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DIETS RICH IN N-3 POLYUNSATURATED FATTY ACIDS ARE ASSOCIATED WITH AMELIORATION OF D-GALACTOSAMINE INDUCED HEPATITIS IN MICE

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Abstract
Liver disorders are often associated with long chain polyunsaturated fatty acid deficiency due to hepatic insufficiency or lack of dietary intake that is hypothesized to further aggravate the condition. Consumption of diets rich in n-3 polyunsaturated fatty acids (PUFA) have been described to be beneficial against diets rich in saturated fatty acids (SFA) in various inflammatory disorders. Hence, we tested the hypothesis that the consumption of dietary fatty acids especially n-3PUFA is associated with the amelioration of hepatitis. We selected two sources of dietary fatty acids i.e. fish oil rich in n-3 PUFA and meat oil rich in SFA. The diet preparation differed only in the type of the oil used. 10% fish oil or meat oil was freshly blended with the pellet diet and fed to the experimental mice for a period of 30 days. Hepatitis was induced in the experimental model i.e. mice (*Mus musculus*) by intraperitoneal injection of D-Galactosamine. Upon induction of hepatitis, the n-3 PUFA group showed elevated levels of total protein while maintaining thiobarbituric acid reactive substances and tocopherol unaltered. In contrast, the SFA group showed decreased level of total protein, reduced glutathione and tocopherol along with an augmentation of TBARS. Degree of augmentation of gamma glutamyl transpeptidase activity and bilirubin concentration upon induction of hepatitis were much lower in the fish oil supplemented groups compared to the control diet fed groups. In spite of induction of hepatitis, the n-3 PUFA group showed well preserved liver architechture with no evidence of hemorrhage, necrosis or hepatocytolysis except mild inflammation. Our present findings indicate a significant alleviation of D-GalN induced hepatitis by dietary n-3 polyunsaturated fatty acids rich fish oil than that of meat oil.

Key Words: PUFA; SFA; Hepatitis; D-GalN; *Mus musculus*.

Introduction
Dietary fatty acids play an important role in various physiological processes and maintaining health. A large number of studies have shown positive health benefits associated with the consumption of n-3 polyunsaturated fatty acids (PUFA) on infant development, cancer, cardiovascular diseases and various mental illnesses such as depression, attention deficit hyperactivity disorder and dementia [1]. On the other hand, a few studies have also suggested that the total dietary fat intake is linked to an increased risk of obesity and diabetes [2]. Diets high in saturated fatty acids (SFA) are correlated with an increased incidence of atherosclerosis and coronary diseases [3]. Thus, some of the most common medical disorders today like cardiovascular disease, hyperlipidemia, obesity, cancer *etc*. are characterized by the altered levels of fatty acids or their metabolites [4].

Liver disorders are often associated with a long chain PUFA deficiency. The impaired PUFA status is mainly due to hepatocellular insufficiency. A low intake of dietary fat is suspected as another reason for a deficiency in plasma PUFAs [5]. The consumption of diets rich in n-3 PUFA has been described to be beneficial in cardiovascular disorders [6] rheumatoid arthritis [7] *etc*. Dietary PUFA have been shown to suppress proinflammatory cytokine production [8] and inhibit lymphocyte proliferation [9]. Cabre and Gassul [10] reported that habitual fish intake protects against hepatic encephalopathy. The objective of the present study was to test the hypothesis that the consumption of dietary fatty acids especially n-3PUFA is associated with the amelioration of hepatitis. We selected two sources of dietary fatty acids i.e. fish oil rich in n-3 PUFA and meat oil rich in SFA (Table1) for our present study. Hepatitis was induced by using D-Galactosamine (D-GalN) as it is very similar to human viral hepatitis.

Materials and Methods
This study was conducted in the animal house of department of Zoology, University of Goa, India. Swiss albino mice, *Mus musculus* (Two months old) of uniform weight (22 ± 0.5 g) were selected for our present study. They were maintained in constant temperature, humidity, 12 hour light dark cycle and received humane care. After acclimatization for a

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period of two weeks, experimental animals were divided into three different groups of (n=12). They were fed with the commercial pellet diet and water *ad libitum.* The experimental protocols and methods of animal maintenance were approved by Animal Ethics Committee of Goa University.

Protocol of Experiments:

Swiss albino mice were divided in to three groups (n=12). At the end of 30 days of feeding half the animals from each group received intraperitoneal saline injection (1ml of physiological saline) for two consecutive days and served as controls while the other half received intraperitoneal D-GaIN injection (0.5mg/g body wt /1ml of physiological saline) and served as liver injury models [11].

