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HEPATOPROTECTIVE EFFECT OF POLYUNSATURATED FATTY ACIDS AGAINST REPEATED SUBACUTE ACETAMINOPHEN DOSING IN RATS

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ABSTRACT

Omega-3 polyunsaturated fatty acids are essential fatty acids offering multiple health benefits. They form an integral part of cell membrane and are indispensable for normal metabolism. Fish oil and flaxseed oil are among few dietary sources of these fatty acids. In present investigation, hepatoprotective potential of omega-3 fatty acids in the form of flaxseed oil and fish oil was assessed against repeated acetaminophen dosing in rats. Administration of flaxseed oil or fish oil prevented hepatic damage with marked improvement in hepatic function and normalization of lipid profiles in serum and liver. Administration of these oils normalized oxidative stress through improvements in levels of anti-oxidant enzymes and oxidative stress markers. Histological analysis showed absolutely normal hepatic architecture in flaxseed oil and fish oil treated animals and signify hepatoprotective effect of these supplements. This study is the first report of analysis of hepatoprotective potential of two different nutritional supplements of omega-3 fatty acids.

KEYWORDS: **Acetaminophen, Flaxseed oil, Fish oil, Hepatotoxicity, Omega-3 fatty acids, Paracetamol**

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INTRODUCTION

Liver diseases are one of the important worldwide health problems[1]. Liver is a unique organ in the body and is central to the regulated metabolism since it performs a vital function of detoxification of endogenous and exogenous compounds[2]. Liver is an organ which is subjected to frequent metabolic insults due to its central role in detoxification, metabolism of drugs and xenobiotics and exposure to insecticides, pesticides and other environmental pollutants[3].Liver toxicity or hepatotoxicity is one of the most studied liver disorders[4]. Most of the hepatotoxic chemicals, including some drugs, damage liver cells mainly by inducing oxidative damage and lipid peroxidation in liver. Acetaminophen or paracetamol is one of such therapeutic chemical which otherwise has an excellent safety profile under prescribed dosage regimen. Acetaminophen is converted into N-acetyl-p-benzoquinoneimine (NAPQI) which is an intermediate in the metabolism of acetaminophen. Accumulation of NAPQI in liver leads to depletion of glutathione, an important antioxidant in liver and causes direct damage to liver cells[5].Due to scarcity of metabolically safe hepatoprotective drugs, there is increasing interest[6] in the alternative medicines for the treatment of liver diseases and associated metabolic derailements[7] which include herbal remedies and dietary supplements[8]. Omega 3 fatty acids or polyunsaturated fatty acids (PUFA) are one of such dietary supplements[9]. Most common and richest sources of PUFA include fish oil and some plant sources like flax seed oil and algal oil[10,11,12,13]. Fish oils are rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) while flaxseed oil is rich in alpha linolenic acid (ALA). The intake of these omega 3 fatty acids is known to offer immunomodulatory, antidiabetic, and cardioprotective benefits[14,15,16]. In view of these remarkable health benefits of PUFA, we undertook the study to evaluate the hepatoprotective action of these omega 3 fatty acids as dietary supplements in the form of flaxseed oil and fish oil.

MATERIALS AND METHODS

(i) Chemicals and reagents

Flax oil (Alpha Lite) was purchased from EnSigns Diet Care Pvt. Ltd. (Pune, MS, India) and contained 50% alpha-linolenic acid (ALA), 20% Oleic acid and 12% Linoleic acid. Fish oil (Maxepa) was purchased from Merck Limited (Goa, India) and contained 60% EPA and 40% DHA. Acetaminophen (Paramol; Ranbaxy Laboratories Ltd.) and Silymarin (Silybon; Micro Labs) were purchased from local pharmacy and dissolved in sterile water to make the stock solution convenient for animal administration.

(ii) Experimental animals:

Three months old male Albino Wistar rats, weighing about 150-200g were obtained from institutional animal house and used in the experiment. Animals were maintained under standard husbandry conditions (Temperature 25±2ºC, 12-h light: 12-h dark cycle) and fed with standard pellet diet (Nutrivet Life Science, Pune, M.S., India) and tap water *ad-libitum*. This study was carried out as per CPCSEA guidelines and after approval of the experimental protocol by the Institutional Animal Ethical Committee (Ref. No: BVDUMC/443/2012-2013).

