Effects of Lecithin and Vitamin E Supplementation on Liver Steatosis and Oxidative Stress Induced by Chronic Ethanol Consumption in Rats

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Summary

Oxidative stress and lipid peroxidation, associated with ethanol, are considered important pathogenic mechanisms in the formation of hepatic steatosis. The objective of the present study was to assess the effects of supplementation with lecithin and vitamin E on the oxidatives stress and hepatic steatosis induced in rats by chronic ethanol consumption. Fifty-two Wistar rats were divided into 4 experimental groups: control (AIN-93 diet), ethanol group (control diet plus a 20% hydroalcoholic solution), ethanol + vitamin E group (addition of 0.6% vitamin E to the diet plus a 20% hydroalcoholic solution); ethanol + soy lecithin group (addition of 5 % soy lecithin to the diet plus a 20% hydroalcoholic solution). At the end of 4 weeks the animals were sacrificed. The results showed a significantly smaller number of animals ($p < 0.05$) classified as having a low degree of steatosis in the ethanol + vitamin E group and ethanol + soy lecithin group compared to the ethanol group. In addition, the ethanol + soy lecithin group had a significantly lower concentration of hepatic fat ($p < 0.05$) than the ethanol group. A significant reduction of hepatic TBARS concentration ($p < 0.05$) was detected in the ethanol + vitamin E group compared to the ethanol group. Hepatic carbonyl concentration was significantly lower in the ethanol + soy lecithin group. However, hepatic GSH was significantly lower in the ethanol + vitamin E and ethanol + soy lecithin groups compared to the control group. In conclusion, supplementation with lecithin and vitamin E attenuated the hepatotoxic effects of chronic ethanol intake and contributed to a reduction of the progression of steatosis status.

Introduction

Alcoholic liver disease (ALD) is due to the abusive consumption of alcohol, the major cause of morbidity and mortality associated with liver disease (*Gramenzi et al., 2006*) and one of the greatest health problems in the world (*Singha et al., 2007*). Over the last few years, several studies have clarified important immunological and metabolic consequences of excessive alcohol consumption that may contribute to liver pathogenesis (*Stewart et al., 2001*).

Increased oxidative stress and the modulation of the redox state of the cell are among the most extensively studied consequences of alcohol consumption (*Gyamfi et al., 2008; Ramírez-Farías et al., 2008*). After ingestion, alcohol is metabolized in the liver by two pathways. The first involves its transformation to acetaldehyde by the action of the enzyme alcohol dehydrogenase (ADH) located in the cytosol and is followed by transformation to acetate predominantly due to the action of the

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mitochondrial enzyme aldehyde dehydrogenase (ALDH) (*Stewart et al., 2001*). The formation of aldehydes is accompanied by the formation of free radicals through the microsomal ethanol oxidation system and its key enzyme cytochrome P450 21E. These metabolic processes result in the reduction of NAD to NADH. Increased NADH significantly affects lipid metabolism, stimulating fatty acid synthesis and the concomitant reduction of the beta-oxidation pathway, promoting the accumulation of fat in the liver (steatosis), the initial stage of ALD (*Stewart et al., 2001; Lieber 2004*). The increased oxidative stress can also promote oxidative damage such as lipid peroxidation, playing an important role in the pathogenesis of ethanol-induced liver injury (*Mantena et al., 2007*).

Phosphatidylcholine (PC) is an essential substrate for the synthesis of very low density lipoproteins (VLDL). In the presence of PC deficiency, VLDL synthesis and excretion are compromised, with the possible accumulation of triglycerides in the hepatocytes due to the reduced excretion or oxidation of fatty acids (*Gyamfi et al., 2008*). Ethanol has a similar effect when it reduces the synthesis of PC, causing alteration of methionine metabolism (*Gyamfi et al., 2008*). Thus, some investigators have tested the hypothesis that lecithin (which contains choline) supplementation may attenuate the ethanol-mediated free radical-induced hepatotoxicity and steatosis (*Navder et al., 1997; Das & Vasudevan, 2006; Das et al., 2007*). Vitamin E is also an important antioxidant frequently tested as a free radical scavenger (*Jordão et al. 2004; Ramíres-Farías et al., 2008*). However, little is known about the effects of supplementation with these compounds on the pathogenic state of liver steatosis induced by chronic ethanol ingestion.

