

Exacerbation of Alcohol-Induced Oxidative Stress in Rats by Polyunsaturated Fatty Acids and Iron Load

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Patere, *et al.*: PUFA and iron load in alcohol-induced stress

The hypothesis that excessive intake of vegetable oil containing polyunsaturated fatty acids and iron load precipitate alcohol-induced liver damage was investigated in a rat model. In order to elucidate the mechanism underlying this synergism, the serum levels of iron, total protein, serum glutamate pyruvate transaminase, liver thiobarbituric acid reactive substances, and activities of antioxidant enzymes superoxide dismutase, catalase in liver of rats treated with alcohol, polyunsaturated fatty acids and iron *per se* and in combination were examined. Alcohol was fed to the rats at a level of 10-30% (blood alcohol was maintained between 150-350 mg/dl by using head space gas chromatography), polyunsaturated fatty acids at a level of 15% of diet and carbonyl iron 1.5-2% of diet *per se* and in combination to different groups for 30 days. Hepatotoxicity was assessed by measuring serum glutamate pyruvate transaminase, which was elevated and serum total protein, which was decreased significantly in rats fed with a combination of alcohol, polyunsaturated fatty acids and iron. It was also associated with increased lipid peroxidation and disruption of antioxidant defense in combination fed rats as compared to rats fed with alcohol or polyunsaturated fatty acids or iron. The present study revealed significant exacerbation of the alcohol-induced oxidative stress in presence of polyunsaturated fatty acids and iron.

Key words: Alcohol, alcoholic liver diseases, cytochrome P450, iron, polyunsaturated fatty acids

Liver cirrhosis is one of the major causes of liver cancer accounting for mortality in western countries^[1]. The great susceptibility of the liver to injury by chemical agents appears to be a consequence of the anatomical position of this organ and central role it plays in the metabolism and disposition of foreign chemicals. Drug-induced liver injury presents a root for liver damage, which raises a major health problem and concern to health care professionals and pharmaceutical industry^[2]. The increased production of reactive oxygen species (ROS) followed by decline in antioxidant activity of the cells results in oxidative stress, which further deregulates the cellular functions^[3]. Liver disease induced by alcohol is a complex multistep chronic disease which typically progresses through stages of alcoholic steatosis (AS), alcoholic hepatitis (AH), alcoholic cirrhosis (AC) to end-stage liver disease^[4]. The recent development in molecular pharmacology reveals that oxidative stress plays a major role in the pathophysiology of numerous diseases. Further alcohol and various food additives have been shown

to initiate and/or exacerbate this phenomenon^[5]. According to WHO 2007 report, globally alcohol causes 1.8 million deaths annually and accounts for 4.0% of disease burden.

Alcohol is soluble in water and fat due to which it can permeate through all tissues and can affect all the organs of the body^[6]. Major pathways for alcohol metabolism in the liver are alcohol dehydrogenase in the cytosol, microsomal ethanol oxidizing system in the endoplasmic reticulum, and aldehyde oxidase in the mitochondria. This results in the generation of free radicals or reactive oxygen species like superoxide, hydroxyl radical, and hydrogen peroxide, which plays a major role in liver damage^[7].

Iron is present in many food products, such as iron enriched grain, cereal products, bread, meat, fish, poultry, vegetables and beans, multivitamins, mineral supplements including those recommended for patients with anemia and in pregnancy. Iron from these products contributes to oxidative stress by acting as catalyst in generating free radicals like superoxide, hydroxyl, ferryl or perferryl species by Fenton reaction^[8]. Diets with high unsaturated

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fats are encouraged today all over the world for its hypocholesterolemic effect^[9]. Thermal oxidation of fats modify its nutritional properties especially when it is rich in polyunsaturated fatty acids (PUFA) which exacerbate alcoholic liver injury by accentuating oxidative stress^[10,11].

Increased consumption of alcohol and diet with higher levels of unsaturated fatty acids and iron via dietary supplements can aggravate alcohol based liver disease. Hence the influence and the pathological consequence of PUFA diet and iron load on the extent and nature of liver damage by alcohol was studied in detail in experimental rodents.

MATERIALS AND METHODS

Epinephrine was purchased from Sigma Aldrich. Absolute Alcohol (AR) was purchased from E-Merk. Carbonyl Iron was obtained as a gift sample from Anshul agencies (ISP corp.), Mumbai, India. Sunflower oil (commercially available) was subjected to two frying cycles of 30 min each at 180° to produce thermally oxidized oil. All other chemicals and reagents used were of analytical grade.

