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Dietary polyunsaturated fatty acids alleviate D-galactosamine induced hepatitis by regulating cytokine production

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Abstract
Dietary fatty acids are able to modulate the immune system through several mechanisms. Dietary polyunsaturated fatty acids are shown to alleviate various inflammatory disorders. The present study aims at finding out the effect of supplementation of two lipid differing in their polyunsaturated fatty acid composition on cytokine expression, liver histology and fatty acid profiles in D-galactosamine induced hepatitis in mice. Two months old swiss albino mice, were fed on diet supplemented with lipid differing in their polyunsaturated fatty acids composition for a period of 30 days. Both experimental and control mice were subjected to hepatitis induction by using D-galactosamine. D-galactosamine administration in the control group of mice showed 30-50% (P<0.05) increase in the expression of IL-2, TNF-α and iNOS. The expression of these mRNA remained low upon induction of hepatitis in the experimental group of mice. These groups of mice showed significant changes in the fatty acid profiles upon induction of hepatitis. Dietary fish oil supplemented group of mice showed almost normal liver histology in spite of D-galactosamine induced hepatitis. Dietary fish oil alleviates D-Galactosamine induced hepatitis in by regulating the hepatic cytokine expression. Novel lipid metabolites may also be involved in the process which needs further investigation.

Keywords: Dietary PUFA; fish oil; cytokine; D-GaIN; hepatitis.

INTRODUCTION

Nutrition is one of the factors that regulate immune response and that is why a balanced and an adequate diet is required for optimal development and functioning of the immune system (Kelley, 2001). Among different nutrients, lipids play a key role in immune function. Dietary fatty acids are able to modulate the immune system through several mechanisms that include alterations in lymphocyte proliferation, cytokine synthesis, phagocytes' activity (Calder *et al.*, 2002). Acute hepatitis due to viral, toxic or autoimmune pathogenesis is characterized by an activation of macrophages and T cells with an increased production of cytokines that leads to parenchymal liver damage and liver dysfunction (Schmocker *et al.*, 2007). Growing evidence indicates that n-3 polyunsaturated fatty acids (PUFA) and their specific lipid mediators can reduce the activity of inflammatory processes (Weylandt and Kang, 2005; Simopoulos, 2002; Okita *et al.*, 2002; De La Cruz *et al.*, 2000).

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Observations in various inflammatory diseases have shown that n-3 fatty acids lower the inflammation susceptibility in general (Pablo and Alvarez *et al.*, 2000; Arita *et al.*, 2005; James *et al.*, 2000). Hence, we hypothesized that the dietary PUFA could also alleviate D-GaIN (D-galactosamine) induced hepatitis by regulating Kuffer cell activation and altering pro-inflammatory cytokine production as well as the lipid profiles of liver tissue. We therefore, evaluated the effect of two diets differing in their PUFA composition and unsaturation index in the pathogenesis of D-GaIN induced hepatitis in mice. We selected two sources of dietary fatty acids namely fish oil, rich in n-3 PUFA and other unsaturated fatty acids with unsaturation index 0.7 and meat oil rich in saturated fatty acid and moderately higher amount of n-6 PUFA with unsaturation index 2.3 for our present study (Pujari and Roy, 2010).

D-GaIN induced hepatitis is a well established model for macrophage dependent liver injury in mice (Freudenberg *et al.*, 1986). D-GaIN is known as a

specific hepatotoxic transcriptional inhibitor leading to an acute cytokine dependent liver inflammation (Sass *et al.*, 2002). TNF-alpha is a critical mediator of apoptotic liver damage in D-GaIN induced hepatitis (Schumann and Tiegs, 1999). The results presented here show that the n-3 PUFA rich fish oil diet developed less severe D-GaIN induced inflammatory liver damage than that of the n-6 PUFA rich diet as evidenced by decreased production of pro-inflammatory cytokines and significantly less severe liver pathology.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice, *Mus musculus*, weighing 22 ± 0.5g were divided into three groups consisting of 12 animals each (Male) and maintained as per the guidelines of Animal Ethic Committee of Goa University, Goa, India. Group 1 was fed with standard pellet diet while Group 2 (FO) and Group 3 (MO) were fed with same standard pellet diet but mixed with 10% fish oil and meat oil respectively for a period of 30 days. The fish oil, enriched with n-3 PUFA mainly eciosapentaenoic acid and docosahexaenoic acid was obtained from M/s. E.Merck, India. The meat oil, enriched with n-6 PUFA mainly arachidonic acid, was extracted and concentrated in our laboratory from the fat tissue of *Capra aegagrus* (Table 1a,1b *[Supplementary data]*). At the end of feeding period, half the animals from each group received saline injection (i.p.) for two consecutive days to serve as controls in each group while the other half received D-galactosamine injection (0.5mg/g body wt / 0.5ml of physiological saline) and served as liver injury models in each group (Anandan and Devaki, 1999). After a gap of two days, animals were sacrificed to collect blood and liver samples.