Group 1. Served as control and received standard pellet diet and water *ad libitum*. This group was further subdivided into saline and GaIN groups depending upon the treatment they received.

Group 2. Fish oil at a dose of 10% freshly blended along with standard pellet diet was given for a period of 30 days. This group was further subdivided into saline and GaIN groups depending upon the treatment they received.

Group 3. Meat oil at a dose of 10% freshly blended along with standard pellet diet was given for a period of 30 days. This group was further subdivided into saline and GaIN groups depending upon the treatment they received.

The composition of these two oils is as shown in Table 1. Cages were cleaned the next day and checked for any left over food. During the entire period of our experimentation we found none or negligible amount of food left over. Animals were starved for 10-12h before sacrificing. Experimental animals were anesthetized to collect blood and liver samples for further analysis.

Methods

(A) Biochemical Tests: Albumin (using bromocresol green reagent), protein (using Lowry's reagent), total and conjugated bilirubin (using Diazo reagent) were estimated by following routine analytical method [12]. Thiobarbituric acid reactive substances were estimated by using TBA-TCA-HCl reagent following the method of Niehaus and Samuelsson [13]. Reduced glutathione was measured by using 5, 5'-Dithiobis, 2-nitrobenzoic acid [14]. Tocopherol and ascorbic acids were measured by using dipyridyl-ferric chloride reagent [15] and dinitrophenyl hydrazine reagent [12] respectively.

(B) Enzyme Tests: Alanine transaminase (ALT) [EC 2.6.1.2] and aspartate transaminase [EC 2.6.1.1] (AST) activities were assayed by using dinitrophenyl hydrazine reagent [16]. Alkaline phosphatase (ALP) [EC 2.6.1.2] activity was assayed by using *p*-nitrophenol reagent [17]. Gamma glutamyl transpeptidase (GGT) [E.C. 2.3.2.2] activity was measured by using glacial acetic acid reagent [18].

(C) Histopathological Tests: Routine laboratory method was followed for histological studies. Liver tissues were perfused with phosphate buffer saline (pH 7.0) and fixed in 10% formaline. The paraffin block was cut into uniform sections of 10µm thickness using a microtome and tissue sections were stained with hematoxylin and eosin for histopathological examination (40 X) under a polarizing microscope (Olympus BX41).

Statistical Analysis: Analyses were performed using the Statistical Package for the Social Sciences, version 7.5 (SPSS Inc, Chicago Ill). Differences between two groups were analyzed by independent samples student *t* test. Differences between various groups were analyzed by one way ANOVA. The level of significance was set to $P< 0.05$.

Results

Table 1: Fatty acid composition of fish oil and meat oil.

Type of fatty acid	Fish oil	Meat oil
14:0	11.62	8.97
16:0	23.27	32.88
18:0	5.76	26.31
20:0	0.86	1.57
18:1	11.90	1.52
20:1	10.69	12.55
22:1	0.12	0.35
18:2n6	3.13	9.25
20:2n6	0.16	0.32
20:4n6	1.73	4.17
18:3n3	0.21	0.10
20:5n3	17.86	0.52
22:5n3	1.83	0.12
22:6n3	10.86	1.37
Sat/Unsat ratio	0.709	2.304
n-3 /n-6	6.127	0.154

Dietary intake of fish oil for a period of 30 days was found to cause 35-40% elevation (P<0.001) in the total protein content including both albumin and globulin fractions in liver and serum. Meat oil diet elevated the serum total protein content (20%, P<0.001) only due to globulin fraction. It is revealed from table 2 that induction of hepatitis in the control diet fed group (Cd) led to about 60% reduction (P<0.001) in the total protein including both albumin and globulin fractions. The total protein content remained unaltered in the fish

oil supplemented group upon D-GalN administration (Fd). On the other hand, induction of hepatitis in the meat oil supplemented group of mice (Md) caused a significant decrease in the total protein content including both albumin and globulin fractions in liver tissue while it did not show any major changes in the serum protein levels.