(iii) Assessment of hepatoprotective activity

Rats were divided into five groups by random assignment of six animals per group. The variation in the average weight of the animals in and between the groups was less than 20%. Hepatotoxicity was induced by oral administration of 1000mg acetaminophen/kg b.w. for 15 days. The treatment protocol is summarized below:

Group I: Healthy control (n=6); fed on a normal diet and water for 15 days.

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Group II: Negative control (n=6); rats were administered acetaminophen (1000mg/kg b.w./day, p.o.) for 15 days.
Group III: Positive control (n=6); rats administered with acetaminophen (1000mg/kg b.w./day, p.o.) 30 minutes after administration of silymarin (100mg/kg b.w./day, p.o.) for 15 days.
Group IV: Treatment group 1 (n=6); rats administered with acetaminophen (1000mg/kg b.w./day, p.o.) 30 minutes after administration of flax oil (500mg/kg b.w./day, p.o.) for 15 days.
Group V: Treatment group 2 (n=6); rats administered with acetaminophen (1000mg/kg b.w./day, p.o.) 30 minutes after administration of fish oil (500mg/kg b.w./day, p.o.) for 15 days.
During the experiment, animals were observed daily for any signs of infection and/or discomfort. After 15 days of the protocol, animals were fasted overnight and blood samples were collected from retro-orbital plexus by capillary glass tubes and animals were humanely scarified. Blood samples were allowed to clot at R.T. for 30 minutes and serum was collected by centrifugation at 2000 rpm for 15 minutes. The animals were immediately dissected and liver was excised, washed in saline, weighed and part of liver was stored in 10% neutral buffered formalin for histological studies while part of the liver tissue was snap frozen in liquid nitrogen for later use.

(iv) Blood biochemical markers assay

Biochemical markers of liver damage and dysfunction (serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin), lipid profile (Total cholesterol, HDL cholesterol, Triglycerides) and total protein were estimated using commercial kits (Coral clinical system, Goa, India). LDL cholesterol (mg/dL) was estimated by using the formula: (Total Cholesterol – HDL Cholesterol) – triglycerides/5 and VLDL Cholesterol was estimated by using the formula: Triglycerides/5.

(v) Liver biochemical markers

Liver tissue was homogenized with PBS buffer (pH 7.0) and the homogenate was centrifuged at 30,000 rpm for 15 minutes. Cell free supernatant was used for assay of total protein, total cholesterol, HDL-cholesterol, triglycerides (Coral clinical system, Goa, India) and antioxidant parameters like Lipid peroxidation (OxisResearch™, U.S.A), Catalase (CAT) (abcam®), Superoxide dismutase (SOD) (Sigma-aldrich U.S.A). Part of the homogenate was deproteinated with metaphosphoric acid (MPA) and 4M triethanolamine (TEAM) reagent and was used for estimation of reduced glutathione (GSH) content in the tissue by using commercially available kits (Cayman Chemical Company). Lipid profile (total cholesterol, HDL cholesterol, triiglycerides) and total protein were estimated using commercial kits (Coral clinical system, Goa, India). LDL cholesterol (mg/dL) was estimated by using the formula: (Total Cholesterol– HDL Cholesterol) – triglycerides/5 and VLDL Cholesterol was estimated by using the formula: Triglycerides/5.

(vi) Histological analysis

The liver tissue was fixed in 10% formalin and embedded in paraffin. Four μm thick sections of paraffin embedded tissue were stained with haematoxylin and eosin (H&E staining), observed under a light microscope at 10X magnifying power and photographed using Image Pro Plus (v5.1.2.59).

(vii) Statistical analysis:

Results are presented as Mean ± Standard Error (SE). Tukey-Kramer Multiple Comparison Test and one way Analysis of Variance (ANOVA) was done to estimate the statistical significance between groups.

