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Liver Necrosis Induced by Acute Intraperitoneal Ethanol Administration in Aged Rats

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It is generally agreed that the deleterious pathophysiological effects of ethanol are caused, at least partially by an increase in free radical production. However, little attention has been directed to the effects of ethanol upon elderly organisms. Male Wistar rats at ages 3, 6, 12, 18 and 24 months were treated either with a single i.p. dose of 35% ethanol (v/v) at 3 g ethanol/kg body weight or an isovolumetric amount of 0.9% saline solution. We then assessed the plasma levels of transaminases and hepatic levels of oxidative stress-related parameters, followed by liver histological evaluation. The younger rats (3 months old) were not affected by the treatment with ethanol with respect to any of the studied parameters except for a lowering of total hepatic GSH and an increase in hepatic thiobarbituric acid reactants (TBARS) formation, while animals older than 3 months were increasingly more affected by the treatment. Acute ethanol treatment elicited the similar responses to those in the 3 months-old group, plus a decrease in the hepatic and plasma levels of β-carotene and the plasma level of α-tocopherol, as well as an increase in the activity of plasma transaminases. In the 12, 18 and 24 months old groups, there was increasing liver necrosis. These findings suggest that liver damage induced by acute ethanol administration in elderly rats may involve a lack of antioxidants.

Keywords: Aging; Alcoholism; Liver injury; Oxidative stress

INTRODUCTION

Acute ethanol administration to young or adult experimental animals, when given at high dosages, leads to the development of transient liver triglyceride accumulation, slight ultrastructural alterations without significant changes in the level of liver damage indicators in serum,[1] and promotion of free radical processes.[2] Oxidative stress has been observed in the liver of rats subjected to acute ethanol intoxication as assessed by different indirect assays.[2,3] These include measurements of the level of thiobarbituric acid reactants (TBARS),[4–6] diene conjugates formation,[7,8] or spontaneous chemiluminescence,[9] both in liver homogenates or in hepatic subcellular fractions. Although these approaches led to conflicting results,[2] the peroxidative effect of ethanol was confirmed by the use of non-invasive techniques, namely, hydrocarbon production both *in vivo*[10] and by the isolated perfused rat liver,[11] biliary efflux of TBARS in the anaesthetized rat,[12] and *in situ* rat liver chemiluminescence.[13,14] Liver oxidative stress induced by acute ethanol administration involves a derangement of antioxidant mechanisms,[6,7,12] seems to be mediated by ethanol-derived acetaldehyde,[11,14] can involve the α-hydroxyethyl

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radical,[15] is significantly diminished by pretreatment with antioxidants such as (+)-cyanidanol-3[13] or methionine,[18] and has been proposed to play a significant role in liver injury after chronic intake.[3]

The aging process constitutes a phenomenon involving a general decline in physiological functions that appears to be the result of cumulative cell damage along with a decreased repair activity, apparently being conditioned by different mechanisms.[16] Among them, the free radical theory of aging suggests that free radical reactions are implicated in the molecular changes related to the aging process as well as cellular events associated with the environment and disease.[17] The unbalance between free radical production and radical scavenging capacity, or sustained repair functions, may lead to the age-associated increases in oxidative damage to different cellular macromolecules and the consequent derangement in their functions, as shown for several tissues including the liver.[16] In view of these considerations, in the current study, we tested the hypothesis that aging may further modify the hepatic morphological and/or functional alterations induced by acute ethanol administration to rats. For this purpose, ethanol-induced changes in the level of serum transaminases and liver histology were determined in rats of different ages, and results were correlated with changes in oxidative stress-related parameters in the liver.

MATERIALS AND METHODS

Animals and Treatments

Male Wistar rats of different ages (3, 6, 12, 18, and 24 months) kept on a standard pellet diet *ad libitum* were administered a single intraperitoneal injection of either 3 g ethanol/kg body weight as a 35% v/v solution in 0.9% w/v NaCl or equivalent volumes of 0.9% w/v NaCl (controls). Studies were performed 6 h after treatment in animals kept in a warm environment (25–28°C). All animals used in this study received humane care according to the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences (National Institutes of Health, Publication No. 86-23).