The objective of the present study was to assess the hepatic damage caused by chronic ethanol consumption and to assess the effects of soy lecithin and of vitamin E in attenuating the rate of lipoperoxidation and the possible development of alcoholic steatosis.

Materials and Methods
Animals and treatments
The study was conducted on 52 Wistar rats from the Central Animal Facilities of the Faculty of Medicine of Ribeirão Preto, University of São Paulo. The experiment was approved by the Ethics Committee for Animal Experimentation of the same institution. The animals were housed in individual cages on a 12/12 hour light/dark cycle and at a mean temperature of 22 ºC and were divided at random into four groups of 13 rats each: Control (C), Ethanol (Et), Ethanol + vitamin E (EtE), and ethanol + soy lecithin (EtSL).

Ethanol was administered as a 20% hydroalcoholic solution added to the water containers of the animals over a period of four weeks (*Gil-Martin et al., 1998*). The diet (AIN-93) for the control group was prepared as described by Reeves *et al.* (*1993*). Vitamin E supplementation consisted of the addition of 20 times the recommended value to the diet for the E and EtE groups. A 5% portion of soy lecithin was added to the diet for the EtSL group (*Aleinyc & Lieber, 2003*). All animals had free access to food and to the hydro-alcoholic solution. Food intake and body weight were monitored every two days throughout the experimental period.

At the end of the 4-week period the animals were sacrificed by decapitation. Blood was collected and centrifuged and plasma was stored at -70º for later analysis. The liver was removed, weighed, immediately frozen in liquid nitrogen (-196 ºC) and stored for later analysis.

Laboratory methods
Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations and total protein were determined with the Labtest® kit (São Paulo, Brazil).

Lipid peroxidation in the liver was measured by the determination of thiobarbituric acid reactive substances (TBARS) (*Buege & Aust, 1978*) expressed as nmol malondialdehyde (MDA)/mg protein. Carbonyl protein oxidation in the liver was assessed by the methods of Odetti *et al.* (*1996*) and

Levine *et al.* (*1990*). Hepatic reduced glutathione (GSH) concentration was determined by the method of Sedlack & Lindsay (1968). Hepatic vitamin E was determined by the method of Jordão *et al.* (2004) and total hepatic fat was determined by the method of Bligh & Dyer (*1959*).

For histopathological analysis, hepatic tissue fragments were embedded in paraffin, cut into 4-µm thick sections and stained with Harris hematoxylin and eosin for the semi-quantitative determination of hepatic steatosis by the method of Oh *et al.* (*1998*) using a standard (400X) light microscope. The presence of steatosis was classified as follows: I - 0 to 25%, II – 25 to 50%, and III – 50 to 75%.

Statistical analysis
Data are reported as means ± SD. Groups were compared by analysis of variance (ANOVA) followed by the Tukey po*st test*. The number of animals and the grades of steatosis were compared by the Chi-square test for qualitative variables. The level of significance was set at $p \leq 0.05$ for all analyses.

Results
Table 1 lists the general characteristics, biomarkers of oxidative stress and hepatic antioxidants of the groups studied. The weight gain of the C group was significantly greater ($p < 0.05$) compared to the remaining groups, with the EtSL group having a significantly lower weight gain than the Et and EtE groups. The same behaviour was observed for food intake, with C animals ingesting significantly more food than all other groups. Regarding the biomarkers of oxidative stress, hepatic TBARS concentrations were significantly reduced ($p < 0.05$) in the EtE group compared to the Et group. Hepatic carbonyl concentrations were significantly lower in the EtSL group and hepatic GSH concentrations were significantly lower in groups Et, EtE and EtSL compared to group C. Supplementation significantly increased vitamin E concentrations in the EtE group compared to all other groups.

Table 1. General characteristics (body weight gain and food intake), hepatic biomarkers of oxidative stress (TBARS and carbonyls) and antioxidants (reduced glutathione and hepatic vitamin E) (mean ± SD) of the control (C), ethanol (Et), ethanol + supplementation with vitamin E (EtE) and ethanol + supplementation with soy lecithin (EtSL) groups.