RESULTS

1. Serum biochemical parameters

In the present study, activities of serum SGOT, SGPT and ALP from negative control group had a sharp and significant increase as

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compared to a healthy control group indicating successful induction of hepatotoxicity by repeated dosing of acetaminophen at subacute concentrations (Table 1). Total bilirubin, protein, cholesterol and triglyceride levels indicated significant elevation in paracetamol treated animals compared to a normal control group, further indicating liver damage. Administration of flaxseed oil and fish oil at a dose of 500mg/kg b.w./day had a beneficial effect on the levels of these biochemical markers in the treatment groups. SGOT, SGPT and ALP activities were found to be elevated (by 44.42%, 20.72% and 78.22%) in the negative control group as compared to healthy control. Treatment with silymarin helped to lower the activities of these enzymes to some extent. Flax oil and fish oil treated groups showed comparable improvements in these parameters and fish oil was found to have a pronounced effect on the activities of these enzymes, in addition to a significant decrease in bilirubin contents, especially in comparison to the positive control group (Table 1). As compared to negative control group, flax oil and fish oil displayed remarkable benefits in relation to improvements of lipid profiles. Flax oil and fish oil treated groups showed 40.14% and 25.90% decrease in total cholesterol, 86% and 139.56% increase in HDL content, 70.86% and 55.09% decrease in LDL content and about 5.5% and 3.3% decrease in VLDL and triglyceride contents as compared to the negative control group. All these variations in the biochemical parameters were found to be statistically significant when compared to healthy, negative control or positive control group (Table 1).