Biochemical Parameters

Animals were euthanized by cervical dislocation, and blood samples were taken from the aorta for the measurement of the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum using commercial kits (Sigma Chemical, St. Louis, MO). The abdominal cavity was opened, and ice-cold 0.9% w/v NaCl was infused through the inferior vena cava to eliminate blood. The livers were removed, weighed, and homogenized in 0.5N $HClO_4$ for the determination of total glutathione (tGSH).[18] Tissue samples were homogenized (25% w/v) in 10 mM potassium phosphate buffer pH 7 containing 140 mM KCl, centrifuged at 900*g* for 20 min at 4°C, and the supernatants were used to determine TBARS formation.[19] Postmicrosomal supernatants were used to measure the activity of superoxide dismutase (SOD),[20] catalase,[21] and glutathione peroxidase (GPx).[22] The total protein content of the studied fractions was measured using the classical method described by Layne.[23]

For the determination of α-tocopherol and β-carotene, lipid extracts were prepared by homogenizing approximately 100 mg of liver in 1 ml of water containing 0.05 ml of 10 mg/ml butylated hydroxytoluene to prevent lipid autoxidation. After the addition of 1 ml of 0.1 M SDS, the mixture was vortexed for 30 s, supplemented with 2 ml methanol plus 4 ml *n*-hexane, vigorously vortexed for 1 min, and centrifuged for 5 min at 1000*g* to separate the organic layer. A 3 ml aliquot of the *n*-hexane layer was dried under N_2 and the residue was redissolved in 0.5 ml methanol/ethanol (1:1 v/v) and filtered through a 0.22 μm-pore membrane.[24] Quantitative measurements of α-tocopherol and β-carotene were made by HPLC of lipid extracts using a Bioanalytical System electrochemical detector (LC-4C) coupled to an isocratic delivery system.[25] Samples were injected through a Rheodyne system (loop: 20 μl) to a C8 Nova-Pak column, 4 μm, with dimensions of 3.9 × 150 mm. Separation was done using a flow rate of 1 ml/min of the mobile phase (20 mM lithium perchlorate in methanol/water 98:2 [v/v]). Electrochemical detection was done using an applied oxidation potential of 0.6 V. Standard solutions of α-tocopherol and β-carotene were prepared by dissolving the pure compounds in 200 μl of ethanol:methanol (1:1).[26] All chemicals and reagents used were obtained from Sigma Chemical (St. Louis, MO), except for HPLC solvents (LiChrosolv®, E. Merck, Darmstadt, Germany).

Histological Studies

Liver samples from groups of control and ethanol-treated rats at different ages were fixed in 10% formaldehyde and paraffin embedded. Sections were stained with hematoxylin–eosin, Masson's trichrome and PAS (with and without diastase digestion) and blindly observed by two different pathologists. Three sections of each liver (submitted to the described staining) were used to evaluate histological alterations. Ten microscopic fields were assessed per liver section studied, using 100 times (10 × 10) magnification.

TABLE I Body weight, food intake, and dietary α-tocopherol in rats of different ages*

Parameter	Age (months)				
	3	6	12	18	24
Body weight (g)	216 ± 3	387 ± 27†	410 ± 16†	416 ± 13†	432 ± 6†
Food intake (g/day)	13.0 ± 0.4	15.5 ± 0.1†	16.0 ± 0.1†	16.4 ± 0.2†	16.5 ± 0.1†
α-Tocopherol intake‡ (mg/day/100 g b wt)	0.18 ± 0.007	0.11 ± 0.008†	0.08 ± 0.005†¶	0.08 ± 0.003†	0.08 ± 0.001†

* Results are means ± SEM for three rats. † Statistical studies: $p < .05$ compared to 3 month-old rats. ‡ Calculated from daily food intake, knowing that Purina lab chow contains 0.020 mg of α-tocopherol/g and the respective body weight. ¶ $p < .05$ compared to 6 month-old rats.

Statistics

Values shown correspond to the means ± SEM for the number of separate experiments indicated. The statistical significance of differences between mean values was assessed by one-way ANOVA followed by the Tukeys' test. Linear regression coefficients and their significance were calculated according to the Pearson's method.

