The in vivo deleterious effects of ethanol

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Abstract

Oxidative stress, which is defined as an imbalance between pro-oxidants and antioxidants, has been demonstrated to mediate the pathogenesis of ethanol-induced injury. Senescence-accelerated mice prone P8 (SAMP8) is considered an excellent model for rodent aging. However, the deleterious effect of ethanol-induced liver injury of SAM P8 has not been established. In this study we investigated the antioxidant enzyme activities in the liver of SAMP8 during chronic ethanol exposure. The mice were orally administered 0, 0.5, 2 and 4g ethanol/kg BW three times/week for 10 weeks. Results showed that ethanol elevated activity of alanine aminotransferase (ALT) slightly and aspartate aminotransferase (AST) levels were increased significantly in ethanol-fed 0.5 and 4 g/kg BW groups. Hematoxylin and eosin staining indicated moderate to severe fatty infiltration but not fibrosis. Ethanol also enhanced the formation of malondialdehyde (MDA) and protein carbonyls in the liver, whereas ethanol treatment resulted in significantly lower activity of hepatic glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD). We conclude that ethanol damaged the liver of SAMP8 by increasing oxidative stress.

Keywords: Senescence-accelerate mice, Oxidative stress, Ethanol.

Introduction

Alcohol is mainly metabolized in the liver. Excessive alcohol use can lead to acute and chronic liver disease including hepatitis, liver cirrhosis and fatty liver [1]. Although diverse mechanisms are involved in the ethanol-induced hepatotoxicity [2, 3], accumulating evidence has supported the importance of oxidative stress mediated by reactive oxygen species (ROS) [4]. Intake of alcohol results in excessive generation of free radicals, which alter the biomembranes and cause damage. ROS leads to the peroxidation of membrane lipids and produces malondialdehyde (MDA) and oxidation of protein to carbonyl group [4].

Alcohol abuse in the elderly is an important public health problem [5, 6]. Laboratory animal studies have also indicated that as people age, the body's ability to metabolize ethanol decreases, and the toxicity of ethanol correspondingly increases [7, 8]. In addition, several studies have shown that the greater susceptibility of older people to the adverse effects of ethanol may be due to the depletion of hepatic antioxidants and a resulting decrease in the ability of the liver to recover from ethanol-induced damage [9]. Therefore, a greater insight is needed into the age-related susceptibility of ethanol-induced damage to the liver and other tissues.

Few animal models are available for studying the effects of ethanol toxicity in aged animals. Depending on the strain and type of animals used, studies have differed widely in their definition of "aged". Aged Sprague Dawley (SD) rats have been defined as 18 months old [10], aged C57BL/6 mice as 18-22 months old [11, 12], BALB/C mice as 14 months old [13], and middle-aged Wistar rats as 16 months old [14]. The senescence-accelerated mouse (SAM) is a novel murine model of accelerated aging derived from the AKR/J strains that was established by Takeda et al. [15]. Senescence-accelerated-prone 8 mice (SAMP8) strain shows a shortened life span and early signs of various indices of aging such as higher oxidative stress and impaired mitochondrial function [15-17]. Therefore, SAMP8 are considered an excellent model for aging.

SAMP8 has been used to study brain aging and antioxidant activities of natural products [18]. To our knowledge, the deleterious effects of ethanol-induced liver injury on SAM P8 have not been established. Therefore, we attempted in the present study to evaluate the validity of the SAMP8 animal model for investigating ethanol-induced liver injury in aged groups.

Materials and methods

Animals and diets

Male and female SAMP8 mice were kind gifts from Toshio Takeda (Kyoto University) and were six months of age and weighted 20-24 g at the start of the study. All of the procedures using animals were approved by the Institutional Animal Care and Use Committee of the Central Taiwan University of Science and Technology. All of the animals were maintained in individual cages on a 12 h: 12 h light: dark

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schedule, with food and water provided ad libitum.

Experimental design

Mice were randomized into four groups: a saline-fed control group, ethanol-fed groups (0.5, 2, 4g/kg BW). Mice were orally given saline or ethanol via intragastric gavage three times per week for 10 weeks. After 10 weeks, the mice were anesthetized and blood samples were collected from the eye socket into a non-heparinized capillary tube. Serum was obtained by centrifugation (500g, 10 min) and then stored at -80°C. Livers were blanched with saline, excised and portions of liver were either fixed in formalin for histology or frozen in liquid nitrogen and stored at -80°C until further analysis.

Biochemical indicators of liver function

Serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed with a clinical analyzer (CR5B2 Hitachi 7250; Hitachi, Ltd, Tokyo, Japan).