	C	Et	EtE	EtSL
Weight gain (g)	244.5±16.7[a]	200.3±27.1[b]	178.9±14.1[b]	154.3±24.2[c]
Food intake (g/day)	23.2±2.1[a]	17.5±6.2[b]	20.5±2.9[a]	14.7±4.6[b]
TBARS (nmol MDA/mg prot)	0.12±0.01[a]	0.12±0.01[a]	0.09±0.01[b]	0.11±0.01[a]
Carbonyls (nmol/mg prot)	5.3±2.4[a]	4.6±2.4[a]	4.3±2.1[a]	2.1±0.8[b]
GSH (nmol/mg prot)	41.1±11.9[a]	31.9±8.3[b]	28.6±5.8[b]	27.5±7.3[b]
Liver vitamin E (µmol/g tissue)	83.9±21.4[a]	106.9±55.9[a]	837.5±438.7[b]	59.4±21.2[a]

[a,b,c] Mean values with different superscript letters were significantly different ($P < 0.05$ by ANOVA followed by Tukey post-test). TBARS: Thiobarbituric Acid Reactive Substances; MDA: Malondialdehyde; GSH: reduced glutathione.

Figure 1 presents the plasma levels of AST and ALT detected in the various groups. Although all ethanol-treated groups presented an increase in AST concentration, no significant differences were found between them. Also, no significant differences were found in plasma ALT concentrations.
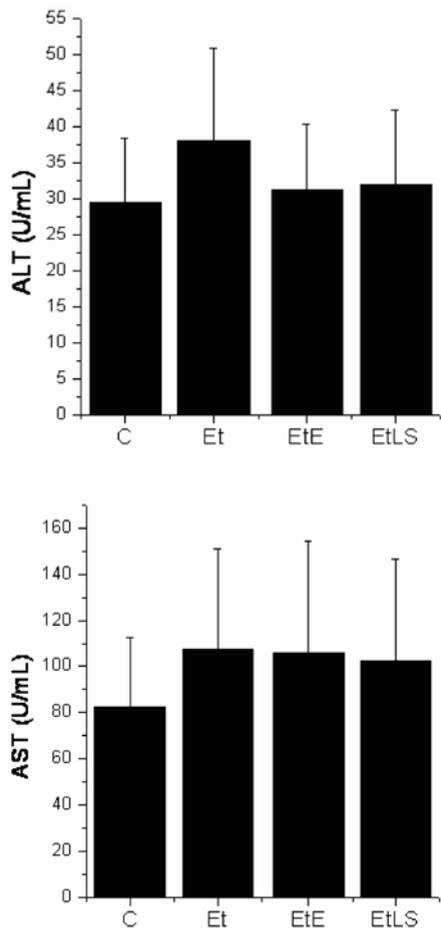
Figure 1. Plasma alanine aminotransferase (ALT) and alkaline phosphatase (AST) activity in control animals (C) and animals of the ethanol (Et), supplementation with vitamin E (E), ethanol + supplementation with vitamin E (EtE), supplementation with soy lecithin (LS), and ethanol + supplementation with soy lecithin (EtSL) groups. Data are reported as means ± SD.

The number of animals classified as having grade II steatosis was significantly lower ($p < 0.05$) in the EtE and EtSL groups compared to the Et group (Table 2). Also, the EtSL group had a significantly lower concentration of hepatic fat ($p < 0.05$) than the Et group.

Discussion
Oxidative stress is a condition in which the cellular production of pro-oxidants exceeds the physiological ability to remove this activity by the endogenous antioxidant system together with the exogenous antioxidants obtained from the diet (*Zhong & Lemasters, 2004*). Oxidative stress and lipid peroxidation are considered to be important pathogenic mechanisms in the formation of hepatic steatosis (*Gyamfi et al., 2008*) and the possible protective action of vitamin E against the progression of this pathogenesis may be due to its action as a free radical scavenger. For this reason, vitamin E supplementation has been studied by several investigators as a form of prevention and treatment of hepatotoxicity induced by ethanol consumption (*Jordão et al., 2004; Yanardag et al., 2007; Ramírez-Farías et al., 2008; Das et al., 2007*). Jordão *et al.* (*2004*) demonstrated that vitamin E deficiency alone induces lipid peroxidation, the acute administration of ethanol affects vitamin E levels and the maintenance of adequate or higher vitamin E levels acts as a protective factor against free radical generation. Yanardag *et al.* (*2007*) also detected a reduction of the biomarkers of lipid peroxidation in the liver and intestine of rats induced by acute ethanol intake after supplementation with vitamin E and vitamin C. In a study of the effect of supplementation with antioxidant vitamins (vitamins A, C and E) during seven days of ethanol intake, Ramírez-Farías *et al.* (*2008*) demonstrated a significant action of vitamin E in removing free radicals and reducing damage to the cell membrane, with a consequent reduction of indicators of liver damage such as AST and ALT. These authors concluded that vitamin E supplementation appears to be more effective than supplementation with vitamin