Experimental animals:

Adult male albino Wistar rats (120-150 g), in-house bred at the Animal House of Bombay College of Pharmacy, Mumbai, India were used for the study. Rats were housed in polypropylene cages lined with husk in standard environmental conditions (temperature 25±2°, relative humidity 55±10% and 12:12 light:dark cycle). The rats were fed on a standard pellet diet (Amrut rat and mice feed, Sangli, India) *ad libitum* and had free access to water. The experiments were performed after approval of the protocol by the Institutional Animal Ethics Committee (IAEC) of Bombay College of Pharmacy and were carried out in accordance with the current guidelines for the care of laboratory animals.

Study of hepatic oxidative stress:

Alcohol levels in the blood were first standardized to maintain the levels within the range 150-350 mg/dl to get reproducible results. Rats were divided in three groups consisting of 4 animals each. The concentrations of alcohol for rats of Group I, II, and III were 10%, 15%, 20% (v/v) respectively during initiation of the study i.e. the animals received alcohol containing water in place of normal drinking

water *ad libitum* for 30 days and the alcohol intake volume was recorded daily. All the animals were pair fed and the amount of alcohol fed was initially 8-10 g/kg/day during first week and was gradually increased to 14-16 g/kg/day at the end of the study as tolerance developed. Blood samples were collected from retro-orbital plexus under mild ether anesthesia and were subjected immediately for estimation of alcohol using headspace chromatography. The serum alcohol concentration, which is equivalent to blood alcohol level was estimated using headspace gas chromatography. The group, which maintained the alcohol level between 150-350 mg/dl was selected for the study. The percentage and volume of carbonyl iron and PUFA was also carefully standardized.

After standardizing the model all animals were randomly assigned into five groups of six animals each and received the following treatments: Group I i.e. vehicle control (VC) group received 1.5% of sodium carboxy methylcellulose (sodium CMC) orally once daily. Group II received increasing concentration of alcohol through drinking water (10-30%), Group III received thermally oxidized sunflower oil (15% of diet), Group IV received carbonyl iron (1.5-2% of diet) and Group V received alcohol, thermally oxidized sunflower oil (PUFA) (15%) and carbonyl iron (1.5-2%) (stress control, SC).

Blood samples were collected from retro-orbital plexus under mild ether anaesthesia at specified time intervals to evaluate the maintenance of blood alcohol levels between 150 and 350 mg/dl. Sunflower oil was subjected to two frying cycles of 30 min each at 180° to produce PUFA. This oil (15% of diet) was administered orally once daily to group III. Carbonyl iron (1.5-2% of diet) was suspended in sodium CMC and was administered orally once daily to group IV. Group V (SC) received increasing concentrations (10-30%) of alcohol through drinking water and carbonyl iron was suspended in PUFA (15% of diet) and was administered orally once daily. All groups were treated for 30 days.

Alcohol and food intake were monitored daily throughout the study (Data not shown). Body weights were monitored weekly and blood samples were collected under mild ether anaesthesia weekly after overnight fasting of animals for blood alcohol analysis. At the end of the experimental period, the animals were fasted overnight and blood was

collected under mild ether anaesthesia for various biochemical estimations. The animals were sacrificed by cervical decapitation. Liver was dissected out, part of liver was fixed in 10% formalin saline for histopathological evaluation and part was perfused with ice-cold saline and stored at -70° for estimating liver parameters.

Blood alcohol levels:

Blood alcohol levels (BAL) were determined randomly in animals receiving alcohol in between and at the end of the study to evaluate the maintenance of alcohol levels in between 150-350 mg/dl. Blood samples were collected from the retro orbital plexus of the animals under mild ether anaesthesia and were sealed and stored in cool conditions after collection. The samples were then centrifuged immediately in cooling centrifuge at 4° and the serum samples were analyzed using head space gas chromatography^[12,13].

Biochemical Analysis:

All serum parameters were estimated using commercially available kits. Serum glutamate pyruvate transaminase (SGPT) was estimated using DNPH colorimetric method (Span Diagnostics). Serum total protein was estimated using biuret method (Erba Diagnostics) and serum iron was estimated using liquid FerroZine[®] method (Thermo Electron Corp.). *In vivo* lipid peroxidation in liver, expressed as thiobarbituric acid reactive substances (TBARS) was estimated according to the method of Ohkawa *et al*^[14]. The antioxidant enzyme superoxide dismutase (SOD) in liver was estimated using epinephrine auto oxidation^[15-17] and the estimation of antioxidant enzyme catalase (CAT) was based on the ability of CAT to induce the disappearance of hydrogen peroxide^[18].