Liver histology

Pieces of liver tissue were fixed in 10% formalin for 48-72 h, processed routinely, and embedded in paraffin. Sections of 2 μm thick were cut and stained with hematoxylin and eosin (HE). Livers were fixed in 10% buffered formaldehyde solution for one week and then embedded in paraffin. The paraffin sections were cut into 2 μm sections, stained with HE, and then observed by light microscopy. For semi-quantitative scoring of fatty infiltration, the following values were used: no visible fat, score 0; <5% of liver surface infiltrated by fat, score 1; 5-25% fat, score 2; 25-50% fat, score 3; and >50% fat, score 4 [19]. The final numerical score was calculated by dividing the sum of the number per grade of affected mice by the total number of examined mice.

Antioxidant status

Superoxide dismutase (SOD)

The SOD level in the liver cytosolic fraction was determined using a Ransod kit purchased by Randox Laboratories (Ardmore, Northern Ireland, UK). Xanthine and xanthine oxidase were used to generate superoxide anion, which reacts with 2-(4-indophenyl)-3-(4-nitro-phenyl)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. Changes in the absorbance were determined at 505 nm during the first 3 min of the reaction. Enzyme activities in the samples were calculated using a standard curve. One enzyme unit of SOD was defined as the amount that inhibits the INT reaction by 50%. Specific activities were defined as units/mg protein.

Glutathione peroxidase (GPx)

The activity of GPx was assayed by the method of Paglia and Valentine [20]. GPx activity was assayed by measuring the rate of NADPH oxidation at 340 nm with a cellular glutathione peroxidase assay kit (Calbiochem). This kit is based on the reduction by GPx of organic peroxide, tert-butyl hydroperoxide in the presence of glutathione (GSH). One unit of GPx activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol of NADPH/min/mg protein.

Catalase (CAT)

CAT activity was assayed according to a slightly modified version of the method of Aebi [21]. Hydrogen peroxide (H_2O_2) disappearance was monitored kinetically at 240 nm. One unit of activity is equal to the μmoles of H_2O_2 degraded/min/mg protein.

Determination of thiobarbituric acid reactive substances (TBARS)

The concentration of TBARS in tissue homogenates was determined according to the method of Ohkawa et al. [22] using malondialdehyde (MDA) from tetramethoxypropane as the standard and double-distilled water as the control. The TBARS concentration of the n-butanol layer was measured spectrophotometrically at 532 nm. The butanol-pyridine phase containing the TBARS was measured at 532 nm. The results were expressed as nmol equivalents of MDA/mg of protein.

Determination of oxidized protein

The amount of liver cytosolic oxidized protein was determined according to the method of Smith [23]. The difference in the absorbance spectra for 2, 4-dinitrophenylhydrazide-protein in guanidine hydrochloride and for a guanidine hydrochloride-protein blank was used to calculate the nmol of 2, 4-dinitrophenylhydrazide incorporated per mg of protein.

Protein assay

The liver cytosolic protein was determined by Lowry's method [24], with bovine serum albumin (BSA) as a standard.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical significance of the differences between groups was checked by one-way ANOVA followed by LSD multiple comparisons. Differences with $P < 0.05$ were considered to be significant.

Results

Liver histology

Fatty infiltration in SAMP8 mice fed with ethanol:

Spontaneous fatty infiltration with numerous vacuoles in hepatocytes was found in SAMP8 control mice (Fig. 1). After being fed 0.5, 2, 4g ethanol/kg BW, affected livers showed moderate to severe fatty infiltration with numerous vacuoles in hepatocytes (Fig. 1), and their scores of fatty infiltration were significantly higher than those of controls (Table1).

Table1. Histopathology scores of fatty infiltration of SAMP8 mice fed with ethanol (0-4g/kg BW) for 10 weeks.

Group	Administration	Mean score of fatty infiltration
	Ethanol 0 g/kg BW	2.9±0.3[a]
	Ethanol 0.5 g/kg BW	3.1±0.6[a]
	Ethanol 2 g/kg BW	3.6±0.5[b]
	Ethanol 4 g/kg BW	3.8±0.6[b]

Data are mean±SD (*n*= 8-10). Values with different alphabetical letters differ significantly (*P* < 0.05).
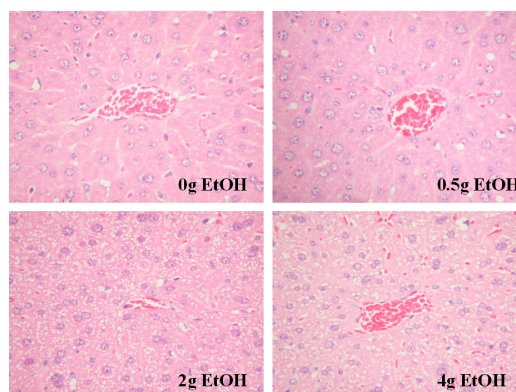

Figure 1. Histopathological changes in livers in SAM P8 mice fed with ethanol (EtOH) 0-4g/kg BW for 10 weeks. HE staining, 400x.