RESULTS

Assessment of biochemical and morphological parameters were performed in animals exhibiting a normal growth and food intake profile during the time period studied (Table I). α-Tocopherol daily intake decreases in rats of 6 and 12 months of age and remains constant thereafter (Table I). In these conditions, acute ethanol administration did not alter serum AST and ALT activities in 3 month-old rats, whereas 6 month-old animals over exhibited a significant and constant increases in AST, as compared to the respective age-control groups (Table II). In the case of ALT, ethanol-induced enhancement in its serum activity reaches a maximum effect in 12 month-old rats that remains constant with increasing age (Table II). Morphological studies revealed that livers from control (Fig. 1A) and ethanol-treated animals at 3 and 6 months (Fig. 1B) of age were between normal ranges. On the contrary, livers from ethanol-treated rats at 12 (Fig. 1C), 18, and 24 (Fig. 1D) months of age exhibited confluent pericentral necrosis with increasing frequency (Table II). Some of these necrotic areas were accompanied by early polymorphonuclear leukocyte infiltration. Hepatic cells from periportal areas were essentially normal (Fig. 1).

Administration of 3 g ethanol/kg led to an increase in the lipid peroxidation potential of the liver (Table III) and a decrease in the content of hepatic tGSH (Table IV), independently of animals age, parameters that are not modified in control rats with increasing age. Contrarily, aging elicited a progressive decrease in the liver content of a α-tocopherol and β-carotene in control rats, starting at 12 months of age (Table IV), parameters that exhibit a significant inverse correlation with increasing age [α-tocopherol vs. age, $r = -0.985$, $p < .01$; β-carotene vs. age, $r = -0.955$, $p < .02$]. Ethanol administration led to a diminution in the content of hepatic α-tocopherol and β-carotene (Table IV), an effect which is age-dependent for α-tocopherol [$r = -0.932$, $p < .02$]. Plasma levels of α-tocopherol also decreased in control animals in an age-dependent manner [$r = -0.978$, $p < .05$], whereas

TABLE II Effect of acute ethanol administration on the serum activity of AST and ALT and on the incidence of liver necrosis in rats of different ages*

Treatment	Age (months)				
	3	6	12	18	24
(A) Serum AST (U/ml)					
Control	73 ± 3 (5)	67 ± 3 (7)	73 ± 2 (7)	78 ± 3 (8)	77 ± 2 (9)
Ethanol	76 ± 3 (5)	233 ± 23 (6)†	214 ± 20 (8)†	198 ± 4 (6)†	183 ± 8 (10)†
% Change	NS	250‡	193‡	143‡	138‡
(B) Serum ALT (U/ml)					
Control	57 ± 2 (5)	62 ± 4 (7)	64 ± 2 (8)	61 ± 1 (6)	63 ± 3 (9)
Ethanol	64 ± 3 (5)	125 ± 6 (7)†	208 ± 12 (8)†,¶	209 ± 13 (7)†,¶	198 ± 8 (10)†,¶
% Change	NS	100‡	226‡	241‡	213‡
(C) Liver necrosis§					
Control	0/3	0/3	0/3	0/4	0/6
Ethanol	0/3	0/3	1/3	3/4	5/6

* Animals were administered either 3 g ethanol/kg or isovolumetric amounts of 0.9% w/v NaCl (controls) for 6 h. Results are means ± SEM for the number of rats used shown in parentheses. † Statistical studies: $p < .05$ compared to 3 month-old ethanol-treated rats. ‡ $p < .05$ compared to the respective age-control group. ¶ $p < .05$ compared to 6 month-old ethanol-treated rats; NS: not significant. § Corresponds to the number of animals showing hepatic necrosis per the total number of rats studied.

