rats fed Fish-LDC ALD diet, namely the ω-3 based diet showed no fatty liver and inflammation. Saff-LDC ALD diet caused higher scores of fatty liver and inflammation, while Fish-LDC ALD diet led to significantly lower scores when compared to LDC-ALD diet. This data suggested that ω-3 in fish oil prevented, while high ω-6 in safflower oil could increase the ethanol mediated fatty liver and inflammation. In addition, among the three dietary control groups, only rats fed Saff-LDC Ctrl diet (without ethanol) showed fat deposition and inflammation which suggested some development of non-alcoholic steatohepatitis in high ω-6 fed rats (Chapter-3, Figure 17, Table 5).

Rats fed LDC-ALD and Saff-LDC ALD diets showed elevation of serum ALT and AST relative to their controls, which demonstrated ethanol mediated liver function failure in these rats (normal ALT ~ 40 IU/L and AST ~ 200 IU/L under our experimental conditions, Chapter-3, Figure 15, 16). In contrast, rats fed Fish-LDC ALD diet did not show ethanol mediated liver function failure relative to its control. Serum ALT/AST levels were also found to be much lower in rats fed Fish-LDC ALD diet in comparison to LDC-ALD fed rats, while comparable levels were observed for rats fed LDC-ALD and Saff-LDC

ALD diets (Figure 15, 16). Again the results suggest that fish oil in diet could prevent the ethanol mediated liver function failure.

Downregulation of pro-inflammatory factors, TNF-α and i-NOS mRNA was observed in rats fed Fish-LDC ALD diet with respect to LDC ALD diet (Figure 19). mRNA of anti-inflammatory cytokine, IL-10 was also found to be increased in rats fed Fish-LDC ALD diet in comparison to rats fed LDC ALD diet. This data suggested that downregulation of TNF-α, i-NOS and increase in anti-inflammatory IL-10 could be the possible mechanisms by which ω-3 PUFA in fish oil prevented ethanol mediated liver inflammation (Figure 19). In contrast mRNA expression of TNF-α was found to be significantly upregulated in rats fed Saff-LDC ALD diet in comparison to rats fed LDC ALD diet (Figure 19), suggesting that administration of safflower oil (high ω-6) along with ethanol could further increase the expression of hepatic TNF-α.

Protein expression of TNF-α was also found to be significantly upregulated by ethanol in rats fed LDC and Saff-LDC diets with respect to their controls (Figure 20). Addition of safflower oil in ethanol diet (Saff-LDC ALD) did not further increase TNF-α protein relative to LDC-ALD diet, while addition of fish oil significantly downregulated TNF-α in rats fed Fish-LDC ALD with respect to LDC ALD diet (Figs. 19, 20). In addition, rats fed Saff-LDC Ctrl diet showed higher expression of TNF-α than both LDC-Ctrl and Fish-LDC Ctrl fed rats. Thus, safflower oil (high ω-6 vs ω-3) could increase TNF-α even in absence of ethanol in diet, resulting in liver inflammation and initiation of NASH in these rats (Figure 20).

Activation of NF-κB was observed when ethanol was administered with LDC diet which demonstrated that ethanol could potentially activate this transcription factor. Since

NF-κB has been implicated in the induction of TNF-α (Figure 8), activation of NF-κB may thus be one of the possible mechanisms of increase in TNF-α mediated by ethanol. Rats fed Saff-LDC ALD diet showed the highest activation of NF-κB among all dietary groups, with significantly higher activation compared to LDC-ALD group as well. This result re-emphasized that Saff-LDC ALD diet could act like a 'double hit' with both ethanol and ω-6 PUFA acting as stimulants to potentiate the ethanol mediated activation of NF-κB. High activation of NF-κB resulted in the high induction of TNF-α in this group (Figure 19-21), consistent to the highest scores for inflammation (Table-5). In contrast, Fish-LDC ALD group showed significantly lower activation of NF-κB (Figure 21) than LDC-ALD diet. This result indicated that fish oil could prevent the ethanol mediated liver inflammation by preventing activation of NF-κB, resulting in lower expression of TNF-α. This observation of inflammation was consistent to the pathology examination scores (Table-5). Interestingly, rats fed Saff-LDC Ctrl diet also significantly induced higher expression of phosphorylated (activated) NF- κB while almost no activation of NF-κB was observed in both LDC Ctrl and Fish-LDC Ctrl fed rats. This result showed that safflower oil could activate NF-κB even in the absence of ethanol in diet (Figure 21), initiating the development of NASH by increasing the transcription of TNF-α, consequently increasing inflammation in rats fed Saff-LDC diet.