Histopathological examination of Liver tissue:

Small pieces of liver tissue were collected in 10% formalin saline for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 microns in thickness were cut and stained with hematoxylin and eosin. Each liver was microscopically examined for distribution of lesions as focal, multifocal, diffuse and no abnormalities detected (NAD). The pattern of liver injury and lesion grading as minimal, moderate and marked were determined in coded slides. The liver sections were also observed for any necrosis/abscesses, leucocyte/lymphocytic infiltration, degenerative changes, structural disruption and any other changes.

Statistical analysis

All values were expressed as mean \pm SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for all *in vivo* parameters. $P<0.01$ was considered statistically significant.

RESULTS

Blood alcohol levels:

The mean BAL was found to be 2.14 ± 0.11 mg/ml (Table 1). The large standard deviations observed in the BAL could be due to cyclical pattern of BAL. Even though BAL was not determined every day in this study, the cyclical pattern of intoxication was obvious from daily observation of the animals given alcohol. The levels were found to be between 150-350 mg/dl and the PUFA and Carbonyl iron diet did not influence the blood alcohol levels.

Serum parameters:

Increase in SGPT levels was observed in group II receiving alcohol *per se* as compared to vehicle control group (Table 2). But SGPT levels were more significantly increased ($P<0.01$) in stress control rats receiving alcohol, PUFA and carbonyl iron in combination with respect to vehicle control, alcohol, PUFA and iron *per se* groups. SC group showed a significant decrease ($P<0.01$) in serum total protein levels at the end of the study as compared to other groups. The decrease was also observed in rats fed alcohol *per se*. In contrast to stress control, no significant decrease was observed in serum protein levels in rats fed PUFA and carbonyl iron *per se*, respectively.

Alcohol fed group showed increase in iron levels whereas no significant increase was observed in PUFA fed group as compared to vehicle control. Stress control and iron group showed significant increase ($P<0.01$) in serum iron levels as compared to vehicle control group. The increase in serum iron levels

TABLE 1: BLOOD ALCOHOL LEVELS

Day	Alcohol conc (mg/ml) [#]
5	1.63 \pm 0.09
10	2.18 \pm 0.23
18	1.97 \pm 0.18
26	2.45 \pm 0.30
30	2.47 \pm 0.21

[#] Values are expressed as mean \pm SEM of four observations.

TABLE 2: SERUM PARAMETERS OF DIFFERENT EXPERIMENTAL GROUPS

Experimental group	SGPT levels (IU/L) [#]	Total proteins (gm/dl) [#]	Iron (µg/dl) [#]
Vehicle control (VC)	23.66±2.38	7.09±0.16	64.21±3.21
Alcohol	42.66±4.21	5.76±0.38	90.26±6.42
PUFA	26.33±2.02	6.78±0.23	61.75±3.78
Iron	35.00±3.56	6.37±0.29	113.58±5.13 ^a
Stress control (SC)	70.66±6.56 ^{a,b}	4.16±0.26 ^{a,b}	153.75±5.99 ^{a,b}

[#]Values are expressed as mean±SEM of six observations, ^aP<0.01 when compared to corresponding values of vehicle control, ^bP<0.01 when compared to corresponding values of alcohol, PUFA and iron *per se*, PUFA: polyunsaturated fatty acid diet, Stress control (SC): alcohol+PUFA+carbonyl iron.

TABLE 3: LIVER PARAMETERS OF DIFFERENT EXPERIMENTAL GROUPS

Experimental group	MDA conc. (µM/g of wet tissue) [#]	Superoxide dismutase (SOD) (U/mg of protein) [#]	Catalase (CAT) (U/mg of protein) [#]
Vehicle control (VC)	0.53±0.01	7.07±0.17	116.70±3.05
Alcohol	0.81±0.06	6.23±0.24	98.01±4.45
PUFA	0.64±0.03	6.89±0.09	109.30±3.09
Iron	0.77±0.05	6.58±0.14	102.45±3.04
Stress control (SC)	1.40±0.08 ^a	4.24±0.37 ^a	68.43±4.09 ^a

[#]Values are expressed as mean±SEM of six observations, ^aP< 0.01 when compared to corresponding values of vehicle control, alcohol, PUFA and iron *per se*, SOD: one unit of SOD activity is the amount of enzyme required to give 50% inhibition of epinephrine auto oxidation, CAT: one unit activity is the mM of H₂O₂ decomposed/min/mg of protein.