Analysis of cytokine mRNA expression

To analyze cytokine expressions, the mice were sacrificed after two days of D-GaIN/ Saline injection and the liver was collected immediately in phosphate buffer saline (pH 7.00) prepared in RNAse free water under sterile conditions (Ausubel *et al.,* 1989). To determine intra hepatic IL-1alpha, IL-2, TNF-alpha and iNOS expressions, total RNA was isolated by using Gene Zol solution (Taurus Scientific Inc, Ohio, USA) according to the manufacturer's specifications. To check the quality of the RNA extract, 4 µl was mixed with the loading buffer and run on 1.2% agarose gel prepared in tris borate buffer TBE (1X) containing ethidium bromide (10 mg/ml distilled water). cDNA was synthesized using Revert AidTM First strand cDNA synthesis kit (Fermentas Inc. Maryland, USA). Quality of the cDNA was checked with the constitutive gene β actin. Reverse transcription PCR was performed by using a thermal cycler (Eppendorf). 4µl of the PCR product was mixed with 2µl of loading dye (Bromophenol blue) and loaded into the wells carefully. It was run on agarose gel (1.2% prepared in TBE 1X) and observed with the help of gel doc system (Alpha Imager TM 1220 Documentation and Analysis system). β actin was used as a housekeeping gene to normalize mRNA levels. Primers used in amplification were as show in Table 1a, 1b *[Supplementary data]*.

Analysis of Lipid Profiles

The lipid extract of liver tissue was fractionated on silica gel thin layer plate (Roy *et al*., 1997). Individual fractions were quantified by routine analytical method. To determine the fatty acid profile in liver tissue, total neutral lipid / total phospholipids fractions obtained through silica gel column were subjected to saponification with 1 ml of 5N NaOH along with internal standard (C17:0) heptadecanoic acid at 80-90ºC for 2 hours followed by acidification and extraction with petroleum ether (40-60ºC). Fatty acid thus obtained were methylated using 2% H_2SO_4 in methanol at 70ºC for 4hrs. Methyl esters thus obtained were analyzed on Agilent 6890 series GLC system equipped with FID detector, using supelco SP2330 fused silica capillary column (30mx25mmlDx0.2µm film thickness) using temperature program. The column was initially maintained at 100 ºC for 5 min, increased by 30 ºC/min to 160 ºC and next by 5 ºC/min to 220ºC and there it was kept isothermal for 10 min. injector and detector ports were maintained at 220ºC and carrier gas (nitrogen) pressure was maintained at 18psi. (Ghafoorunissa, 1989). Fatty acids were identified by frequent comparison with authentic standards obtained from Sigma Aldrich Chemical Co., USA. The concentrations were expressed as nmole%.

Histology

Liver samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.0) for 6-8 hour at 4ºC and post fixed in 1% OsO_4 for 1-2 hours at 4ºC. The tissues were dehydrated by using grades of alcohols and en-bloc stained with 2% uranyl acetate at 4ºC for 1-2 hours. After clearing in propylene oxide, the tissues were left in a mixture of 1:1 propylene oxide: embedding medium (Araldite Cy 212) overnight on a rotator at room temperature followed by two changes in pure embedding medium, for 6 hours on a rotator at room temperature. The liquid embedding medium containing the tissues was polymerized in an oven at 60 ºC for 48 hours. Ultra thin sections of 60nm thickness were taken using a glass knife and placed on a copper grid. The grids were stained in uranyl acetate for 1-2 hours washed well in water, dried and then stained in lead citrate for 5-7 minutes and washed immediately. The sections were viewed under the

electron microscope (Decnai, G2 Bio-Dwin) and areas of interest were photographed (Frasca and Parks**,** 1960).