Dietary lipid intake led to an elevation in ascorbic acid (2-5 fold, *P*<0.001), tocopherol (10-15%, *P*<0.001) and GSH (15-60%, *P*<0.001) while it lowered TBARS (15-25%, *P*<0.001) (Fig. 1). D-GalN administration in the control diet fed Cd group caused depletion of ascorbic acid (20-30%, *P*<0.0001), tocopherol (35%, *P*<0.0001) and GSH (45-50%, *P*< 0.0001) while leading to a significant 1.5 fold elevation in the TBARS (Figure2). Dietary lipid intake maintained higher levels (50% to 3 fold, *P*<0.0001) of ascorbic acid in both liver and serum in spite of induction of hepatitis (Fig 3). Fd group of mice maintained the tocopherol and TBARS unaltered in both liver and serum. However, Md groups had significantly higher levels of TBARS (60% to 2 fold), lower levels of tocopherol (20 -25%) and GSH (10-30%).

Table 2: Effect of dietary fatty acids on albumin and globulin fractions of protein upon D-GalN induced hepatitis in mice, *Mus musculus*. Data are represented as mean of six values ± SE.

	LIVER (mg/100mgtissue)						SERUM (mg/ml)					
	Group 1		Group 2		Group 3		Group 1		Group 2		Group 3	
	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GalN
Protein	6.960± 0.198	2.950± 0.221 *	10.010± 0.182	7.930± 0.156*	6.600± 0.224	2.920± 0.243*	3.510± 0.030	3.327± 0.030*	4.740± 0.020	4.660± 0.040	4.265± 0.090	4.086± 0.079
Albumin	3.410± 0.193	1.310± 0.096*	4.608± 0.089	2.586± 0.077*	4.134± 0.187	1.816± 0.101*	2.850± 0.042	2.600± 0.036*	3.451± 0.164	2.662± 0.066*	2.870± 0.037	2.590± 0.015*
Globulin	3.548± 0.206	1.648± 0.284*	5.405± 0.200	5.346± 0.157	2.475± 0.287	1.106± 0.240*	0.660± 0.042	0.727± 0.036	1.288± 0.164	1.998± 0.066*	1.395± 0.117	1.497± 0.088
A/G ratio	0.961	0.794	0.852	0.483	1.670	1.641	4.318	3.576	2.679	1.332	2.057	1.730

Table 3: Effect of dietary fatty acids on enzyme activities upon D-GalN induced hepatitis in mice, *Mus musculus.* Data are represented as mean of six values ± SE.

Enzymes	LIVER						SERUM					
	Group 1		Group 2		Group 3		Group 1		Group 2		Group 3	
	Saline	GaIN	Saline	GaIN	Saline	GaIN	Saline	GaIN	Saline	GaIN	Saline	GaIN
ALT (K/min/mg protein)	9.278± 0.30	16.376 ±0.38*	7.956± 0.15*	9.898± 0.19	8.634± 0.38	10.391± 0.24*	13.770± 0.68	30.530± 2.30*	12.640± 0.44	11.720± 0.35	12.570± 0.37	15.490 ± 0.36*
AST (K/min/mg protein)	102.200 ±0.96	166.320 ±6.62*	82.470± 1.38*	90.790± 1.716*	81.237± 0.97*	87.557± 1.27*	117.25± 6.12	236.55± 8.35*	90.470± 2.87*	86.620± 4.85*	80.351± 0.61*	77.846± 2.28*
ALP (IU/mg protein)	16.656± 0.35	24.694± 0.62*	17.540± 0.30	18.990± 0.23	16.045± 0.27	20.060± 0.12*	17.310± 2.10	30.530± 2.30*	20.360± 2.47	19.812± 0.19	16.945± 0.29	23.740± 1.38*
GGT (IU/mg protein)	7.146± 0.27	16.720± 0.26*	6.835± 0.06	8.935± 0.23*	7.181± 0.07	10.091± 0.150*	7.255 ±0.08	17.836± 0.17*	6.886± 0.10*	9.140± 0.20*	7.471± 0.11	11.673± 0.31*

Table 4: Effect of dietary fatty acids on total and conjugated bilirubin concentration upon D-GaIN induced hepatitis in mice, *Mus musculus*. Data are represented as mean of six values ± SE.