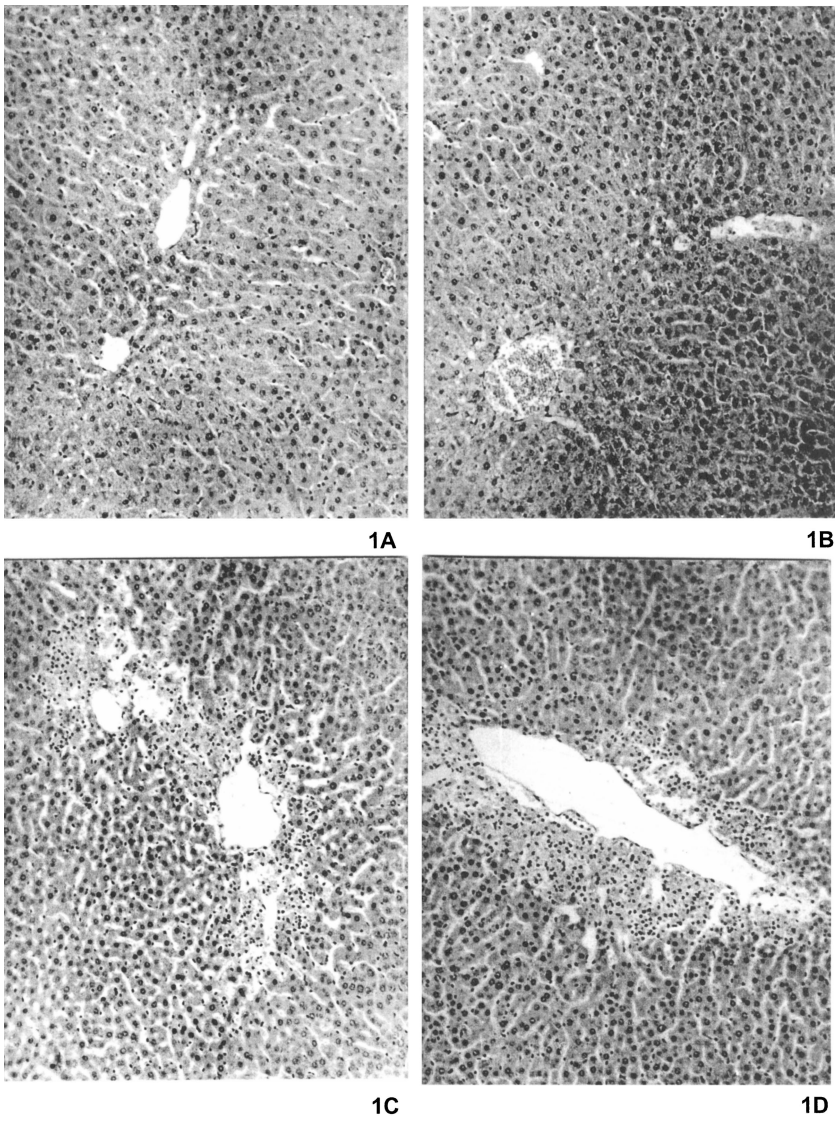

FIGURE 1 (A) Liver normal histologic aspect from a control animal. H&E, stain, × 150; (B) Liver from an ethanol-treated rat at 6 months. The perivenular area is essentially normal. H&E, stain, × 150; (C) Liver from an ethanol-treated rat at 12 months. The centrolobular area shows liver cell necrosis with early polymorphonuclear leukocyte infiltration. H&E, stain, × 150; (D) Liver from and ethanol-treated rat at 24 months. The centrolobular area exhibits confluent hepatic necrosis with early polymorphonuclear leukocyte infiltration. H&E, stain, × 150.

that of ethanol administration is independent of age (Table V). Liver antioxidant enzymes, namely, SOD, catalase, and GPx, do not change with increasing age, whereas ethanol administration elicited a decrease in the activity of catalase, an effect that is significant in 6, 12, and 24 month-old animals, without altering SOD and GPx activities (Table VI).