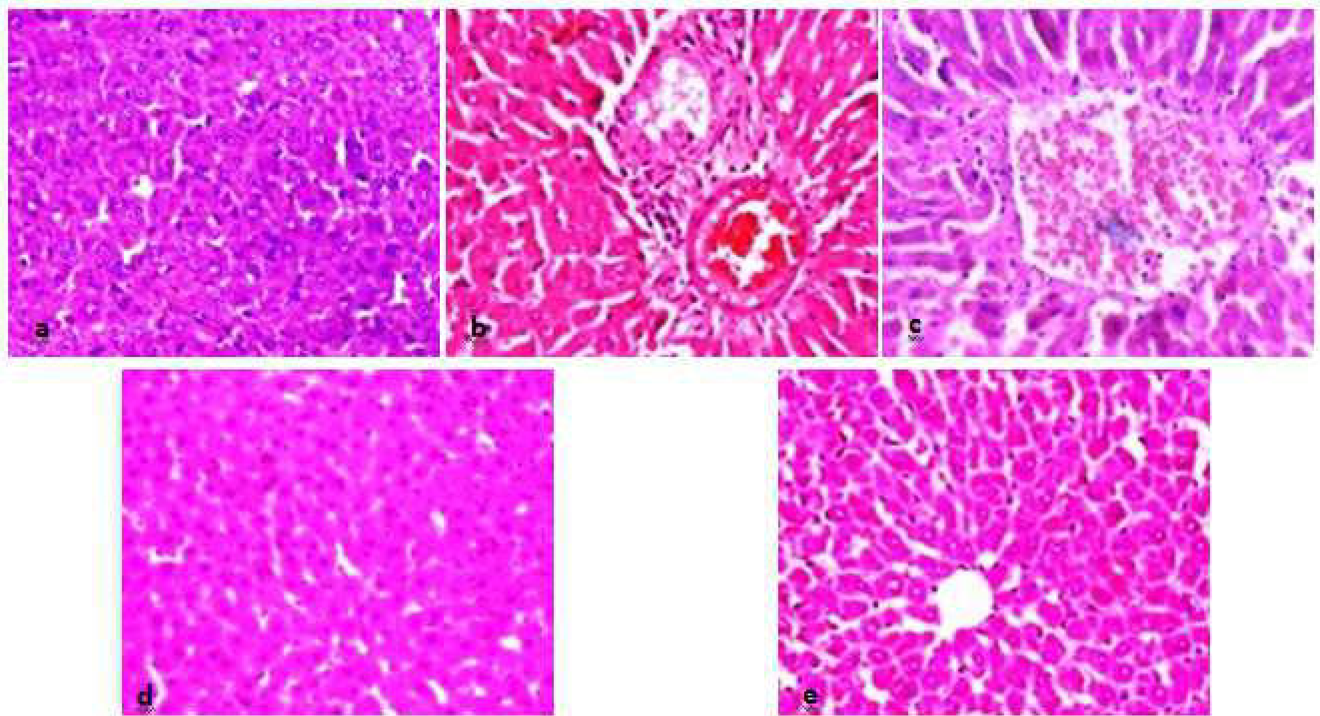
2. *Liver biochemical parameters:*

Flax oil and fish oil supplementation was found to be significantly effective in ameliorating the oxidative damage through decrease in the extent of lipid peroxidation and increase in the contents of reduced glutathione which acts as a principal antioxidant compound in liver (Table 2). The extent of lipid peroxidation (MDA levels) was significantly decreased in flax oil (65.20% reduction) and fish oil (84.62% reduction) treated animals as compared to paracetamol treated animals while the levels of reduced glutathione (GSH) were significantly increased in flax oil (about 145%) and fish oil (about 160%) supplemented groups as compared to paracetamol treated animals (62.5% reduction as compared to healthy animals). Flax oil and fish oil supplementation outperformed silymarin treatment with respect to improvements in SOD and Catalase activities (Table 2). The total protein content of liver tissues was significantly improved in flax oil and fish oil treated groups as compared to acetaminophen group and the contents were statistically indifferent, and hence comparable to silymarin treated animals. The lipid profile of liver also displayed significant improvements in flax oil and fish oil treated groups when compared with negative control group. Flax oil and fish oil treated groups displayed 36.10% and 32.73% decrease in total cholesterol, 35.93% and 18.63% increase in HDL, 77.64% and 52.96% decrease in LDL and about 56% and 62% decrease in VLDL and triglycerides as compared to the negative control group. It can be noted here that, total cholesterol, HDL, VLDL and triglyceride contents in flax oil and fish oil treated groups were at par with the positive control group.

3. *Liver Histology*

The hepatoprotective effects of flax oil and fish oil were also evident from the prominent variations in liver histology (Fig 1 a-e). The healthy control group (Fig 1a) displayed normal liver histology while the acetaminophen treated group (Fig 1b) showed swollen or occasionally apoptotic hepatocytes with coarse granular cytoplasm and compressed sinusoids. The group also displayed a few nucleated cells indicating spontaneous regeneration of hepatocytes. The group treated with the standard drug silymarin (Fig 1c) had near normal histology with mild congestion of central vein and mildly swollen hepatocytes. Both flax oil and fish oil treated groups (Fig 1d-e) exhibited strikingly normal liver histology without any anatomically detectable anomalies.

Figure 1
Liver histology of healthy, acetaminophen treated and flax/fish oil treated animals. Note drastic variations in the architecture of liver tissue in different groups



Hematoxylin and eosin stained cross sections of paraffin embeded liver tissues of rats from control and experimental groups (40X). Liver from healthy (a) group shows the normal architecture. Sections of liver from acetaminophen treated group (b) show ***swollen or occasionally apoptotic hepatocytes with coarse granular cytoplasm and compressed sinusoids with few nucleated cells indicating spontaneous regeneration of hepatocytes***. Liver from silymarin treated group (c) shows ***near normal histology with mild conjunction of central vein and mildly swollen hepatocytes***. The liver histology of ***animals treated with flax oil (d) and fish oil (e) show completely normal liver architecture without any anatomically detectable anomalies.***

Table1

Serum Levels of Liver marker enzymes, bilirubin and lipid profile of control and experimental animals. Group I is healthy control while Group II and III served as negative and positive control respectively. Group IV and V were treated with *500mg/kg b.w. flax oil and fish oil* (p.o.) respectively.

Group	SGOT (U/mL)	SGPT (U/mL)	ALP (U/mL)	BIL (mg/dL)	Lipid Profile				
					TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	TG (mg/dL)
I	132.83±8.51	150.46±4.38	30.17±3.36	0.71±0.19	56.86±8.88	22.32±9.25	18.71±5.05	15.83±3.98	79.17**±8.12
II	191.84^{a**} ±6.90	181.63^{a***}±2.97	53.75^{a***}±3.20	1.59^{a*}±0.08	91.06^{a***}±0.77	6.32^{a***}±1.95	57.20^{a***}±1.75	27.54^{a***}±0.40	137.72^{a***}±0.82
III	131.88^{b**}±14.15	173.15^{a***}±2.32	26.04^{b***}±2.73	1.15±0.20	43.61^{a*,b***}±8.84	9.29^{a***}±2.24	19.16^{b***}±6.05	15.16^{b***}±4.69	75.82^{b***}±9.58
IV	146.60^{b*}±11.96	177.56^{a***}±0.66	42.61^{c**}±1.55	1.23±0.32	54.51 ^{b***}±3.85	11.79^{a**}±4.04	16.67 ^{b***}±6.93	26.06^{a**,c***}±3.31	130.28^{a**,c***}±6.77
V	100.83^{b***}±3.77	168.13$^{a**, b*}$±2.75	41.90^{c**}±3.88	0.78±0.06	67.47 ^{b,c***}±6.11	15.14^{b*}±2.21	25.69 ^{b***}±9.49	26.64^{a**,c***}±5.94	133.22$^{a**, c***}$±12.14