No significant change in gene expression of any of the pro-fibrotic factors tested was observed in rats fed Saff-LDC-ALD diet in comparison to LDC-ALD diet, indicating that addition of safflower oil did not induce fibrosis in these rats during the 8-week feeding period tested in this study. In contrast, rats fed Fish-LDC ALD diet showed decreased gene expression of TGF-β with respect to LDC ALD diet (Figure 19), indicating that fish oil

could possibly prevent the onset of ethanol mediated liver fibrosis via downregulation of TGF-β. This study provides a starting point for further investigation of downregulation of TGF-β as a possible mechanism in prevention of ethanol mediated fibrosis by fish oil or other diets rich in ω-3 PUFAs. Feeding of ethanol for much longer periods may be required to accurately achieve the pathological state of fibrosis, and study the effect of ω-3 or ω-6 PUFAs in alteration of several fibrotic factors to clearly understand their role in ALD.

Gene expression of transcription factor, PPAR-α involved in beta-oxidation of fatty acids was determined since beta-oxidation mechanisms can potentially alter the fatty acid concentration in liver. Saff-LDC ALD diet showed significant downregulation of PPAR-α gene expression relative to its control (Figure 30). Lower PPAR-α expression could dysregulate fatty acid oxidation resulting in accumulation of fatty acids and triglycerides which possibly led to development of fatty liver in these rats. Increase in fatty acid concentration in their liver may have contributed to the increased lipid peroxidation leading to generation of pro-inflammatory metabolites. However, no significant alteration in the PPAR-α gene expression was observed in rats fed LDC-ALD and Fish-LDC ALD diets relative to their controls (Figure 30). This study provides a new research direction to further evaluate the alteration in expression of enzymes transcribed by PPAR-α, such as liver fatty acid-binding protein (L-FABP), lipoprotein lipase (LPL) and acyl-coA oxidase (ACOX) to fully understand the role of this transcription factor in PUFA mediated alteration of ALD.

Both gene and protein expression of lipid peroxidation enzymes, COX-2 and 5-LOX were elevated in livers of rats fed LDC-ALD and Saff-LDC ALD diets with respect to their controls (Figs. 26-29). Importantly, protein expression of COX-2 and 5-LOX in liver of rats fed Saff-LDC ALD diet was found to be increased in comparison to LDC ALD

fed rats which demonstrated that safflower oil further increased the ethanol induced increase in COX-2 and 5-LOX protein (Figure 28, 29). Unlike protein, no alteration was observed in the gene expression of COX-2 and 5-LOX in Saff-LDC ALD fed rats relative to LDC ALD fed rats, which suggested the possible role of post-transcriptional modifications induced by safflower oil in alteration of these enzymes. In contrast, both gene and protein expression of COX-2 and 5-LOX showed significant downregulation in liver of rats fed Fish-LDC ALD compared to rats fed LDC-ALD diets (Figs. 26-29). This data indicated that fish oil could decrease the ethanol induced expression of COX-2 and 5-LOX, resulting in decrease in lipid peroxidation mediated by these enzymes.