Statistical Analysis

Data are represented as means ± SEM. Statistical analysis was done using Student's "t" test and two factor ANOVA. P≤ 0.05 was considered the criterion of significance.

RESULTS

Cytokine expressions

Dietary supplementations of lipid reduce the expression of IL-1α, IL-2 and TNF- α by 20-40% in mice. While dietary supplementation of fish oil reduces the expression of iNOS by 22%, the supplementation of meat oil increases the expression of the same by 5% (P <0.05) D-GaIN administration in the control mice showed 30-50% (P<0.05) increase in the expression of IL-2, TNF-α and iNOS over the control saline injected group of mice. Induction of hepatitis also marginally elevated the expression of IL-1α in mice liver. Upon administration of D-GaIN in dietary lipid supplemented group of mice, the expression of IL-1α, IL-2 and TNF-α were lowered by 10-30% (P<0.001) over the same of the control saline injected group of mice (Table 2). Hepatitis induced fish oil supplemented group of mice still maintained the iNOS expression lowered by about 6% (P<0.001) but hepatitis induced meat oil supplemented group of mice showed about 30% elevation (P<0.001) in the iNOS expression (Table 2) than the control saline injected group of mice.

Transmission Electron Microscopy

Transmission electron microscopy studies revealed the normal liver architecture of control group of mice fed with standard pellet diet. The number of mitochondria and number of vacuoles were found to be normal in this group of animals. In the fish oil supplemented group of mice normal endoplasmic reticulum, intact nuclear membrane and normal mitochondria (Fig. 1a, 1c) were observed. However, the number of mitochondria was slightly higher in this group of mice. Presence of few vacuoles in the fish oil supplemented mice indicated mild fat deposition. Meat oil supplemented group of *Mus musculus* showed normal endoplasmic reticulum with more number of vacuoles, normal nucleus and normal mitochondria (Fig. 2a, 2c). Control group of mice upon administration of D-GaIN showed more vacuoles along with damaged ER, deformed mitochondria and abnormal nuclear membrane (Fig. 3b, 3d). Fish oil supplemented group of mice upon induction of hepatitis, showed damage to a mild extent, the nucleus was partly swollen however, the nuclear membrane was normal, number of free ribosome was found to be abnormal and endoplasmic reticulum was damaged (Fig. 1b, 1d) to a lesser extent. Upon induction of hepatitis the meat oil supplemented group of mice showed more number of free ribosome and damaged endoplasmic reticulum, large number of normal mitochondria, more number of vacuoles with normal nuclear membrane (Fig. 2b, 2d).

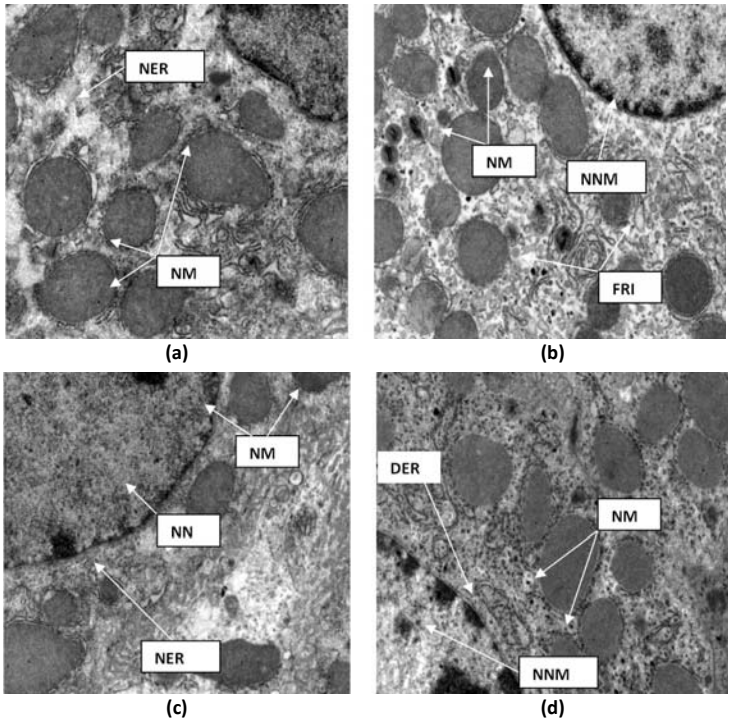

Figure 1: Electron microscopy of liver section of mice fed with fish oil supplemented diet.
a,c : showing the liver sections of fish oil fed saline treated group of mice; b,d: showing the liver sections of fish oil fed D-GaIN treated group of mice.