Table 2. Steaotohepatitis and total hepatic fat of control animals (C), of animals of the ethanol group (Et), ethanol + supplementation with vitamin E group (EtE) and ethanol + supplementation with soy lecithin group (EtSL).

| Groups | Degrees of steatosis | | | Hepatic fat (mg/g tissue) |
	I	II	III	
C	8[a]	5[a]	-	123.4 ± 27.8
Et	6[a]	7[a]	-	141.4 ± 28.3[#]
EtE	10[b]	1[b]	2	139.6 ± 44.7
EtSL	12[b]	1[b]	-	106.9 ± 17.9[#]

Values are expressed as means ± SD. I - 0-25% presence of steatosis; II - 25-50% presence of steatosis; III - 50-75% presence of steatosis. [ab] Mean values within a column for groups with different letters were significantly different ($P < 0.05$, chi-square test by). [#] Mean values followed by this symbol were significantly different ($P < 0.05$ by ANOVA followed by the Tukey post-test).

A in attenuating lipid peroxidation and protecting the liver against the dysfunction and damage caused by alcohol consumption. In the present study, vitamin E supplementation proved to be effective in reducing lipid peroxidation (TBARS) induced by chronic ethanol consumption over a period of 4 weeks, an effect that was not observed regarding the biomarkers of protein oxidation (carbonyls). It is interesting to note that the opposite effects were observed in the EtSL group, in which lecithin supplementation had no significant protective effect against TBARS formation, in contrast to the significant effect against carbonyl formation (Table 1). These results demonstrate a selective action of these two compounds in attenuating oxidative stress and the progression of hepatic pathogenesis.

Increased free radical formation in the liver causes disorders of lipid metabolism. Thus, steatosis is an important manifestation of alcohol abuse in the liver, characterized by lipid accumulation inside the hepatocytes and considered to be the initial stage of progression of fibrosis. The cause of hepatic steatosis is a disequilibrium between lipid synthesis and lipid removal and/or consumption (*Zhong & Lemasters, 2004*). PC, a precursor of methionine and choline metabolism, is an essential substrate for VLDL synthesis. When these substrates are deficient, VLDL production and/or excretion may be compromised, with a consequent accumulation of triglycerides in the hepatocytes and a reduction of fatty acid export (*Innis et al., 2007; Gyamfi et al., 2008*). Ethanol intake reduces PC synthesis and alters the metabolism of methionine, causing disorders of fat metabolism. Also, the accumulation of fatty acids in the hepatocytes may damage cell membranes and promote oxidative stress (*Gyamfi et al., 2008*). For this reason, the hypothesis has been proposed that supplementation with PC or its precursors may reduce the accumulation of fat in the liver and attenuate its pathogenic effects (*Navder et al., 1997; Das & Vasudevan, 2006a; Das et al., 2007*). Navder *et al*. (*1997*) detected positive effects of supplementation with soy-derived PC in rats that ingested alcohol for 3 weeks. These authors reported