	LIVER (mg/100mgtissue)						SERUM (mg/ml)					
	Group 1		Group 2		Group 3		Group 1		Group 2		Group 3	
	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GalN	Saline	GaIN
Total Bilirubin	1.030± 0.014	6.085± 0.148*	1.191± 0.130	3.000± 0.135*	1.015± 0.052	3.118± 0.172*	0.990± 0.100	11.670± 0.640*	0.998± 0.080	3.270± 0.090*	0.986± 0.019	3.248± 0.093*
Conjugated bilirubin	0.660± 0.021	4.788± 0.173*	0.632± 0.023	2.008± 0.021*	0.630± 0.034	1.868± 0.160*	0.530± 0.130	8.830± 0.730*	0.543± 0.220	2.600± 0.090*	0.538± 0.029	2.116± 0.066*

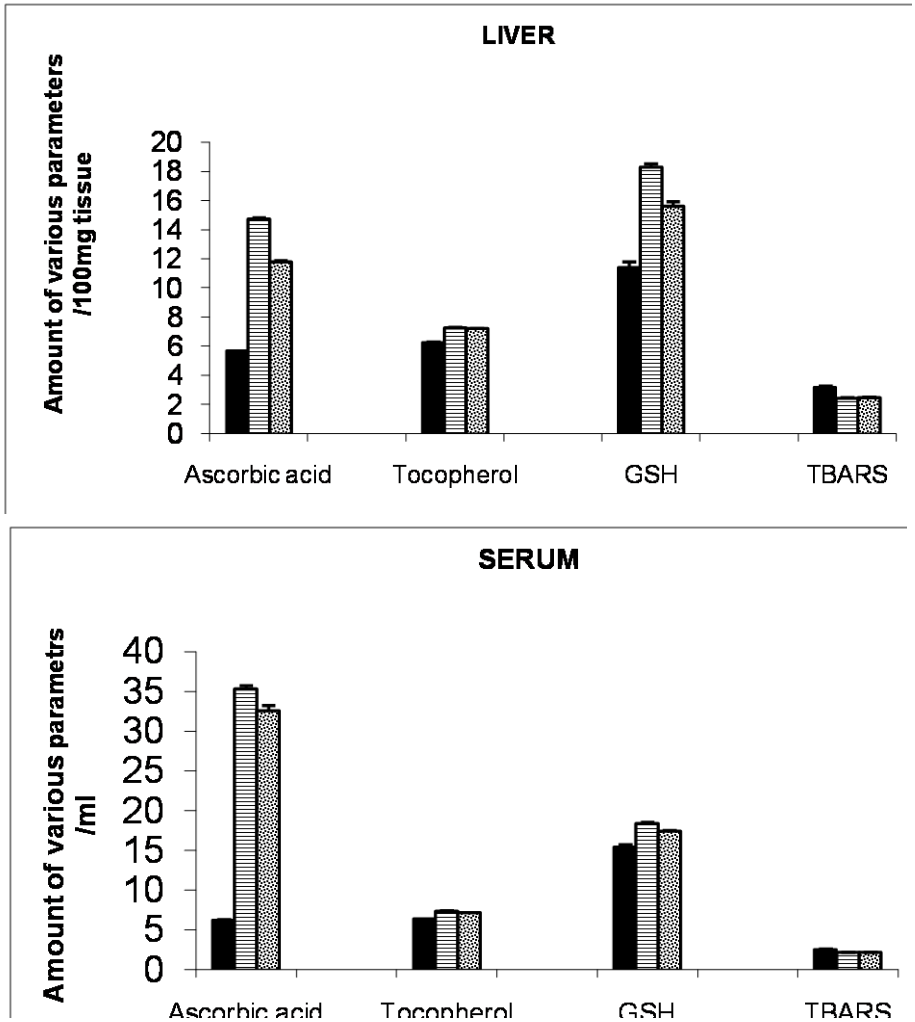

Fig. 1: Effect of supplementation of dietary lipids on various parameters like Ascorbic acid (µmol), tocopherol (nmol x10), reduced glutathione (µmol), TBARS (nmol MDA) of mice, *Mus musculus*. Mice were fed *ad libitum* with commercial pellet diet supplemented with 10% fish oil or meat oil for 30 days. Cs (without lipid supplementation); Fs (supplemented with fish oil); Ms (with meat oil supplementation). All these mice received 1 ml of physiological saline for two days. Values are mean ± SE of six oservations. All the changes were significant (P<0.05) compared to the Cs group.