Lipid profiles

Lipid Composition of Liver

Dietary supplementation of fish oil or meat oil reduces the concentration of cholesterol ester, triglycerides but increases the concentration of diglycerides and monoglycerides in mice. Supplementation of meat oil in the feed of mice did not reduce the concentration of free cholesterol and free fatty acid (Table 3 *[Supplementary data]*). Induction of hepatitis in the control diet fed group of *Mus musculus* led to significant increase in the concentrations of triglycerides, free fatty acid, free and esterified cholesterol, total glycolipid along with about 40% decrease in the concentration of monoglycerides and total phospholipid (Table 3 *[Supplementary data]*). Dietary supplementation of lipid in the form of fish oil or meat oil for a month prior to

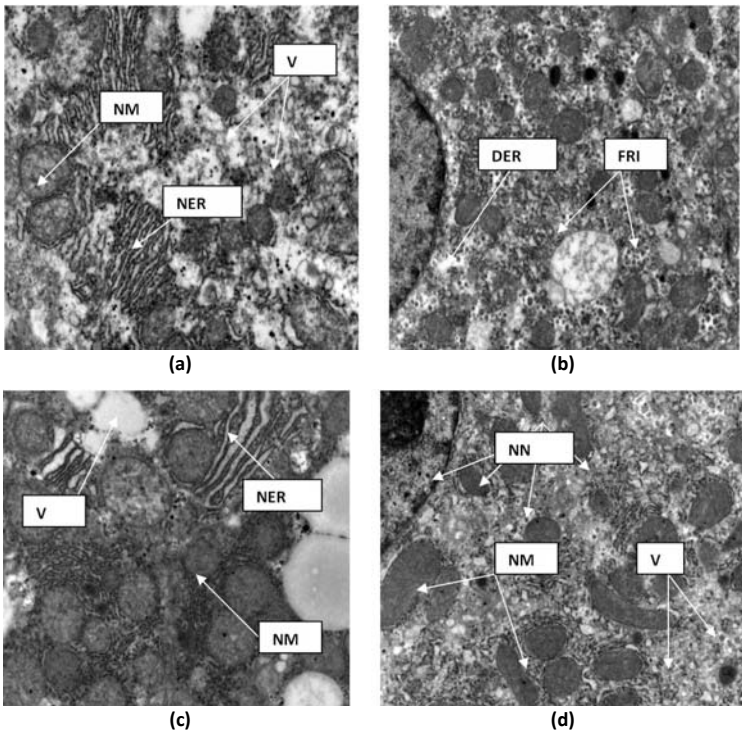

Figure 2: Electron microscopy of liver section of mice fed with meat oil supplemented diet.
a,c: showing the liver sections of meat oil fed saline treated group of mice; b, d: showing the liver sections of meat oil fed D-GaIN treated group of mice.

injection of D-GaIN ameliorates the above mentioned effect of hepatitis in mice.

Fatty acid Profiles

Intake of fish oil or meat oil through the feed, the relative concentration of palmitic acid (C 16:0) and stearic acid (C 18:0) significantly lowered but the relative concentration of oleic acid (C18:1), linolenic acid (C18:2), arachidonic acid (C 20:4) and other polyunsaturated fatty acids elevated in the neutral lipid fraction of mice hepatic lipid (Table 4). Almost a similar trend was observed in the fatty acid profile of phospholipid. The changes in relative composition of fatty acids in cytoplasmic lipid (neutral lipid) and membrane lipid (phospholipid) fractions, upon induction of hepatitis in control diet fed group of mice are not in same direction. While the relative concentrations of palmitic acid was significantly brought down along with increase in the relative concentration of oleic, linoleic, arachidonic and docosahexaenoic (C22:6) acids in the neutral lipid fraction, the relative concentration of palmitic, stearic and eciosapentaenoic (C20:5) acids were significantly elevated up along with 30-70% decrease in the relative concentration of oleic, linoleic, eciosatrienoic (C20:3), arachidonic acids in the membrane lipid or phospholipid fractions due to induction of hepatitis (Table 4 and Table 5). Dietary intake of fish oil /meat oil prior to induction of hepatitis could alleviate the changes in the fatty acid profiles of neutral as well as phospholipid fraction in mice.