Biochemical indicators of liver function

Ethanol administration affected biochemical markers of liver function. As shown in Table 2, AST levels were increased significantly in ethanol-fed 0.5 and 4 g/kg BW groups, whereas, ALT levels were increased but not differ significantly.

Table 2. Biochemical indicators of liver function in serum of SAMP 8 mice following chronic ethanol administration.

Treatment[a]	AST(U/L)[b]	ALT(U/L)[b]
Ethanol 0 g/kg BW	107±37[a]	54±17
Ethanol 0.5 g/kg BW	227±56[b]	77±32
Ethanol 2g/kg BW	138±46[ac]	78±51
Ethanol 4 g/kg BW	183±58[bc]	93±63

[a]SAMP8 mice were orally fed ethanol (0-4 g/kg BW) three times/week for 10 weeks.
[b]Data are means±SD ($n = 8$-10). Values in the same column not sharing a common superscript differ significantly ($P < 0.05$).
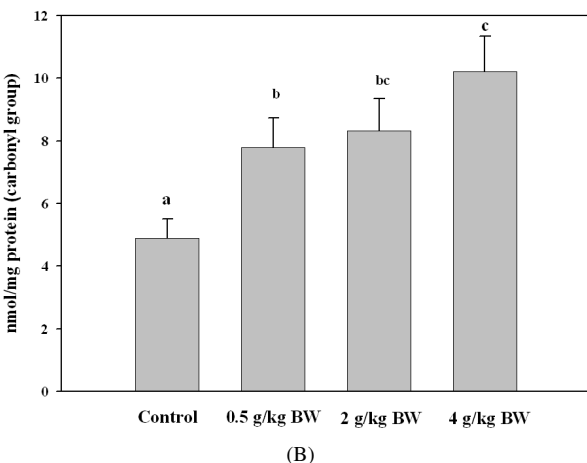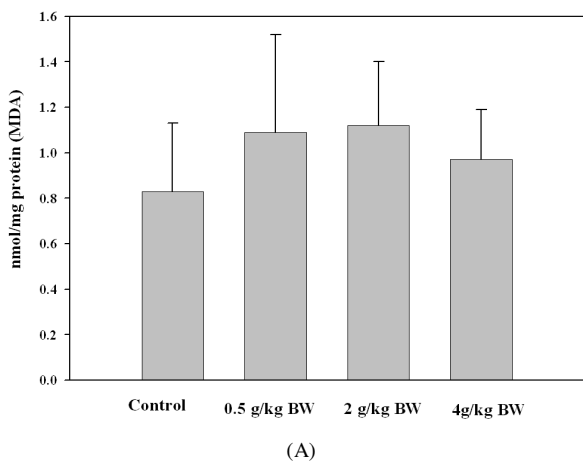AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Figure 2. Levels of MDA (A) and protein carbonyl (B) in liver tissue after chronic ethanol administration (0-4g/kg BW) for 10 weeks. Data are means±SD ($n = 8$-10). Values with different alphabetical letters differ significantly ($P < 0.05$).

Lipid peroxidation and protein oxidation of the liver

The chronic administration of ethanol (0.5-4 g/kg BW) led to an increase in the levels of hepatic MDA and carbonyl groups, indicating an increased amount of lipid peroxidation and protein oxidation in the liver (Fig. 2A and 2B).

