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Effect of Wheatgrass on Membrane Fatty Acid Composition During Hepatotoxicity Induced by Alcohol and Heated PUFA

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Abstract Alcoholism is a broad term used for problems related to alcohol, medically considered as disease, specifically an addictive illness, abuse, and dependence. It is the major cause of liver disease in western countries. Alcoholic liver disease encompasses the hepatic alterations leading to fatty liver, hepatitis, and fibrosis or cirrhosis. Fried food items prepared with repeatedly heated polyunsaturated fatty acid (PUFA) exacerbate the disturbances induced by alcohol. The use of herbs to treat diseases is almost universal. Wheatgrass (WG) is used as a supplemental nutrition because of its unique curative properties. As it has antioxidant property, it prevents cancer, diabetes, and acts as liver cleanser. The present study was undertaken to evaluate the efficacy of WG on preserving membrane integrity in liver damage induced by alcohol and heated PUFA (ΔPUFA).The rats were divided into four groups. The animals in group 1 served as normal (standard diet), group 2 served as hepatotoxic (alcohol + ΔPUFA), group 3 served as treated (alcohol + ΔPUFA + WG), and group 4 served as WG control. The compositions of membrane fatty acid, total phospholipids, phospholipase A, C (PLA and PLC) were analyzed in liver to evaluate the effects of WG. Changes in fatty acid composition, decrease in phospholipids levels, and increase in PLA, PLC were observed in the diseased group. Restoration effect was seen in WG-treated rats. Histopathological observations were in correlation with the biochemical parameters. From the results obtained, we conclude that WG effectively protects the liver against alcohol and ΔPUFA-induced changes in fatty acid composition and preserves membrane integrity.

Keywords Fatty acid composition · Phospholipids · Hepatotoxicity · Wheatgrass

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Introduction

Alcoholism is generally used to mean compulsive and uncontrolled consumption of alcoholic beverages, which is detrimental to health, personal relationships, and social standing of the drinkers. Chronic consumption of alcohol leads to secretion of inflammatory cytokines, oxidative stress, lipid peroxidation, acetaldehyde toxicity, membrane damage and finally apoptosis (Bruha et al. 2012).

Nowadays, the use of vegetable oils rich with polyunsaturated fatty acid (PUFA) in the diet is rapidly increasing. Traditional cultures always followed mixed pattern of oil usage, and they exhibited excellent health. PUFAs are linked by multiple double bonds, highly unstable, and vulnerable to oxidation. Refined oils represent damaged fats and are detrimental to health. Processed oils are unstable, and oxidation can still occur once these fats are in the body. In addition to the dangers of oxidation, concerns are about the omega-6 content of polyunsaturated oils. Studies suggest that maternal *n*-6 PUFA status during pregnancy influences offspring's adiposity in childhood (Moon et al. 2013), and the omega-6 fatty acids could accelerate the growth of prostate tumor cells (Hughes-Fulford 2005). A recent report explained that lower ratio of omega-6/omega-3 fatty acids is preferable to reduce the risk of many of the chronic diseases of high prevalence in western societies (Simopoulos 2002).

Usually people take alcohol along with fried food items made up of repeatedly heated sunflower oil rich in PUFA. Partially hydrogenated oils are used for preparing snack foods. A report suggests that the plasma lipid peroxidation is elevated five times when oil is repeatedly heated. The difference is attributed to the higher contents of PUFA present in the oil, which are more prone to oxidation. A previous study suggests that it is recommended not to heat cooking oil more than once in view of its possible deleterious effect on health (Jaarin and Kamisah 2012).

Combined alcohol and PUFA ingestion aggravates the effects of alcoholic liver damage. When alcohol fed rodents are maintained on diets containing PUFA, they develop fatty liver, inflammation, and fibrosis. Products of lipid peroxidation that result from the ethanol-induced oxidation of PUFA are believed to stimulate stellate cell proliferation and collagen deposition, leading to fibrosis and the diets containing PUFA may cause an overproduction of these aldehydes, enhancing the damage (Varma et al. 2004; Latha et al. 2010; Aruna and Rukkumani 2006).

In India, more than 70 % of the population use herbal drugs for their health. Using "reverse pharmacological" approach, several institutes carry out basic and clinical researches on the potential health benefits of herbal drugs. There are many successful examples in this direction. Indian medicinal plants, herbal drugs, and herbal health supplements are also rich sources of beneficial compounds including antioxidants and components that can be used as functional foods. Newer approaches and collaboration with research and technology will establish traditional health principles and open door in providing solutions for various illnesses (Vaidya and Devasangayam 2007).