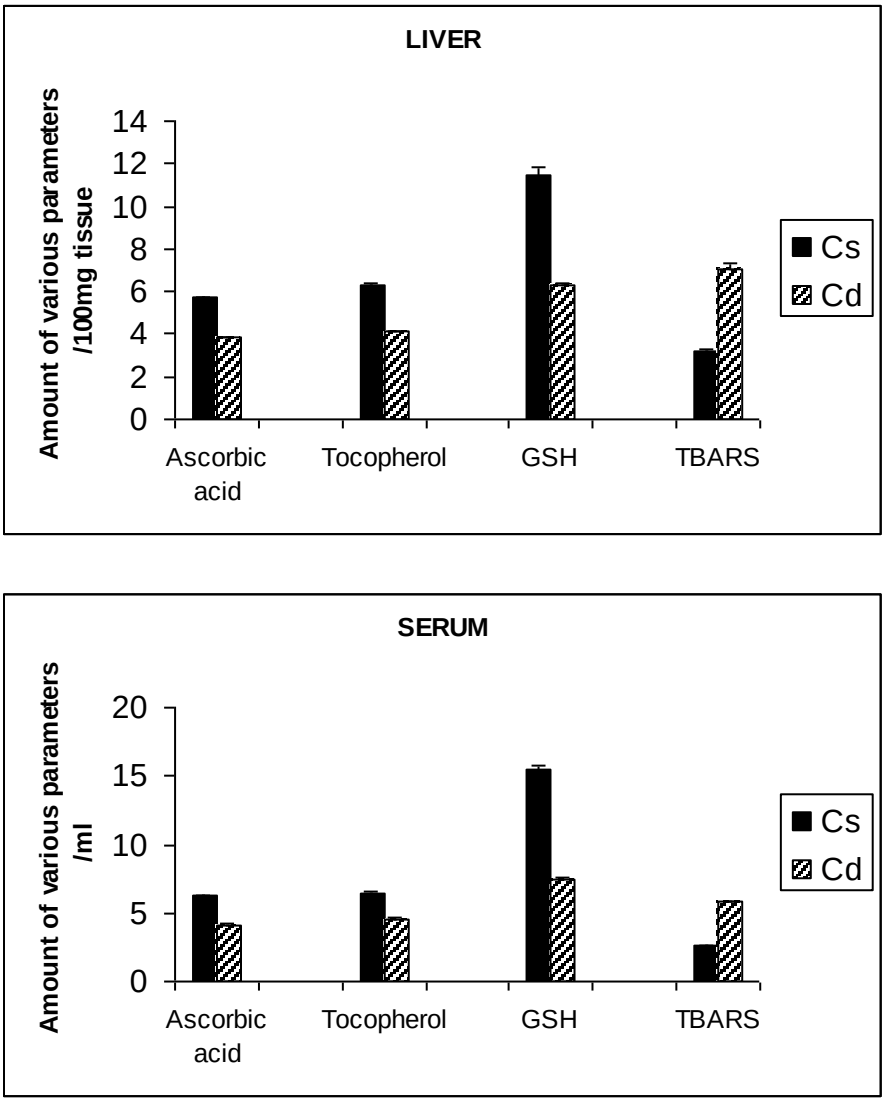

Figure 2 : Effect of D-GaIN induced hepatitis on various parameters like ascorbic acid (µmol), tocopherol (nmol x10), reduced glutathione (µmol), TBARS (nmol MDA) in mice, *Mus musculus.* Cs (without any lipid supplementation and received 1ml physiological saline injection for two subsequent days); Cd (without any lipid supplementation and received 5mg D- GaIN/ g body wt /animal in 1ml physiological saline injection for two consecutive days). Values are mean ± SE of six oservations. All changes were significant (P<0.05) compared to the Cs group.

Dietary intake of fish and meat oil significantly brought down the activity of ALT by 5-15% (P<0.05) in liver and AST activity by 20-30% (P<0.05) in both liver and serum. Both the diets did not affect ALP activity either in liver tissue or in serum (Table 3). No significant changes in the GGT activity were noticed upon fish or meat oil intake. Induction of hepatitis markedly increased the activity of AST and ALT by 60%- 2 fold (P<0.001) in Cd group. It caused about 50-75% (P<0.001) elevation in ALP activity and about 2.5 fold (P<0.0001) increase of GGT activity in Cd group. Fd group showed only about 15% elevation in the liver ALP activity while maintaining it unaltered in serum where as the Md group showed 20-40% elevation in its activity. We observed 25-60% elevation (P<0.0001) in the GGT activity in both Fd and Md groups of mice, however the degree of augmentation was much lower compared to the Cd group.