DISCUSSION

The assessment of parameters associated with oxidative stress revealed that acute ethanol administration enhances the lipid peroxidative potential and depresses tGSH levels in the liver of young rats (3 months old), without altering the content of α-tocopherol and β-carotene, the activity of SOD, catalase, and GPx, or serum transaminases. These features of acute ethanol ingestion are in agreement with previous observations,[4−14,27] and suggest that young animals are less susceptible to oxidant damage probably due to efficient antioxidant and repair mechanisms. Previous studies of the influence of age on acute ethanol-induced liver oxidative stress showed enhanced total liver TBARS and decreased tGSH contents in 3, 6, and 10 month-old animals,[27] in agreement with data presented in this work. However, these ethanol-induced changes were abolished in 13 month-old rats, probably due to the higher dose of ethanol (5 g/kg; 6 h) used.[27] Those results were interpreted in terms of a high and rapid tGSH depletion in the liver

TABLE III Effect of acute ethanol administration on the formation of TBARS in the liver of rats of different ages*

Treatment	Age (months)				
	3	6	12	18	24
Control	2.74 ± 0.15 (5)	2.86 ± 0.19 (7)	2.72 ± 0.11 (7)	3.26 ± 0.30 (9)	3.26 ± 0.14 (8)
Ethanol	4.71 ± 0.08 (5)	4.99 ± 0.30 (8)	4.50 ± 0.11 (8)	4.62 ± 0.19 (8)	5.61 ± 0.29 (8)
% Change	72†	74†	66†	42†	72†

* Animals were administered either 3 g ethanol/kg or isovolumetric amounts of 0.9% w/v NaCl (controls) for 6 h. Results are expressed as ηmol/mg protein/120 min and correspond to means ± SE for the number of rats used shown in parentheses. † Statistical studies: $p < .05$ compared to the respective

TABLE IV Effect of acute ethanol administration on the content of hepatic tGSH, α-tocopherol, and β-carotene in rats of different ages*

Treatment	Age (months)				
	3	6	12	18	24
(A) tGSH (μmol/g liver)					
Control	7.18 ± 0.19 (5)	7.40 ± 0.32 (8)	7.80 ± 0.52 (8)	7.58 ± 0.39 (7)	7.68 ± 0.24
Ethanol	4.12 ± 0.15 (5)	3.49 ± 0.20 (8)	4.13 ± 0.13 (8)	4.33 ± 0.20 (8)	4.34 ± 0.17
% Change	−43†	−53†	−47†	−43†	−44†
(B) α-Tocopherol (ηmol/g liver)					
Control	79 ± 2 (6)	80 ± 4 (6)	70 ± 2 (7)‡	65 ± 6 (6)¶	58 ± 2 (6)§
Ethanol	78 ± 1 (6)	61 ± 1 (5)‖	45 ± 2 (7)#	37 ± 1 (6)**	28 ± 1 (6)††
% Change	NS	−24†	−36†	−43†	−52†
(C) β-Carotene (ηmol/g liver)					
Control	6.24 ± 0.08 (6)	6.95 ± 0.30 (6)	3.61 ± 0.23 (6)‡	2.76 ± 0.10 (6)¶	1.67 ± 0.16 (6)§
Ethanol	6.62 ± 0.14 (6)	3.97 ± 0.32 (6)‖	1.57 ± 0.07 (6)#	1.22 ± 0.09 (6)**	1.16 ± 0.14 (6)**
% Change	NS	−43†	−57†	−56†	−31†

* Animals were administered either 3 g ethanol/kg or isovolumetric amounts of 0.9% w/v (controls) for 6 h. Results are means ± SEM for the number of rats used shown in parentheses. † Statistical studies: $p < .05$ compared to the respective age-control group. ‡ $p < .05$ vs. 3 and 6 month-old control rats. ¶ $p < .05$ vs. 3, 6, and 12 month-old control rats. § $p < .05$ vs. 3, 6, 12 and 18 month-old control rats. ‖ $p < .05$ vs. 3 month-old ethanol-treated rats. # $p < .05$ vs. 3 and 6 month-old ethanol-treated rats. ** $p < .05$ vs. 3, 6, 12 month-old ethanol-treated rats. †† $p < .05$ vs. 3, 6, 12, and 18 month-old ethanol-treated rats; NS: not significant.