DISCUSSION

Acute hepatitis due to viral, toxic or autoimmune pathogenesis is characterized by an activation of macrophages and T cells with an increased production of cytokines that leads to parenchymal liver damage and liver dysfunction (Schmocker *et al.*, 2007). Production of appropriate amounts of TNF-α and IL-1 are beneficial but their overproduction can lead to inflammatory conditions (Simopoulos, 2002). Animal and human studies have shown that production of cytokines can be reduced by n-3 fatty acids (Blok *et al.*, 1996, Calder, 2003). About 20-40% decrease (P<0.001) in the IL-1α, IL-2, TNF-α expression was observed upon dietary supplementation of lipids (Table 1a,1b *[Supplementary data]*). The expression of iNOS was decrease by 20% (P<0.001) with fish oil supplementation but the same was increased by 5% (P<0.001) with meat oil supplementation. n-3 fatty acids could dampen the inflammatory response in liver tissue by probably resulting Kuffer cell activation and cytokine production which is also reflected by almost normal liver architecture (Fig. 1). Numerous studies have described that dietary fatty acids are involved in the modulation of immune system through mechanisms that modify immune response (Pablo and Alvarez, 2000).

D-GaIN induced hepatitis is a well established model of hepatitis showing activation of macrophages and cytokine release factors crucial also in human hepatitis of various causes. D-GaIN administration caused 30-50% (P<0.05) increase in the expression of IL-2, TNF-α and iNOS in the control diet fed group of mice and also marginally elevated the expression of IL-1α in mice liver (Table 1 *[Supplementary data]*) which was also reflected as the damaged endoplasmic reticulum, deformed mitochondria and abnormal nuclear membrane (Fig. 3b,3d). Increased expression of the proinflammatory cytokines such as IL-1 and TNF-α has been reported earlier in atherosclerotic lesions (Plutzky, 2001). Cytokines such as TNF-α have been implicated in the pathogenesis of organ failure after shock and sepsis (Shakhov *et al.*, 1990). Pro-inflammatory cytokines especially TNF-α and several other interleukins are also thought to play a role in cell injury of acute hepatitis (Felver *et al*, 1990). An increase in the TNF-α is one of the early events in liver damage in alcoholic hepatitis and steato-hepatitis (Tilg and Diehl *et al.*, 2000). TNF-α has been implicated in liver

damage in alcoholic hepatitis and steatohepatitis. In D-GaIN induced hepatitis, TNF- α is a critical mediator of apoptotic liver damage. The expression of IL-1α, IL-2 and TNF-α in the dietary lipid supplemented groups remained lowered by 10-30% (P<0.001) in spite of D-GaIN induced hepatitis. Growing evidence indicates that n-3 PUFA and their specific lipid mediators can reduce the activity of inflammatory processes (Weylandt and King, 2005). Schmocker *et al*. (2007) have reported earlier that fish oil supplementation lowers TNF-α and IL-1 production in mononuclear cells. High concentrations of n-3 PUFA has been reported to reduce the activation of nuclear factor kappa B (NF-kB) which leads to a decreased production of TNF-α (Babcock *et al*., 2002). We observed that the dietary fish oil ameliorated effect of D-GaIN induced hepatitis by decreasing the increased activities of liver function enzymes like, alanine amino transferase, aspartate amino transferase, alkaline posphatase, gama glutamyl transpeptidase and by increasing the decreased activities of super oxide dismutase, catalase and glutathione peroxidase (Pujari and Roy, 2010). Recent studies have implicated the n-3 PUFA derived lipid mediators such as resolvin E1 to be responsible for the decreased levels of pro-inflammatory cytokines (Arita *et al*., 2005). Fish oil supplemented group of mice still maintained the iNOS expression lowered by about 6% (P<0.001), however, meat oil supplemented group of mice showed about 30% elevation in the expression of iNOS mRNA upon induction of hepatitis. PUFA may dampen the inflammatory response in liver tissue probably by regulating Kuffer cell activation and suppressing cytokine production.