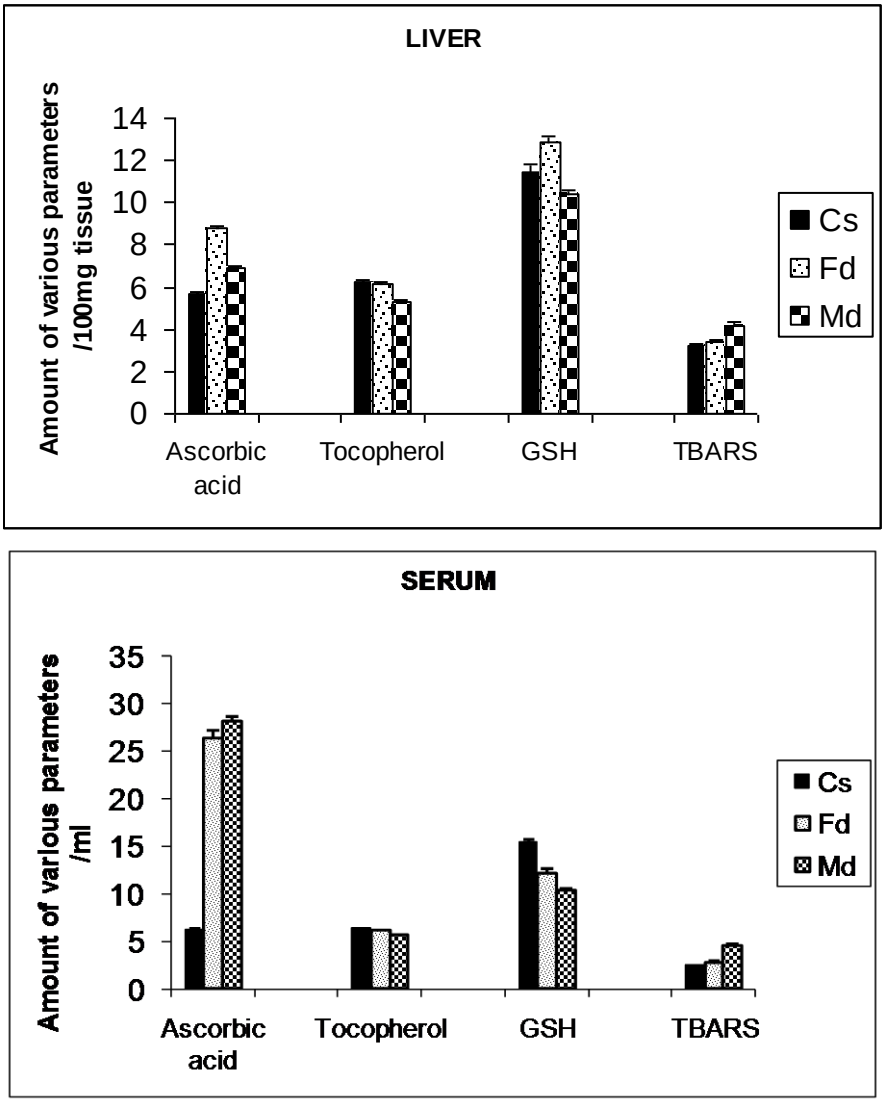

Figure 3: Effect of supplementation of dietary lipids in D-GaIN injected mice *Mus musculus.* on various parameters like ascorbic acid (μmol), tocopherol (nmol x10), reduced glutathione (μmol), TBARS (nmol MDA). Fd (with 10% fish oil supplementation for one month prior to D-GaIN injection); Md (with 10% meat oil supplementation prior to D-GaIN injection at a dose of 5mg/ g body wt /animal in 1ml physiological saline injection for two subsequent days). Values are mean ± SE of six oservations. All changes other than* were significant (P <0.05) compared to Cs. group.

It can be noticed from Table 4 that dietary intake of both fish and meat oil for a period of 30 days, maintained the bilirubin levels unaltered. Induction of hepatitis led to a marked increase in the total and conjugated bilirubin concentration in liver and serum (6-15 fold P<0.001). Dietary lipid supplemented groups also showed 3-5 fold increase (P<0.001) in the bilirubin concentration however the degree of augmentation was much lower compared to the Cd group.

Figure 4a revealed the normal histology of liver consisting of hepatic lobules, central vein and portal triad in the Cs group. Almost normal architecture of liver along with mild fatty changes was noticed in fish oil supplemented Fs group (Fig 4c).Dietary intake of meat oil caused fatty degeneration and mild inflammation in liver architecture where, abundant vacuolated foamy cytoplasm was seen (Fig 4e). D-GaIN administration caused complete distortion of normal liver histology with severe degenerating changes in the Cd group (Fig 4b). Md group showed severe damages due to D-GaIN administration as was evident by the inflammatory and fatty changes (Fig 4f). However, we found alleviation of D-GaIN effect in the Fd group of mice where, the normal architecture of liver tissue was completely well preserved with no evidence of hemorrhage, necrosis or hepatocytolysis except mild inflammation (Fig 4d).