Wheatgrass, a successful plant in the track of evolution, has an amazing ability to concentrate nutrients from the soil. Scientists have established that it has to be cultivated carefully and harvested at the "jointing" stage, when the content of chlorophyll, enzymes, and other nutrients are at their peak. Extracts of wheatgrass have been shown to inhibit ascorbate-Fe^{2+}-induced lipid peroxidation in rat liver mitochondria and have shown higher ORAC values than many natural extracts or vegetables (Shermer 2008). One study highlights the potent antioxidant properties of wheatgrass and proves that supplementation provides better protection against lipid peroxidation by decreasing the oxidative stress (Shyam et al. 2007). Wheatgrass supplementation also shows good improvement in anemic treatments (Sri Jaya and Gayathri 2009) and reduces myelotoxicity (Bar-Sela et al. 2007). Wheatgrass juice is shown to be an effective alternative of blood transfusion, and it is encouraged for terminally ill cancer patients. The present study was designed to analyze the protective role of wheatgrass on membrane integrity during alcohol and ΔPUFA-induced hepatotoxicity (Dey et al. 2006).

Materials and Methods

Animals

Male albino rats, Wistar strain of body weight 140–160 g bred in Central animal House, Pondicherry University, were used in this study. The animals were fed on the standard pellet diet (Hindustan Lever Limited, Mumbai, India). Water was given ad libitum. The standard pellet diet comprised 21 % protein, 5 % lipids, 4 % crude fiber, 8 % ash, 1 % calcium, 0.6 % phosphorus, 3.4 % glucose, 2 % vitamins, and 55 % nitrogen-free extract (carbohydrates). It produces a metabolizable energy of 3,600 K Cal.

The animals were housed in plastic cages under controlled condition of 12-h light/12-h dark cycles, 50 % humidity, and at 30 ± 2 °C. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council for Medical Research, Hyderabad, India and approved by the Institutional Animal Ethical Committee, Pondicherry University.

Materials Used

Absolute ethanol was obtained from Hayman Private Limited, England. Sunflower oil marketed by Gold Winner was purchased from local market, Puducherry, India. Sunflower oil was subjected to heating at 180 °C for 30 min, twice. The oil was analyzed by gas chromatography and found to contain altered fatty acid composition. All chemicals and solvents used were of the highest purity and analytic grade.

Experimental Design

The animals were divided into four groups of six rats each.

Group 1 (normal)	Rats were given standard pellet diet.
Group 2 (alcohol + ΔPUFA)	Rats were given 20 % ethanol orally, using intragastric tube + 15 % heated sunflower oil mixed with standard pellet diet once daily.
Group 3 (alcohol + ΔPUFA + WG)	Rats were given 20 % ethanol orally, using intragastric tube + 15 % heated sunflower oil mixed with standard pellet diet +wheatgrass (75 mg/Kg b. Wt.) orally as a suspension once daily.

Table 1 Levels of phospholipids

S. no.	Groups	Liver mg/100 g tissue
1.	Normal	1,738.802 ± 20.28[a]
2.	Alcohol + ΔPUFA	865.0,193 ± 8.52[b]
3.	Alcohol + ΔPUFA + WG	1,273.602 ± 107.61[c]
4.	WG control	1,725.243 ± 21.86[a]

Values are mean ± SD from six rats in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at $p \leq 0.05$

Group 4 (WG) Rats were given wheatgrass (75 mg/Kg b. Wt.) orally as a suspension once daily.

At the end of the experimental period (45 days), the rats were sacrificed after an overnight fast by cervical dislocation. Liver tissues were removed, cleared off blood, and immediately transferred to ice-cold containers containing 0.9 % NaCl for various estimations.

Biochemical Estimations

For analysis, tissue lipids were extracted according to the method of Folch et al. (1957). Total phospholipids content were estimated by the method of Zilversmit and Davis (1950). The membrane-remodeling enzymes, Phospholipase A, C, were determined by the method of Rimon and Shapiro (1959) and Kleiman and Lands (1968), respectively. The preparation and analysis of fatty acid methyl esters (FAMEs) from these liver tissues were performed according to the method described by Sherlock (2000) and Sahin (2000).The FAMEs were cleaned in anhydrous sodium sulfate and then transferred into GC sample vial for analysis. FAMEs were separated by gas chromatography (HP 6890N, Agilent Technologies, USA). FAMEs profiles of the tissues were identified by comparing the commercial Eucary data base with MIS Software package (MIS Ver. No. 3.8, Microbial ID. Inc., Newark, DE, USA). Histopathological observations were performed by hematoxylin and eosin (H&E) stain.