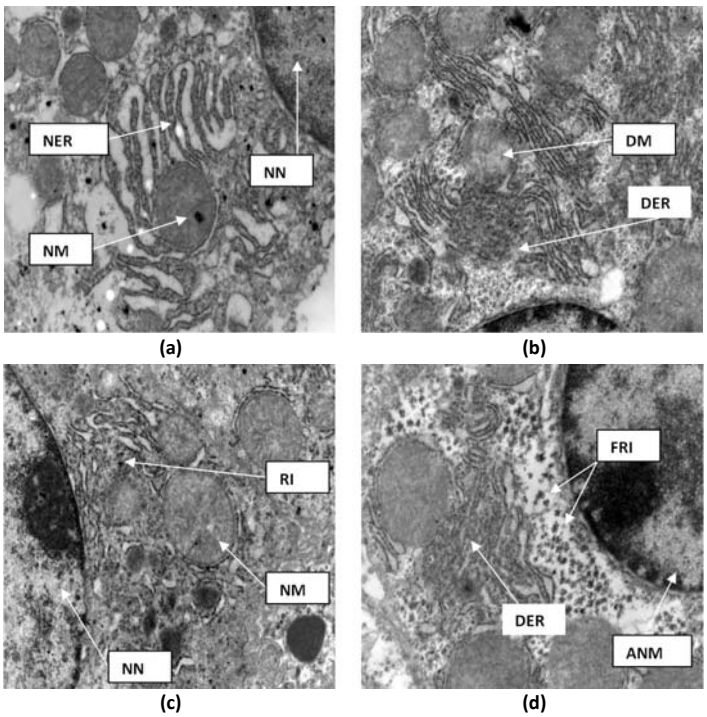

Figure 3: Electron microscopy of liver section of mice fed with control diet. a,,c: showing the liver sections of control diet fed saline treated group of mice; b,d: showing the liver sections of control diet fed D-GaIN treated group of mice.

Changes in the source of lipid consumed in the diet may modify the fatty acid composition of many cell types, including those involved in the development of inflammatory and immunologic diseases (Pablo and Alvarez *et al*., 2000; James *et al*., 2000). Induction of hepatitis in the control diet fed group of mice showed a significant elevation in the concentrations of triglycerides, diglycerides, glycolipids and free fatty acids (Table 3 *[Supplementary data]*) which was also reflected in the electron micrographs which showed fat accumulation (Fig. 3). Increased free fatty acids can be attributed to the hyper metabolic state (Legaspi *et al*., 1987). Puri *et al*. (2007) have earlier reported that the concentrations of diglycerides increase in human nonalcoholic fatty liver diseases. Liver disorders are often associated with the deficiency of essential fatty acid or polyunsaturated fatty acid (Celmmesen *et al*., 2000; Okita *et al*., 2002). D-GaIN administration in the control diet fed group of mice led to massive alteration in the fatty acid profiles of cytoplasmic lipid (Table 4 *[Supplementary data]*) and phospholipid or membrane lipid (Table 5 *[Supplementary data]*). 30-70% decrease in the relative concentration of oleic, linoleic, eciosatrienoic (C20:3), arachidonic acids in the membrane lipid or phospholipid fractions indicate that these fatty acids were mobilized towards the production of eicosanoids which might causes the damage in the normal architecture of the liver. It has been reported earlier that lipid profiles are different in the cirrhosis patients (Jiang *et al*., 2006). Hepatic cellular damage impairs lipid metabolism and leads to the changes in the lipid profiles. It has been well documented that chronic liver dysfunction might interfere with lipid metabolism *in vivo* and could change plasma lipid and lipoprotein patterns (Miller, 1990). We also observed 15 to 20% elevation in the levels of serum cholesterol and triglycerides along with 18% increase in serum LDL and VLDL in mice upon D-GaIN administration (unpublished data). There is substantial evidence to suggest that increased intake of EPA and DHA has a positive impact on fat metabolism (Soria *et a*l., 2002). It is known that lipids and lipoprotein metabolism could be regulated by cytokines and in addition these cytokines could also decrease lipolysis *in vivo* (Cheung *et al*., 1990).

The results presented here indicate that increasing hepatic content of n-3 PUFA could decrease inflammatory activity in hepatitis. This may be due to the altered cytokine expression in liver tissue. Thus

intake of fish oil rich in omega 3 polyunsaturated fatty acid may be helpful to prevent the hepatitis.

Abbreviations

D-GaIN: D-Galactosamine; PUFA: Polyunsaturated fatty acids; IL: interleukin, TNF: Tumor necrosis factor; iNOS: inducible Nitric oxide synthase.

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