Activities of liver antioxidant enzymes

Fig. 3 describes the measured activities of hepatic

antioxidant enzymes. Mice treated chronically with ethanol showed that activities of catalase (Fig. 3A), GPx (Fig. 3B) and SOD (Fig. 3C) were markedly lower than controls.
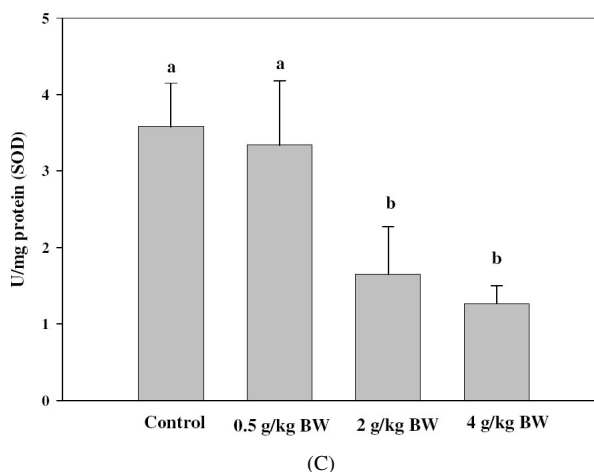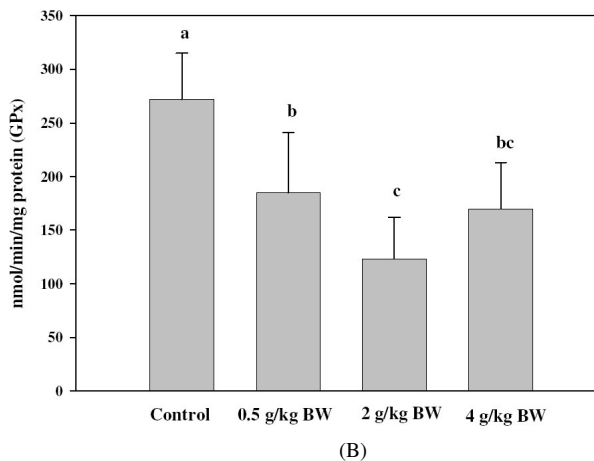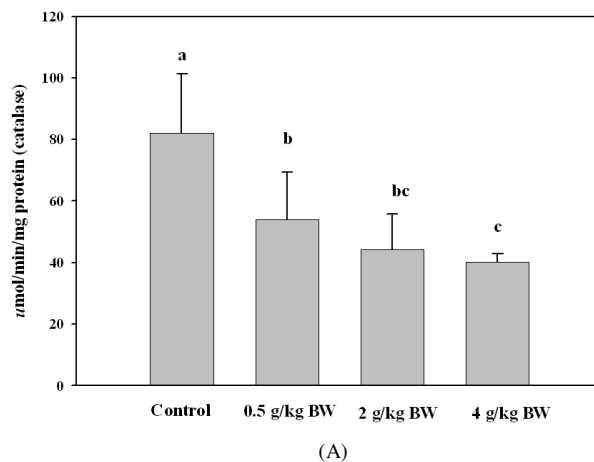

(A)

(B)

(C)

Figure 3. Levels of hepatic antioxidant enzymes in SAMP8 mice after chronic ethanol administration (0-4g/kg BW) for 8 weeks.(A) catalase; (B) GPx; (C) SOD. Data are means ± SD ($n = 8$-10). Values with different alphabetical letters differ significantly ($P < 0.05$).

Discussion

Liver is the major organ of ethanol metabolism, and the role of oxidative stress is significant in the pathogenesis of ethanol related disease [1]. The older rats are remarkably more susceptible to ethanol induced oxidative damage than the

younger ones [25]. We found that even at a relatively low dose (0.5-4 g/kg) and infrequent exposure (3 times/week) to ethanol, SAMP8 mice showed clinical histopathological toxicity. Hepatotoxicity was also induced and was clearly evident as a histological change reflected in fatty accumulation, accompanied by slight increases in the levels of serum AST and ALT. Moreover, ethanol led to an increase in lipid peroxidation and protein oxidation in the liver, and a decrease in the activities of hepatic antioxidant enzymes, such as SOD, catalase and GPx. These findings reflect increased oxidative stress in the liver.

Oxidative stress is an early event in alcoholism that often occurs before liver function tests (AST, ALT) show any abnormalities [26]. ROS are potentially damaging to cellular constituents, and cells use defense mechanisms to keep ROS accumulation under control. The principal defenses are SOD and GPx [27]. Glutathione is the most abundant intracellular thio based antioxidant present in milli molar concentration, and it serves as a significant first line of defense against the oxidative stress and it plays an important role in maintaining the integrity of cells [28]. Previous studies [29] have shown that delivering the mitochondrial isoform of SOD using recombinant adenovirus prevents liver injury in rats receiving ethanol intragastrically. Moreover, thiol-containing proteins appear to be targets of free radicals. Chronic ethanol feeding significantly increases the level of protein carbonyl groups [30]. The present study reports findings similar to those observed in different experimental mouse models of ethanol exposure [31-33], and it confirms the pathogenic role of oxidative stress in the liver. We suggest that SAMP8 can be used as an animal model for investigating the oxidative stress of ethanol. Further work is needed to provide more insight into the age-related susceptibility of ethanol-induced liver damage.

Conclusion

We concluded that ethanol damaged the liver of SAMP8 by increasing oxidative stress. The use of the SAM model of ethanol-induced liver injury may provide substantive information leading to a more fundamental understanding of ethanol-induced oxidative stress in the livers of aging mice, as well as clinical treatments.

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