We further studied the relationship between free fatty acid concentration in liver, their metabolism via lipid peroxidation and severity of ethanol induced liver injury. Acute administration of AA not only increased the sensitivity of detection of free radicals generated in vivo, but also enabled us to study the metabolism of AA in the voluntary oral ethanol intake rat model of ALD. High basal arachidonate levels were observed in livers of rats fed LDC and Saff-LDC diets in comparison to Fish-LDC diet wherein very low levels of free AA, while high EPA and DHA were detected (Chapter-5, Figs. 24, 25). Hepatic arachidonate levels correlated closely with severity of liver pathology. Increase in AA as substrate for lipid peroxidation led to generation of inflammatory mediators, e.g. PGs, HETEs and AA-derived free radicals which may cause inflammation in liver of rats fed LDC-ALD and Saff-LDC ALD diets. In contrast, high EPA and DHA in rats fed Fish-LDC diet generating PGE_3 as metabolite that possess anti-inflammatory activity from their COX or LOX mediated peroxidation [57, 68, 69, 71] (Chapter-5, Figs. 24, 25, 33). Another

possible mechanism for reducing hepatic free AA concentration by fish oil may involve the reduction in uptake of AA by hepatic membrane transporters.

Increase in AA-peroxidation because of high substrate (AA) and enzyme levels (COX-2 and 5-LOX) led to increased generation of AA-derived PGs, HETEs and free radicals in livers of rats fed Saff-LDC ALD diet which led to AA and ethanol mediated liver inflammation (Figure 27-29, 33, 34, Table-5). In contrast, lower AA-peroxidation led to decrease in generation of AA-derived PGs, HETEs and free radicals, thus decreasing liver inflammation (Chapter-3, 5: Figure 27-29, 33, 34, Table-5) in livers of rats fed Fish-LDC ALD diet. Fish-LDC fed rats not only showed the lowest radical generation in bile than those fed Saff-LDC diet, but also missed some radical adducts, e.g. the proposed HNE-type (m/z 350, LC/ESR peak 5) and COX-AA derived radical adduct (m/z 311, LC/ESR peak 6) (Figure 39). Thus, addition of fish oil in diet could prevent the generation of radicals derived from auto-oxidation and COX mediated pathways, possibly by lower COX-2 expression and reduced hepatic AA-concentration due to decrease in its uptake in liver.

Results from Chapter-3 and 4, including liver histopathology, liver function tests and gene/protein expression of inflammatory and fibrotic factors, provided evidence of fatty liver and inflammation in rats fed Saff-LDC ALD and LDC-ALD diets. In contrast, livers of rats fed Fish-LDC ALD diet were found to be free of fatty liver and inflammation (Chapter-3, 4: Figure 17-19, Table 5). The rate and extent of lipid peroxidation assessed by the generation of reactive lipid species in livers strongly correlated with the results obtained from histopathology examination, which clearly suggested that there is a relationship between lipid peroxidation and liver inflammation. High AA concentration and its

peroxidation in rats fed Saff-LDC ALD and LDC-ALD diets (rich in ω-6 PUFAs) led to generation of pro-inflammatory metabolites, fatty liver and inflammation in these rats. In contrast, low AA concentration and its peroxidation in rats fed Fish-LDC ALD diet (rich in ω-3 PUFAs) led to prevention of fatty liver and inflammation in these rats. In this study, we observed a relationship between the type of dietary PUFAs and severity of ethanol induced liver injury. Fish oil (rich in ω-3 PUFAs) was found to exert anti-inflammatory effect, while safflower oil (rich in ω-6 PUFAs) was shown to promote the ethanol induced liver steatosis and inflammation.

Recent studies suggest that in addition to the important role played by lipid peroxidation and pro-inflammatory cytokines, the extent of liver damage may undergo change by the various elimination mechanisms operating in the liver. Our studies further suggest that efflux transporters, MRP-2 and ABCA1 may play a role in alteration of disease state in alcoholic steatosis and liver inflammation. Ethanol mediated decrease in MRP-2 protein (Chapter-6, Figure 48) possibly led to accumulation of pro-inflammatory lipid peroxidation metabolites, e.g. eicosanoids, radical adducts and conjugates (e.g. cysteine conjugates) in liver. This could result in increased inflammation in liver of rats fed LDC-ALD and Saff-LDC ALD diets as observed in liver histo-pathology studies (Chapter-3, Table-5). Saff-LDC ALD diet significantly downregulated MRP-2 protein in comparison to rats fed LDC ALD diet, indicating that safflower oil could further decrease MRP-2 expression. In contrast, Fish-LDC ALD diet significantly upregulated MRP-2 protein expression in comparison to rats fed LDC ALD diet (Chapter-6, Figure 48). This result suggested that fish oil in diet not only prevented the ethanol mediated downregulation, but also increased the expression of MRP-2 protein in ethanol induced inflammatory condition