that PC attenuates recent manifestations of alcohol toxicity and that this attenuation is associated with increased fatty acid oxidation and improvement of mitochondrial injury. These authors also concluded that the beneficial effects of PC supplementation in the initial stage of alcoholic liver injury can prevent or reduce the progression of steatohepatitis and/or cirrhosis of the liver. Das & Vasudevan, (*2006b*), in a study on lecithin as a form of treatment or prevention of liver damage caused by chronic ethanol intake for 4 weeks, concluded that lecithin administered in combination with the vitamin-B complex has promising effects both in terms of the treatment and prevention of hepatotoxicity. However, Das & Vasudevan (*2006a*), even though they detected beneficial effects of lecithin supplementation (500 mg lecithin/kg weight/day) in rats subjected to chronic ethanol intake for 4 weeks, concluded that treatment with tocopherol acetate yielded more satisfactory results than lecithin. These investigators also reported that antioxidants are promising therapeutic agents since they combat oxidant agents considered to represent primary mechanisms of hepatotoxicity. Das *et al*. (*2007*) studied the efficacy of supplementation with vitamin E, lecithin alone and lecithin plus the vitamin-B complex in the treatment of rats subjected to 12 weeks of ethanol intake. These investigators demonstrated that vitamin E and lecithin in combination with the vitamin-B complex were more effective in reducing levels of the plasma transaminase enzymes ALT and AST, interleukin (IL)-10, gamma interferon (INF-gamma) and the concentration of TBARS, in addition to increasing GSH concentration and superoxide dismutase (SOD) activity compared to the use of lecithin alone. These authors also concluded that the administration of vitamin E and lecithin in combination with the vitamin-B complex is a promising therapeutic conduct for the control of immunomodulatory activities involved in liver damage processes. In the present study, soy lecithin (a natural compound of the phospholipid class containing choline) was used because of its possible lipotrophic effect (*Das & Vasudevan, 2006a*). One of the parameters used to assess steatosis was histopathological analysis of the liver. This parameter revealed a significantly reduced number of animals classified as having a low degree of hepatic steatosis (grade II) in the EtSL and EtE groups compared to the Et group (Table 2). In addition, hepatic fat concentration was significantly lower in the EtSL group compared to the Et group (Table 2). Thus, in addition to demonstrating the selective ability of attenuating hepatotoxicity by lecithin and vitamin E supplementation, the present study demonstrated an effective protective action of lecithin and vitamin E in preventing the progression of fat accumulation in the liver and the consequent steatosis status.

Reduced glutathione (GSH) is an important antioxidant and the most effective one in the liver. In addition to acting against lipid peroxidation, GSH has other protective activities such as regeneration of oxidized vitamin E and detoxification of reactive aldehydes such as MDA generated during lipid peroxidation (*Villanueva et al., 2006*). GSH depletion is considered to be an important mechanism altering the metabolism of methionine (*Das & Vasudevan, 2006a; Villanueva et al., 2006*). In the liver, methionine metabolism and the antioxidant GSH are linked by the transsulfuration process, which converts methionine to cysteine, the GSH precursor amino acid (*Prudova et al., 2006*). In the present study, the reduction in GSH concentration in the Et group compared to C seems to demonstrate this sensitivity. However, in contrast to some previous reports (*Gyamfi & Wan, 2006; Villanueva et al., 2006; Yanardag et al., 2007; Das et al., 2007*), in the present study lecithin and vitamin E supplementation did not prevent the ethanol-induced reduction of GSH. Several authors have demonstrated that transsulfuration and remethylation processes are regulated according to the availability of methionine, which is required for several transmethylation reactions (*Stipanuk, 2004*). Thus, lecithin supplementation may increase the bioavailability of choline and betaine and modulate the S-adenosylmethionine/S-adenisylhomocyscine (SAM/SAH) ratio, possibly affecting GSH synthesis,

especially when GSH requirements increase since this compound is quite sensitive to situations of toxicity (*Stipanuk, 2004; Villanueva et al., 2006*).

ALT is localized in the liver and its levels in plasma increase in situations of hepatic alteration. The plasma levels of AST, another enzyme localized in the hepatocytes and used together with ALT for the assessment of hepatic diseases, increase during alcohol intake and in the presence of acute liver injuries. Plasma transaminase values are altered in the presence of hepatocyte rupture. Although in the present study AST values were elevated in all groups treated with ethanol, no statistically significant difference was detected between the groups studied. These transaminases are good biomarkers of acute injury in which their values are elevated, but chronic ethanol consumption may induce adaptations in the level of these enzymes, whose values again become equilibrated (*Bonnefont-Rousselot et al., 1998*).

In conclusion, supplementation with soy lecithin reduced the degree of steatosis and the hepatic fat induced by chronic ethanol intake. Vitamin E prevented the formation of lipid peroxidation, an important mechanism in the progression of hepatic pathogenesis, even though it did not prevent the ethanol-induced GSH reduction. These data suggest that lecithin and vitamin E can attenuate the hepatotoxic effects of ethanol in their initial stage and act against the progression of steatosis. Further studies are needed in order to determine the effects of combined supplementation with lecithin and vitamin E for the prevention and treatment of the pathogenic effects induced by chronic alcohol consumption in the liver.

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