TABLE 4: BODY WEIGHTS OF RATS OF EXPERIMENTAL GROUPS

Experimental group	Body weight (g) [#]				
	Day 1	Day 7	Day 14	Day 21	Day 28
Vehicle control (VC)	137.33±3.15	150.00±2.92	163.16±3.15	175.33 ±3.76	188.50±3.31
Alcohol	134.50±3.42	148.00±3.65	161.00±3.55	173.83±4.36	186.50±4.04
PUFA	136.00±3.07	149.50±3.72	164.16±4.26	177.33±4.04	190.33±4.08
Iron	133.00±3.18	139.16±3.13	152.00±3.29	164.00±2.92	173.5±2.74
Stress control (SC)	135.33±3.59	140.00±4.79	139.83±3.48 ^a	139.66±3.73 ^a	135.16±4.12 ^a

[#]Values are expressed as mean±SEM of six observations, ^aP<0.01 when compared to corresponding values of vehicle control.

TABLE 5: LIVER WEIGHT TO BODY WEIGHT RATIO OF RATS OF DIFFERENT EXPERIMENTAL GROUPS

Experimental group	Liver weight: Body weight [#]
Vehicle control (VC)	0.033±0.12
Alcohol	0.036±0.11
PUFA	0.033±0.10
Iron	0.035±0.20
Stress control (SC)	0.045±0.17 ^a

[#]Values are as mean±SEM of six observations, ^aP<0.01 when compared to corresponding values of vehicle control.

of stress control group was higher as compared to alcohol, PUFA and iron *per se* fed groups.

Liver parameters:

There was a significant elevation ($P<0.01$) in the levels of liver TBARS in stress control group as compared to vehicle control. Alcohol and iron *per se* fed rats also showed increase in TBARS levels. There was a significant decrease ($P<0.01$) in SOD and CAT activities of stress control group as compared to vehicle control group (Table 3). The decrease in CAT activity was also observed in alcohol *per se* fed rats.

Body weight and liver to body weight ratio of different treated groups:

Vehicle control rats showed steady increase in their body weight while alcohol, PUFA and iron in combination produced significant loss in body weight as compared to vehicle control rats during the study. No statistical difference in body weight was observed in the alcohol, PUFA and iron *per se* fed rats and vehicle control rats during the duration of the experiment (Table 4). There was significant increase ($P<0.01$) in liver to body weight ratio (LW:BW) of SC rats as compared to vehicle control (Table 5).

Histopathological examination of liver:

Histopathological examinations of liver sections from different groups are shown in the fig. 1. Vehicle control rats were within normal limits, the hepatocytes appeared normal. Minimal to mild fatty change, predominantly microvesicular and centrilobular in type was observed in alcohol *per se* group. Alcoholic hepatitis and hepatic fibrosis were not seen. Iron fed rats showed mild to moderate diffuse granular degeneration. Minimal diffuse granular