TABLE V Effect of acute ethanol administration on the plasma levels of α-tocopherol in rats of different ages*

Treatment	Age (months)				
	3	6	12	18	24
Control	25 ± 1 (6)	25 ± 1 (6)	12 ± 1 (6)†	13 ± 1 (7)‡	10 ± 1 (10)‡
Ethanol	25 ± 1 (6)	19 ± 2 (6)¶	13 ± 1 (8)§	10 ± 1 (6)‖	7 ± 1 (6)#
% Change	NS	−24**	−38**	−23**	−30**

* Animals were administered either 3 g ethanol/kg or isovolumetric amounts of 0.9% w/v Nacl (controls) for 6 h. Results are means ± SE for the number of rats used shown in parentheses. † Statistical studies: $p < .05$ vs. 3 and 6 month-old control rats. ‡ $p < .05$ vs. 3, 6, and 12 month-old control rats. ¶ $p < .05$ vs. 3, month-old ethanol-treated rats. § $p < .05$ vs. 3, and 6, month-old ethanol-treated rats. ‖ $p < .05$ vs. 3, 6, 12 month-old ethanol-treated rats. # $p < .05$ vs. 3, 6, 12 and 18 month-old ethanol-treated rats. ** $p < .05$ compared to respective age-control group; NS: not significant.

TABLE VI Effect of acute ethanol administration on the activity of liver SOD catalase, and GPx in rats of different ages*

Treatment	Age (months)				
	3	6	12	18	24
(A) SOD (U/mg protein)					
Control	199 ± 6 (5)	214 ± 10 (7)	216 ± 8 (8)	225 ± 9 (7)	181 ± 6(10)
Ethanol	185 ± 6 (5)	201 ± 6 (8)	203 ± 3 (8)	209 ± 7 (7)	161 ± 8 (10)
% Change	NS	NS	NS	NS	NS
(B) Catalase (U/mg protein)					
Control	338 ± 10 (5)	347 ± 27 (8)	379 ± 10 (8)	341 ± 21 (8)	330 ± 14 (7)
Ethanol	272 ± 7 (5)	212 ± 20 (7)	249 ± 17 (8)	276 ± 14 (7)	244 ± 14 (7)
% Change	NS	−39†	−34†	NS	−26†
(C) GPx (U/mg protein)					
Control	0.65 ± 0.03 (5)	0.56 ± 0.03 (8)	0.53 ± 0.02 (8)	0.58 ± 0.03 (7)	0.53 ± 0.02 (8)
Ethanol	0.60 ± 0.04 (5)	0.53 ± 0.09 (8)	0.48 ± 0.02 (8)	0.53 ± 0.02 (8)	0.51 ± 0.05 (7)
% Change	NS	NS	NS	NS	NS

* Animals were administered either 3 g ethanol/kg or isovolumetric amounts of 0.9% w/v NaCl (controls) for 6 h. Results are means ± SE for the number of rats used shown in parentheses. † Statistical studies: $p < .05$ compared to respective age-control group; NS: not significant.

induced by ethanol, followed by a rapid increase in glutathione resynthesis that could allow the recovery of the antioxidant potential in these conditions.

Acute ethanol administration leads to the appearance of confluent necrosis in pericentral areas only in 12, 18, and 24 month-old animals (Fig. 1). The absence of ethanol-induced liver lesions in 6 month-old rats may be due to the small number of samples studied, since serum transaminases activities are increased in this experimental group. Acute ethanol-induced liver necrosis in older rats is primarily located in the perivenular zone (zone 3) of the hepatic acinus as demonstrated in human alcoholics.[28] This area is particularly susceptible to toxic aggressions and exhibits a 30-fold higher concentration of cytochrome P450IIE1, isozyme involved in the biotransformation of several hepatotoxins including ethanol, than periportal regions.[29] These morphological alterations are accompanied by substantial diminution in the liver content of GSH, α-tocopherol, and β-carotene as well as in plasma α-tocopherol levels. The combined data presented seem to suggest that the hepatotoxicity of acute ethanol administration is highly influenced by the age-related derangement of liver antioxidants availability, which is clearly seen in elderly control rats (12 up to 24 months old). Hepatic depletion of α-tocopherol, and β-carotene, and lycopene has also been reported in alcoholics[30,31] and in experimental animals subjected to different models of prolonged ethanol ingestion.[32–35] Thus, liver damage induced either by acute ethanol administration in elderly rats or by prolonged ethanol consumption in adult alcoholics could possibly involve the lack of soluble antioxidants.

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