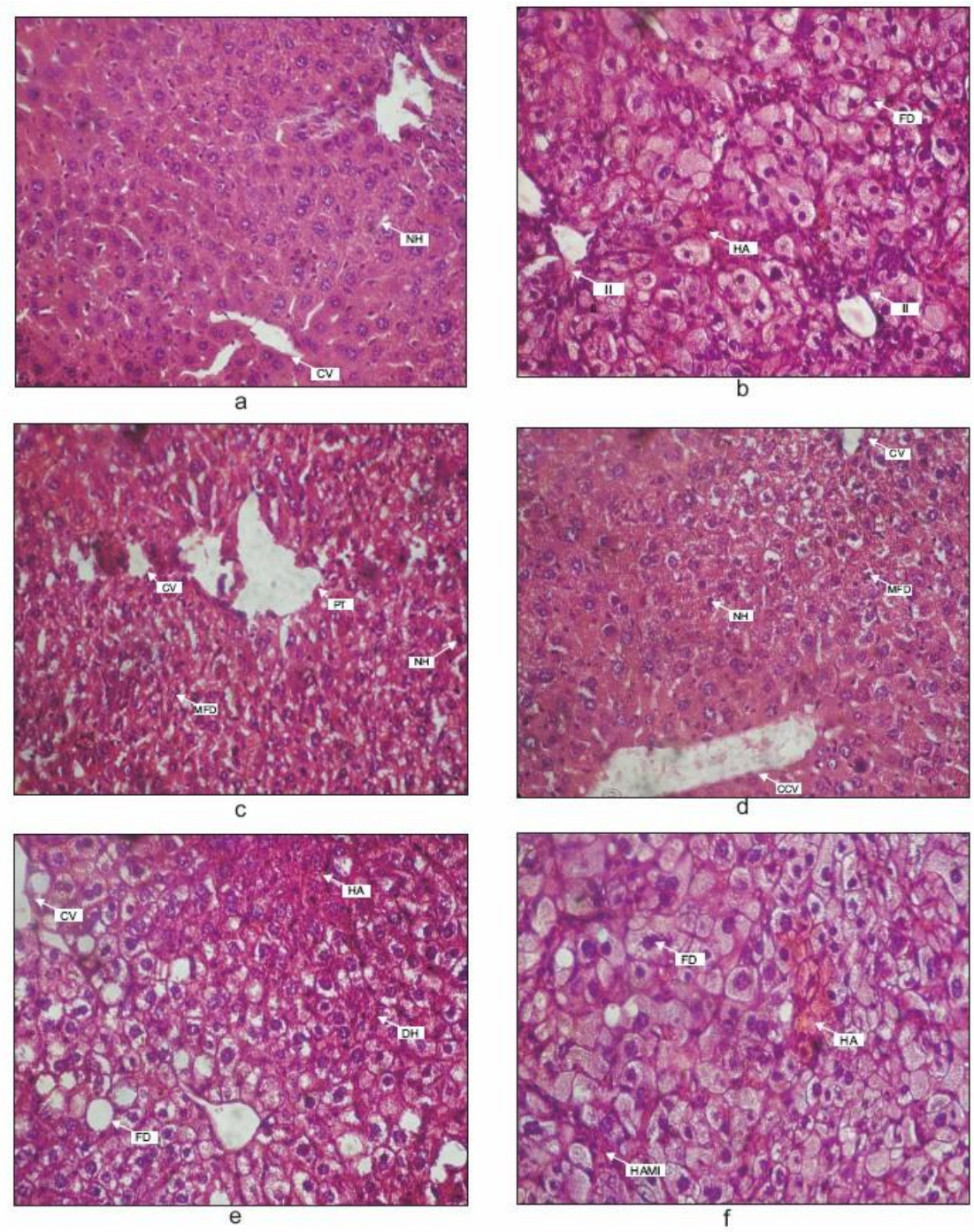

Figure 4 : Effect of dietary lipid supplementation on the liver architecture of mice *Mus musculus.* **a**: mice without supplementation of lipid and received saline injection; **b**: mice without supplementation of lipid and received D-GaIN injection; **c**: mice with 10% fish oil supplementation and received saline injection; **d**: mice with 10% fish oil supplementation and received D- GaIN injection; **e:** mice with 10% meat oil supplementation and received saline injection; **f**: mice with 10% meat oil supplementation and received D- GaIN injection; CV-Central Vein; H-Hepatocytes; HA-Haemorrhagic area; I I –Inflammatory Infilterate; MFD-Mild Fatty Degeneration ; CCV-Congestion of central vein; DH-Derangement of Hepatocytes ; FD-Fatty Degeneration