ANOVA Table

SV	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms
T	26276	6568.9	3524.2	881.1	2910.3	727.6	3.108	0.8	7756.6	1939.2	907.64	226.9	6898.5	1724.6	919.19	229.8	22980	5744.9
R	14328	573.1	1244.3	49.8	1393.6	55.7	5.751	0.2	1049.1	41.9	578.40	23.1	1653.8	66.2	422.13	16.9	10553	422.1
Total	40604	NA	4768.5	NA	4303.9	NA	8.859	NA	8805.7	NA	1486.0	NA	8552.3	NA	1341.3	NA	33533	NA
F	11.462		17.701		13.051		3.378		46.210		9.808		26.070		13.609		13.609	

*Values are a mean of three replicates and expressed as Mean ± Standard Error. The values in a column are marked significant as a(Significant against Healthy Control), b (Significant against Negative Control), and c (Significant against Positive Control) by Tukey-Kramer Multiple Comparisons Test. *P≤0.05; **P≤0.01; *** P≤0.001; Degrees of freedom: Treatments=4; Residuals=25; Total=29; SV: Source of Variation; T: Treatments; R:Residuals; F: F statistic;* SGOT: *serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; BIL: Total bilirubin; TC: Total Cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides; ANOVA: Analysis of Variance; Ss: Sum of Squares; Ms: Mean Square.*

Table2

Oxidative stress markers and and lipid profile of control and experimental animals. Group I is healthy control while Group II and III served as negative and positive control respectively. Group IV and V were treated with _500mg/kg b.w. flax oil and fish oil_ (p.o.) respectively.

Group	SOD (U/mg)	Total Protein (mg/g Tissue)	CAT (mU/g tissue)	µM MDA	µM GSH	Lipid Profile				
						TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	TG (mg/dL)
I	68.56 ^{a***}±1.06	59.58±1.23	0.84±0.01	11.74±0.50	0.88±0.04	23.56±0.73	15.64 ±0.31	4.54±1.03	3.37±0.55	16.87±2.73
II	42.10 ^{a***}±3.10	37.58^{a***}±1.16	0.38 ^{a***}±0.03	22.10 ^{a***}±0.67	0.33 ^{a**}±0.10	26.09±0.83	8.21^{a***}±0.26	10.78^{a***}±0.95	7.12 ^{a**}±1.04	35.58^{a**}±5.18
III	20.22 ^{a,b***}±0.66	56.91^{b***}±0.85	0.69 ^{a,b***}±0.03	17.09 ^{a,b***}±0.43	0.61±0.13	18.90^{a,b***}±0.33	11.61 ^{a,b***}±0.18	1.54 ^{b***}±0.54	5.74±0.58	28.71±2.88
IV	50.71 ^{a,b,c***}±1.53	53.19$^{a*, b***}$±1.23	0.90 ^{b,c***}±0.02	7.69 ^{a,b,c***}±0.71	0.81 ^{b*}±0.01	16.67 ^{a,b***}±0.64	11.16 ^{a,b***}±0.57	2.41 ^{b***}±0.50	3.10 ^{b**}±0.62	15.48 ^{b**}±3.10
V	68.55 ^{a,b,c***}±0.09	54.25 ^{b***}±1.81	1.38 ^{a,b,c***}±0.05	3.40 ^{a,b,c***}±0.26	0.86 ^{b**}±0.13	17.55 ^{a,b***}±0.42	9.74 ^{a***,c*}±0.44	5.07 ^{b***,c*}±0.73	2.74 ^{b**,c*}±0.52	13.68 ^{b**,c*}±2.60

ANOVA Table

SV	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms	Ss	Ms
T	13771	3442.8	1772.2	443.1	3.185	0.8	1317.4	329.3	1.290	0.3	399.96	99.9	185.76	46.4	313.01	78.2	88.2	22.05	2205.0	551.2
R	405.96	16.2	250.51	10.0	0.05452	0.01	43.465	1.7	1.373	0.1	57.323	2.2	21.604	0.8	91.370	3.6	70.7	2.829	1768.4	70.7
Total	14177	NA	2022.7	NA	3.240	NA	1360.9	NA	2.633	NA	457.29	NA	207.36	NA	404.38	NA	158.9	NA	3973.4	
F	212.02		44.215		365.17		189.44		5.875		43.608		53.739		21.411		7.793		7.793	