in liver. Downregulation of MRP-2 protein by both ethanol and safflower oil possibly led to decrease in elimination and thus accumulation of pro-inflammatory lipid peroxidation metabolites. This could lead to increased ethanol induced liver inflammation in rats fed LDC-ALD and Saff-LDC ALD diets (Chapter-3, Table-5). In contrast, upregulation of MRP-2 by fish oil led to increased elimination of pro-inflammatory metabolites of lipid peroxidation, resulting in reduced ethanol induced liver inflammation in rats fed Fish-LDC ALD diet (Chapter-3, Table-5).

Hepatic efflux transporter ABCA1 protein was also found to be downregulated by ethanol, since LDC-ALD and Saff-LDC ALD rats showed lower protein expression relative to their controls (Chapter-6, Figure 49). Also, Saff-LDC ALD diet significantly downregulated ABCA1 protein in comparison to rats fed LDC ALD diet. This showed that safflower oil combined with ethanol caused a greater decrease in ABCA1 protein expression than by ethanol administered alone. In contrast, Fish-LDC ALD diet significantly upregulated ABCA1 protein expression in comparison to rats fed LDC ALD diet. This data suggested that fish oil in diet not only prevented the ethanol mediated downregulation, but could also increase the expression of ABCA1 protein in ethanol induced inflammatory condition in liver.

Downregulation of ABCA1 protein by both ethanol and safflower oil possibly led to decrease in elimination and thus accumulation of fatty acids and triglycerides in liver of rats fed LDC-ALD and Saff-LDC ALD diets (Chapter-6, Figure 49). This could lead to increased ethanol induced fatty liver (mediated by triglycerides) and lipid peroxidation of free fatty acids which subsequently caused inflammation in liver of these rats (Chapter-3, Table-5). In contrast, upregulation of ABCA1 protein possibly led to increased elimination

of fatty acids and triglycerides from the liver which prevented the development of ethanol induced fatty liver and inflammation in rats fed Fish-LDC ALD diet (Chapter-3, Table-5). Thus, we observed that alteration in expression of hepatic efflux transporters, MRP-2 and ABCA1 protein closely correlated with liver pathology mediated by both PUFAs and ethanol in ALD. This study provides a strong basis to further evaluate the role of MRP-2 and ABCA1 and their modulation by dietary PUFAs in ALD.

7.2. Overall results

Overall, our results suggest that Fish-LDC diet (rich in ω-3 PUFAs) prevented the development of ethanol induced fatty liver (steatosis) and significantly reduced liver inflammation. In contrast, LDC and Saff-LDC diets (with moderate to high ω-6 and low ω-3, respectively) promoted the development of ethanol induced fatty liver (steatosis) and significantly increased liver inflammation. ω-3 PUFA (Fish-LDC diet) showed lower hepatic AA concentration possibly due to reduced hepatic uptake of AA. By preventing the upregulation of COX-2 and 5-LOX, Fish-LDC diet can reduce hepatic AA-peroxidation and generation of pro-inflammatory AA-derived metabolites, such as PGs (Series-2), HETEs and free radicals.

In addition, the high EPA and DHA concentration in liver of rats from feeding Fish-LDC diet corresponded to increase in generation of anti-inflammatory EPA/DHA-derived PG (Series-3). By decreasing the activation of NF-κB and reducing induction of pro-inflammatory and pro-fibrotic cytokines, such as TNF-α and TGF-β, ω-3 PUFA also significantly reduced inflammation and prevented the onset of fibrosis. Additionally, Fish-LDC diet upregulated hepatic efflux transporters, MRP-2 and ABCA1. Since MRP-2 is involved in elimination of lipid peroxidation metabolites and GSH conjugates, its

upregulation by ω-3 PUFA rich Fish-LDC diet possibly prevented their hepatic accumulation, thus reduced inflammation.