Statistical Analysis

All the data were analyzed using the SPSS 7.5 -Windows Students version software. Statistical analysis was done by analysis of variance (ANOVA) followed by Tukey's test. $p \leq 0.05$ was considered to be statistically significant.

Results

Total phospholipid levels were reduced in liver of alcohol and heated PUFA group, and increased phospholipids levels nearing control levels were observed on treatment with wheatgrass (Table 1). The membrane-modeling enzymes PLA, PLC showed elevated activity in diseased group and were controlled on treatment with Wheatgrass (Table 2). In membrane fatty acid composition analysis, 16:0,16:1,18:0,18:1,18:2 fatty acids were elevated significantly in the diseased group and controlled on treatment with wheatgrass. 20:4 fatty acid levels were reduced in alcohol and heated PUFA group but raised on treatment with wheatgrass (Table 3).

Table 2 Activities of phospholipases A and C in liver

S. no.	Groups	Phospholipase A Unit*/mg protein	Phospholipase C Unit**/mg protein
1.	Normal	0.721 ± 0.013[a]	0.703 ± 0.092[a]
2.	Alcohol + ΔPUFA	1.613 ± 0.019[b]	1.550 ± 0.046[b]
3.	Alcohol + ΔPUFA + WG	1.101 ± 0.012[c]	1.279 ± 0.087[c]
4.	WG	0.711 ± 0.017[a]	0.731 ± 0.032[a]

Unit*—mEq. of ester hydrolyzed/min

Unit**—mM of phosphorylcholine formed/min

Values are mean ± SD from six rats in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at $p \leq 0.05$

Histopathological Observations

Histology of the liver sections (Fig. 1) of control rats showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus, and visible central veins. The liver sections of alcohol and heated PUFA group rats exhibited intense centrilobular necrosis, vacuolization, micro-vesicular fatty changes and fatty accumulation. Deformation of central vein with the loss of boundaries was also seen. The liver sections of treatment group showed normal hepatic architecture with moderate accumulation of fatty lobules. Normal histology was preserved in wheatgrass control group.

Discussion

Phospholipids are the major structural components of cellular membranes. Chronic alcoholism alters the lipid bilayer and its composition by interacting with phospholipids. Alcohol increases the fluidity of the hepatic membrane, and PUFA changes the PUFA content of the membrane. In our study, the levels of phospholipids were markedly decreased in alcohol and PUFA-fed rats. The main metabolite of ethanol, acetaldehyde, is more liposoluble and produces membrane agitation by increasing the free radical formation, lipid peroxidation, and initiating the

Table 3 Changes in phospholipids' fatty acid composition in liver (% of fatty acid/g tissue)

S. no.	Groups	16:0	16:1	18:0	18:1	18:2	20:4
1.	Normal	23.468 ± 0.49[a]	4.430 ± 0.17[a]	17.565 ± 0.29[a]	4.763 ± 0.15[a]	18.975 ± 0.09[a]	36.143 ± 0.40[a]
2.	Alcohol + ΔPUFA	47.960 ± 0.50[b]	11.503 ± 0.34[b]	22.390 ± 0.50[b]	9.695 ± 0.33[b]	24.593 ± 0.20[b]	6.760 ± 0.17[b]
3.	Alcohol + ΔPUFA + WG	25.935 ± 1.14[c]	6.323 ± 0.46[c]	18.235 ± 0.68[c]	7.068 ± 0.05[c]	18.155 ± 0.26[c]	22.318 ± 0.85[c]
4.	WG	22.783 ± 0.09[a]	5.058 ± 0.09[a]	16.650 ± 0.54[a]	4.075 ± 0.07[a]	15.010 ± 0.14[a]	34.445 ± 0.63[a]

Values are mean ± SD from 6 rats in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at $p \leq 0.05$