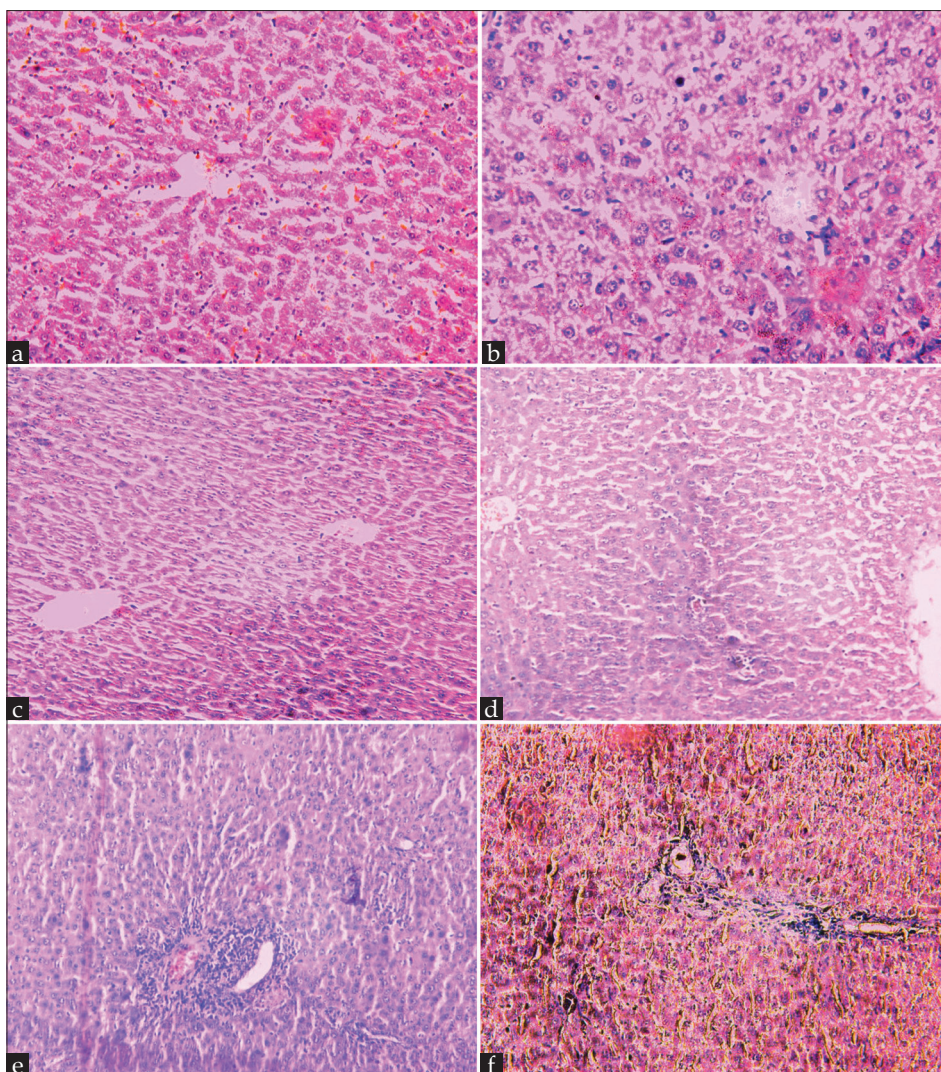


Fig. 1: Histopathological examination of different liver sections of different treated groups

(a) Vehicle control liver section with no abnormalities; (b) Alcohol fed liver section of rat showing mild to moderate diffuse microvesicular fatty change and degeneration; (c) Liver section of PUFA fed rat with minimal diffuse granular degeneration; (d) Liver section of iron fed rat with mild to moderate diffuse granular degeneration; (e and f) Liver sections of stress control rats. The liver section in (e) shows macrovesicular fatty change and mild periportal multifocal lymphocytic/leucocytic infiltration. (f) Shows necrosis, bile pigmentation and necrotic granulomas (200 \times).

degeneration with no major detectable changes was observed in PUFA fed rats. Extensive hepatic damage with necrosis and bile pigmentation was seen in stress control rats. Fatty change, predominantly macrovesicular centrilobular type was also evident in some liver sections of stress control rats. Mild periportal multifocal lymphocytic/leucocytic infiltration was most pronounced in the liver sections of this group of animals.

DISCUSSION

This study was taken up to study the influence of PUFA diet and iron load on alcohol-induced damage in experimental rodents. Rats fed with

alcohol develop fatty changes in liver but they do not develop the more severe forms of liver injury seen in humans like hepatitis and cirrhosis possibly due to short life span^[19]. Alcohol intake level does not always correlate with the severity of alcoholic liver disease, and it is therefore important to know whether certain environmental or genetic factors influence the severity of disease. Histochemical stains from liver of patients with alcoholic liver disease (ALD) revealed increase iron due to which researchers believe that this increase in hepatic iron contributes significantly to the evolution of disease^[20].

However, a study in rats of the interaction of iron and alcohol did not demonstrate significant hepatic fibrosis

after 4 weeks of alcohol feeding. Previous work with iron-loaded and alcohol fed rats did not result in significant fibrosis over a 26 week feeding protocol^[21]. Research has revealed that iron and PUFA with alcohol can increase the severity of hepatotoxicity^[22]. It has been reported that rats, unlike humans, required a high PUFA diet in addition to alcohol to obtain a reproducible level of liver injury^[23,24]. Hence in the current study potential interaction of iron and PUFA in alcohol induced hepatic damage was examined.