Discussion

The main finding of this study was a remarkable degree of amelioration of D-GaIN induced hepatitis associated with the dietary intake of n-3 PUFA rich diets as compared to the SFA rich diets. Previous findings have shown that a long chain PUFA deficiency is often observed with various liver disorders [19]. Hepatocellular insufficiency or a low intake of fat can lead to the deficiency in plasma PUFAs [20]. We made

use of fish oil and meat oil as the sources of dietary lipids which are rich in unsaturated or saturated fatty acids and also have different MUFA and PUFA compositions (Table1). D-GalN induced hepatitis is used as an experimental model for being very similar to human viral hepatitis in its structural and morphological appearance [21].

D-GalN administration in the control diet fed group led to a marked decrease of about 60% (P<0.001) in total protein including albumin and globulin fractions (Table2) and 6-15 fold increase in bilirubin concentration suggesting a severe liver damage. This may be due to an increased protein catabolism or suppression of protein synthesis leading towards hepatotoxicity. Sun *et al*. [22] have reported that a high dose of D-GalN causes haemorrhage due to UTP depletion and inhibition of protein synthesis. Within 30 minutes of D-GalN administration a large number of UTP derived metabolites accumulate leading to the depletion of hepatic UTP and inhibition of macromolecular biosynthesis of RNA, protein, glycogen etc [23]. About 20-35% reduction (P<0.001) in the antioxidant level and 40-45% reduction (P<0.0001) in GSH level along with a marked increase in TBARS (Fig 2) due to D-GalN administration was observed in the control diet fed group of mice. Sandhir and Gill [24] reported a similar kind of reduction in GSH in rats exposed to ethanol. A marked increase (2.5fold) in the liver function tests and GGT activity in the control diet fed group confirmed hepatic damage. ALT and AST are cytoplasmic in location and are released into the circulation after cellular damage [25]. Increased activity of ALP can be due to the disturbance in secretory activity or the transport of metabolites [26]. Gamma glutamyl transpeptidase enzyme is most sensitive indicator of liver disease and a number of chemicals are known to increase its activity by the induction of microsomal enzyme [27]. This may be due to the fact that the depletion of GSH may induce hepatic GGT activity through an increased synthesis of its mRNA [28]. It is clear from table 2 that D-GalN administration in the control diet fed group led to GSH depletion.

Dietary intake of fish oil enhanced total protein (40%, P<0.001) including albumin and globulin fractions (Table2) in *Mus musculus.* Dietary lipid intake enhanced antioxidant levels, decreased TBARS (Fig 1). Both Fd and Md groups showed a significant elevation (25-60%, P<0.001) in the GGT activity in liver and serum however, the degree of augmentation was much lower compared to the induction of hepatitis in control diet fed group indicating a better functioning of liver, more particularly with that of fish oil intake.

Dietary intake of fish oil maintained elevated levels of protein, ascorbic acid without any alteration in the levels of TBARS and tocopherol upon D-GalN administration. In contrast, intake of meat oil brought about 60% decrease (P<0.0001) in the liver total protein, while maintaining higher levels of ascorbic acid (Figure 3) upon D-GalN administration. Md group of mice also showed higher levels of TBARS which may indicate peroxidation of fatty acids upon D-GalN administration. Dietary intake of lipids reduced any changes in GGT activity upon induction of hepatitis (Table 3). The hepatic damage caused by D-GalN like ballooning, congestion or inflammatory infiltrations were lowered with the dietary intake of n-3 PUFA rich fish oil.

Conclusion

Our present findings indicated a remarkable alleviation of D-GalN induced hepatitis with the dietary intake of n-3 PUFA rich fish oil than that of meat oil. Though meat oil also has MUFA and PUFA of ω-6 series their beneficial effects might be counteracted by the presence of high levels of other SFAs unlike fish oil which has a very low level of SFAs. The hepatoprotective effects of fish oil might be due to eicosanoid dependent or eicosanoid independent i.e. cytokine regulatory actions which need further investigation.

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