Upregulation of ABCA1 is also known to be involved in elimination of free fatty acids and triglycerides. Fish oil by downregulating ABCA1 prevented increase in lipid peroxidation and development of fatty liver **(Figure 50)**.

On the other hand, ethanol administered with diets rich in ω-6 PUFAs (LDC-ALD and Saff-LDC ALD diets) showed high hepatic AA concentration. Saff-LDC ALD caused further upregulation of ethanol induced COX-2 and 5-LOX. This led to increased AA-peroxidation and generation of pro-inflammatory AA-derived PGs (Series-2), HETEs and free radicals, thus increasing liver inflammation in these rats. By increasing the activation of NF-κB and induction of pro-inflammatory cytokine TNF-α, Saff-LDC diet can exacerbate liver inflammation. Saff-LDC ALD diet also caused significant downregulation of hepatic efflux transporters, MRP-2 and ABCA1 protein compared to LDC-ALD rats. This led to hepatic accumulation of lipid peroxidation metabolites, fatty acids and triglycerides which could further increase lipid peroxidation, development of fatty liver and inflammation in these rats (Figure 50).

Even in the absence of ethanol, safflower oil diet could trigger the accumulation of fat and inflammation in liver and initiate the pathological condition called non-alcoholic steatohepatitis. The schematic below shows the summary of the proposed pathways by which ω-3 and ω-6 PUFAs may exert their effects in development of ALD.