Metabolism of alcohol follows zero order kinetics; i.e. irrespective of blood concentration, a constant amount is degraded in unit time. Thus the rate of consuming governs whether a person will get drunk^[25]. Due to the development of tolerance after alcohol consumption, it is required to sustain the blood alcohol levels to induce liver damage and to get reproducible results. It must be emphasized that the percentage and volume intake of alcohol, PUFA and iron by the rats were carefully predetermined and standardized. This current study has proven the significant exacerbation of the alcohol induced liver damage in the presence of PUFA and iron.

Alcohol induced liver damage is a well known phenomenon and the obvious sign of injury is the outflow of cellular enzymes into the plasma/serum^[26]. In this study increased serum SGPT levels were observed in alcohol *per se* fed rats. When the rats were co-administered PUFA and iron along with alcohol there was a more significant increase in SGPT levels. Induction of Cytochrome P₄₅₀ 2E1 (CYP450 2E1) due to alcohol could be related to the concentration of PUFA and iron in the diet^[27]. The increased serum levels of SGPT in stress control rats might be due to increased volatile and other non volatile agents, which are produced during the thermal oxidation of oil and due to iron which acts as co-factor to catalyze lipid peroxidation (LP) induced by hepatotoxic compound, such as alcohol^[28].

Total serum protein levels decrease below normal range in different clinical conditions associated with nephrotic syndrome, malnutrition, cirrhosis of liver and other liver diseases in which liver cells are severely damaged^[29]. There was significant decrease in total protein level in alcohol, PUFA and carbonyl iron in combination fed rats but no significant decrease was observed in alcohol, PUFA and carbonyl iron *per se* fed rats. Non-transferrin bound iron is markedly

increased in serum and taken up by the hepatocytes, but it is less efficiently excreted into the bile in liver diseases due to chronic alcohol and iron load^[30]. Increased levels of serum iron were observed in alcohol *per se* fed rats whereas the levels were significantly increased in iron *per se* and alcohol, PUFA and iron in combination fed rats.

Lipid peroxidation of PUFA in membranes has been implicated as a mechanism by which iron and alcohol causes liver damage^[30,31]. It was found that lipid peroxidation (LP) indices (TBARS) were significantly increased in liver of stress control rats. These increased levels of TBARS may be due to increased susceptibility of the liver tissue to the combined effect of toxic metabolites formed from the alcohol, carbonyl iron (CI) and PUFA. Induction of CYP 2E1 by alcohol results in enhanced acetaldehyde production, which impairs the defense system against the oxidative stress^[32]. Results showed decreased activity of antioxidant enzyme CAT in the liver of alcohol *per se* fed rats. The decreased activity of SOD and CAT was more significant in stress control rats. It was observed that the average weight gain by rats during experimental period was significantly reduced in stress control rats. The observed weight reduction might be due to impaired function of the cells and also due to injuries caused to various organs^[33]. The liver to body weight ratio (LW:BW) was significantly increased in stress control rats and was also evident in histopathological evaluation in which necrosis, bile pigmentation and lymphocytic/leucocytic infiltration was observed as compared to the vehicle control, alcohol, PUFA and iron *per se* groups.

PUFA *per se* did not induce hepatotoxicity as all the serum and liver parameters were normal as compared to vehicle control parameters. Increased levels of TBARS were observed in liver homogenates of rats fed iron *per se*, so it can be said that chronic iron overload may injure the liver. Alcohol *per se* showed increased levels of SGPT, iron, liver TBARS and decreased activity of CAT in liver homogenates. Further it was observed that carbonyl iron, PUFA and alcohol in combination intensified liver damage which was observed through dramatic increased levels of SGPT, serum iron, and liver TBARS. The levels of serum proteins and the activities of antioxidant enzymes like SOD and CAT in liver tissue homogenate were also drastically decreased. The weight gain by the rats was decreased and

liver to body weight ratio of the rats was found to be increased at the end of the study. In addition, the extent of damage was clearly evident in the histopathological evaluation of rats.

Thus, this study probed and proved the significant potentiation of liver damage by alcohol, PUFA and iron in combination. Low concentrations of iron and PUFA that do not induce damage by themselves can act as priming or sensitizing factors and precipitate alcohol induced liver damage. This corroborates the hypothesis that iron and PUFA content in diet act as critical modulators of the pathological liver damage induced by alcohol. Additional detail metabolic and epidemiologic studies are needed to better understand the relation between PUFA, iron status and alcoholic liver disease.

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