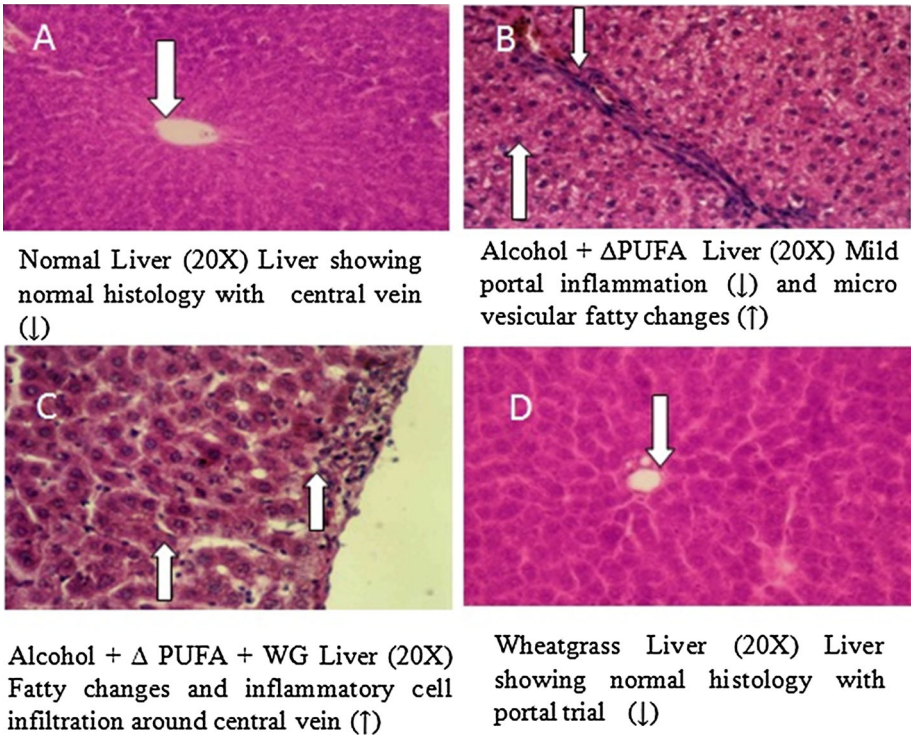

Fig. 1 Histopathology

immunologic reactions resulting in decreased phospholipids (Ingolfsson and Olaf 2011; Zima 1993). Reports have shown that consumption of deep-fried food items prepared with repeatedly heated oil rich in PUFA leads to lipid peroxidation, because of the increased susceptibility of PUFA to oxidation (Jaarin and Kamisah 2012; Kirpich et al. 2012; Aoun et al. 2012). Our findings are in agreement with their study that the repeatedly heated PUFA increased the lipid peroxidation, altered the membrane integrity resulting in the decreased phospholipids content of the membrane. The products of lipid peroxidation are reactive molecules and thus are the potent damagers of cellular molecules (Rukkumani et al. 2004). The peroxidation products are the reason for the chemical susceptibility of individual fatty acids to oxidation which ultimately results in differences in membrane composition (Hulbert 2005). In wheatgrass treatment group, we observed increased phospholipids level. This may be due to the antioxidant property of wheatgrass which might have reduced the free radicals and prevented lipid peroxidation, and membrane damage, and thus preserved the phospholipids content of the membranes (Garima et al. 2012a, b; Sethi et al. 2010).

Phospholipases are a group of enzymes that catalyze phospholipids cleavage. Processing of phospholipids' by these membrane-modeling enzymes converts these molecules into lipid mediators or secondary messengers. In our study, we observed the increased activities of phospholipase A and phospholipase C (PLA, PLC) in alcohol and PUFA-fed group rats.

Previous studies have shown that alcohol modulates PLA activity by increasing intracellular Ca^{2+} ion concentration (Oide et al. 2000). PLA is an important tool in regulation of phospholipids acyl turnover for membrane repair and production of inflammatory mediators. The increase in PLA activity has been suggested to reduce the

proportion of unsaturated acyl composition of selected membrane phospholipids and help in developing resistance to the disordering of ethanol (Kode et al. 2007). An increase in PLA activity after exposure to ethanol has been reported by Aruna et al. (2005a, b). This increased PLA activity results in excessive release of arachidonic acid (AA), which then enters into CYP450, cyclooxygenase, and lipoxygenase pathway and acts as a precursor of the eicosanoid complexes, leukotrienes, thromboxanes, and prostaglandins resulting in the production of various inflammatory mediators during chronic ethanol toxicity, which are known to be associated with ethanol-induced liver injury (Balsinde et al. 2002). Cytochrome P450 exacerbates PLA2- and AA-dependent injury and promotes lipid peroxidation or production of metabolites that alter Ca^{2+} homeostasis (Caro and Cederbaum 2006).