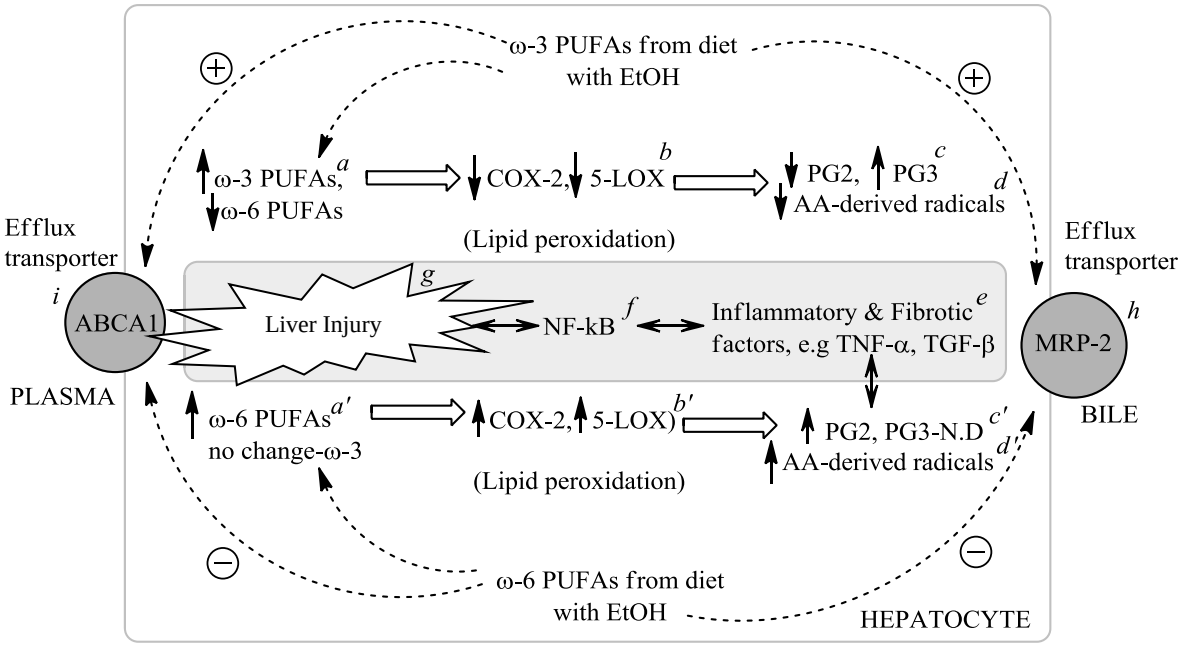


Figure 50. Proposed mechanistic pathways by which ω-3 and ω-6 PUFAs may alter ethanol mediated liver injury. Some of the possible mechanisms involved PUFA metabolism by lipid peroxidation, generation of pro- or anti-inflammatory metabolites and elimination mechanisms operating in the liver. Note that a, a' correspond to LC/MS data of liver fatty acid profile (Figure 24, 25), b, b' correspond to data from gene/protein expression studies using RT-PCR, western blot (Figure 26-29), c, c' correspond to LC/MS data of eicosanoids, derived from peroxidation of AA (Figure 33, 34), d, d' correspond to free radical concentration determined using offline ESR and LC/ESR (Figure 36-39), e and f correspond to gene/protein expression evaluated using RT-PCR, western blot and ELISA (Figure 18-21), g corresponds to histology and serology data (Figure 15-17, Table-5), h and i correspond to protein expression studies done using western blot (Figure 48, 49).

7.3. Clinical implications and future directions

Our study demonstrated that fish oil and other dietary sources rich in ω-3 PUFAs may be further investigated as potential nutritional therapy in prevention of ethanol induced hepatic steatosis (fatty liver) and inflammation. Since hepatic steatosis has been shown to predispose the liver to further injury by fibrosis and cirrhosis, fish oil as a dietary supplement may protect the further progression of ethanol induced hepatic steatosis to fibrosis and cirrhosis [5-11]. Possible mechanisms involved in the protective effect of fish

oil include reduction in AA-peroxidation to decrease AA-derived pro-inflammatory and increase EPA and DHA-derived anti-inflammatory metabolites, decreased TNF-α expression and increased protein expression of efflux transporters MRP-2 and ABCA1. In addition, results from this study suggested that safflower oil in diet (and possibly other sources high in ω-6 PUFAs) could not only aggravate ALD but also trigger the development of non-alcoholic steatohepatitis. The strategy of using acute administration of AA prior to sample collection not only increased the sensitivity of detection of free radicals generated in vivo, but also enabled us to study the metabolism of AA via lipid peroxidation during ethanol induced liver pathology.

The role of ω-3 PUFA rich diets as potential therapeutic interventions in ALD can further be confirmed using fat-1 transgenic mice model to eliminate the confounding factors of diet. Fat-1 transgenic mice express a Caenorhabditis elegans desaturase (fat-gene), leading to endogenous formation of ω-3 PUFAs from ω-6 PUFAs without using dietary supplementation [191, 192]. Due to this capability, fat-1 mice have higher tissue content of ω-3 PUFA which makes them the ideal models to study the alteration in inflammatory conditions, such as hepatitis, pancreatitis, and colitis mediated by ω-3 and ω-6 PUFAs [190, 193, 194]. Furthermore, ex-vivo model of hepatic liver injury with co-cultures of hepatocytes, kupffer cells and stellate cells may be developed to further confirm the identities and pathways involved in generation of several PUFA derived free radicals. These free radicals identified may then be further subjected to studies involving signal transduction pathways triggered by these reactive species in ALD. Gene silencing studies could also be performed for elucidating the precise role of individual hepatic uptake and efflux transporters in alteration of liver injury.

The information gained from this study provides new insights into the effects of ω-3 and ω-6 PUFA rich diets in alteration of ethanol and/or PUFA mediated hepatic steatosis and inflammation. We found that AA-peroxidation may play critical role in development of ALD from its association with pro-inflammatory metabolite generation. To our knowledge, this is the first study to provide a basis to further explore the role of hepatic membrane transporters as novel targets involved in modulation of lipid peroxidation and liver inflammation in ALD.

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