*Values are a mean of three replicates and expressed as Mean ± Standard Error. The values in a column are marked significant as a(Significant against HC), b (Significant against N), and c (Significant against PC) by Tukey-Kramer Multiple Comparisons Test. *P≤0.05; **P≤0.01*; *** P≤0.001; Degrees of freedom: Treatments=4; Residuals=25; Total=29*; *SV: Source of Variation; T: Treatments; R:Residuals; F: F statistic; SOD*: *Superoxide dismutase*; *CAT*: *Catalase*; *MDA*: *Malondialdehyde*; *GSH*: *Reduced Glutathione*; *TC: Total Cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Triglycerides;* ANOVA: Analysis of Variance; *Ss: Sum of Squares; Ms: Mean Square*.

DISCUSSION

Acetaminophen or paracetamol is one of the well known safe drugs when administered in therapeutic dosage. The overdose of acetaminophen is known to cause a potentially fatal, centrilobular hepatic necrosis[17]. Acetaminophen is metabolized through different pathways in liver which include sulfation, glucuronidation and P450 activation[18]. Acetaminophen is metabolically activated by cytochrome P450 enzymes and converted into highly reactive metabolite called N-acetyl-p-benzoquinone imine (NAPQI). The toxic effects of NAPQI are nullified by binding to a non-protein sulfhydryl tripeptide reduced glutathione (GSH), which is metabolically important endogenous antioxidant in liver. The overdose of acetaminophen leads to rapid utilization and decrease in the contents of GSH and consequently, accumulation of toxic NAPQI which exerts its effects mainly through oxidation and protein binding[19]. In addition, significant increase in oxidative stress is also considered as one of the potential reasons behind acetaminophen induced hepatotoxicity since the decrease in the contents of GSH leads to decrease in the efficiency of the major peroxide detoxification enzyme, glutathione peroxidase, in liver[20]. This oxidative stress may lead to oxidation of polyunsaturated fatty acids present in biomembranes, thereby the destruction of structural and functional organization of these membranes. Serum bilirubin estimation helps in assessing the liver function. Bilirubin is a yellow pigment produced during heme metabolism[21]. The type of liver injury is determined by measuring the presence of hepatocellular enzymes in liver like SGOT, SGPT and ALP. The increased levels of these enzymes indicate mitochondrial damage and cell membrane damage[22]. The abnormally high activity of liver enzymes (SGOT, SGPT and ALP) and increased bilirubin levels observed in the present study are indicative of acetaminophen induced liver injury which is quite apparent from the histological analysis also. Omega 3-fatty acids have been shown to have beneficial effects against a number of different pathologies[23]. Fish oil and flax oil are the rich sources of omega 3-fatty acids and are consequently potent antioxidants. Dietary supplementation of fish oil is known to ameliorate hepatotoxicity due to cisplatin[24]. The fish (cod liver) oil pretreatment of rats has been shown to protect liver against toxicity due to acetaminophen[25]. In addition to fish oil as a source of omega 3-fatty acids, raw and baked flaxseed products have been shown to induce hypolipidemic, hypoglycemic and hypocholesterolemic effects which may be attributed mainly to seed oil which is rich in ALA[26,27,28].

CONCLUSIONS

The present study clearly demonstrates the ameliorating effects of fish oil and flax oil on hepatic injury caused by repeated subacute acetaminophen dosing in rats. The protective effects of fish oil and flax oil are mainly attributed to long chain polyunsaturated fatty acids like ALA, EPA and DHA. These fatty acids have been shown to have antioxidant properties and play an important role in overall metabolism. The supplement of these oils may counter the liver toxicity at biochemical level as well as at cellular levels by participating in the repair and regeneration of altered membrane structures due to increased oxidative stress. It is important to note here that, repeated acetaminophen dosing has been shown to cause physiological adaptation to the drug overdose in rats[29] and hence the experiments should be designed with the dosage most unlikely to lead to physiological adaptation, which otherwise may be mistaken as hepatoprotective activity of the drug or supplement under consideration[30].The dietary supplements of these oils may prove to be beneficial, especially in clinical cases where the prescribed drugs are known to be hepatotoxic on long term use.

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