Ca^{2+}-sensitive domain is homologous among PLC isoenzymes, and hence ethanol ingestion increases the PLC activity. PLC normally cleaves phospholipids and produces phosphatidyl inositol 4, 5-bisphosphate, and subsequently, diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3). DAG binds to the membrane, and IP3 diffuses through the cytosol and bind to IP3 receptors, specifically, calcium channels, in the smooth endoplasmic reticulum. This causes the cytosolic intracellular concentration of calcium to increase. Calcium and DAG together activate protein kinase C, which phosphorylates other molecules, and affect a cascade of signaling pathways leading to the repair of the lipid bilayer (Ellis et al. 1998; Aruna et al. 2004). Hence, the increased PLC in alcohol + PUFA group may be a defense mechanism to combat the membrane damage caused by alcohol and PUFA. In our study, wheatgrass treatment decreased the activation of PLA, C. This may be because of the reduction in lipid peroxidation and associated membrane modifications.

Penetration of alcohol into the lipid bilayer results in disordering of the composition of fatty acids in phospholipid bilayer. In our study, the levels of 16:0 (palmitic acid), 16:1 (palmitoleic acid), and 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic) were increased in alcohol and PUFA-fed groups, but 20:4 (Arachidonic acid (AA) was significantly reduced in this group. Ethanol disturbs the fatty acid metabolism by inhibiting 5, 6 delta desaturase activity. Inhibition of desaturases leads to accumulation of 18:2 (linoleic acid) and results in the increased levels of monounsaturated fatty acid (MUFA), such as 16:1 and 18:1. Ethanol blocks the anabolic pathway of arachidonic acid, and moreover AA is diverted for the formation of inflammatory markers during ethanol and PUFA ingestion (Aruna et al. 2005a, b). Blockage in anabolic pathway of AA and utilization for inflammatory markers production may be the reason for the depleted level of AA, observed in alcohol and PUFA-ingested group in our study. The increased PUFA intake increases the degree of unsaturation of the membrane (Rajakrishnan and menon 2002), and as a compensating effect, the levels of saturated fatty acids, like 16:0 (palmitic acid) and 18:0 (stearic acid), are increased for maintaining the fluidity of phospholipids bilayer. Apart from this, interaction of linoleic with nine delta desaturase boosts up the production of palmitic and stearic acids. Hence, we observed the increased levels of palmitic acid and stearic acid in our study as reported in earlier studies (Aruna et al. 2005a, b).

The fatty acid composition was near normal when wheatgrass was given along with alcohol and PUFA. Hepatoprotective effects of plants against ethanol-induced hepatotoxicity have been reported previously by several investigators (Bhawna and Upendra 2009; Gujrati et al. 2007; Pramyothin et al. 2007; Jaishree and Badami 2010). Wheatgrass contains alkaloids, tannins, saponins, and sterols (Garima et al. 2012a, 2012b; Kothari et al. 2008) and is also rich in antioxidants such as polyphenols and flavonoids. Wheatgrass has shown higher free radical scavenging activities, higher elemental content (Kulkarni et al. 2006), and higher oxygen radical absorbance capacity (Lachnicht et al. 2002; Kulkarni et al. 2006). The various phytochemicals present in wheatgrass could have scavenged the free radicals produced during the metabolism of ethanol and PUFA and thus decreased the membrane alteration. Study from our lab also confirmed the presence of phenolics, flavonoids, and other compounds having the reducing activities, such as squalene, phytol, and α, β amyrin to be present in the wheatgrass. Wheatgrass can also interact with acetaldehyde, thereby reducing the latter's toxicity effects on membrane phospholipids. Hence, in our study, wheatgrass maintained the membrane fatty acid's composition by regulating the phospholipases. We conclude that wheatgrass can effectively prevent the oxidative stress-induced changes in the phospholipids' fatty acid composition of the membrane and restore the membrane integrity.

Conclusion

The above study suggests that wheatgrass extract shows direct hepatoprotective effect by inhibiting lipid peroxidation in phospholipids bilayer, controls the activity of PLA, C, and reduces the changes in the fatty acid composition of membrane. Thus, it maintains and preserves the membrane integrity against ethanol and heated PUFA-induced liver toxicity in rats.

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