EFFECT OF ETHANOL ON LIVER ANTIOXIDANT DEFENSE SYSTEMS: A DOSE DEPENDENT STUDY

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ABSTRACT

Alcohol induced oxidative stress is linked to the metabolism of ethanol. In this study it has been observed that administration of ethanol in lower concentration caused gain in body and liver weight, while higher concentration of ethanol caused lesser gain in body and liver weight. Ethanol treatment enhanced lipid peroxidation significantly, depletion in levels of hepatic glutathione and ascorbate, accompanied by a decline in the activities of glutathione peroxidase and glutathione reductase, and increased in hepatic glutathione s-transferase activity. Interestingly catalase activity increased in lower concentration of ethanol exposure, and decreased in higher concentration. Superoxide dismutase activity was also increased on ethanol exposure. But, ethanol feeding did not show any effect on glucose-6- phosphate dehydrogenase activity. Ethanol ingestion perturbs the antioxidant system in a dose and time dependent manner.

KEY WORDS

Ethanol, Antioxidants, Oxidative stress, Glutathione, Superoxide dismutase

INTRODUCTION

Alcohol consumption is associated with a number of changes in cell function and the oxidant-antioxidant system (1). Ethanol associated endotoxaemia and subsequent release of inflammatory mediators may cause hepatocyte injury via oxyradical-dependent and -independent mechanisms. These cytotoxic processes may lead to lethal hepatocyte injury. Cells are protected against oxidation by the action of certain enzymes, vitamins, and other substances, known collectively as antioxidants (2, 3). As a consequence of the constant oxidative challenge, cells have evolved antioxidant systems to counter the prooxidant fluxes. Notable among these antioxidant mechanisms are enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. These enzymes react directly with the oxidizing radicals to yield non-radical product. When the balance between the ROS (reactive oxygen species) production and antioxidant defence is lost, "oxidative stress" results, which through a series of events deregulates the cellular functions leading to various pathological conditions (3).

Different studies at National and International levels

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show that alcohol consumption will lead to liver damage (3, 4, 5, 6, 7); but the exact dosage and other contributing factors, particularly oxidative stress are not well studied. The risk of severe liver damage is dose dependent. In spite of intense investigation, the precise mechanisms leading to alcoholic liver disease are still imprecisely known. This is due in part to the lack of a reliable animal model. To understand the antioxidant status of alcoholic liver damage this study was undertaken.

MATERIALS AND METHODS

Materials

Ethanol was purchased from Bengal Chemicals, Kolkata. All chemicals used in this study were of analytical grade.

Animal Selection

10-week-old male albino rats of Wistar strain weighing 100- 120g were used. The animals were housed in plastic cages inside a well-ventilated room. The room temperature was maintained at $22\pm 2^{\circ}\text{C}$ with a 12-h light/dark cycle. All rats had free access to a standard diet containing 18% proteins (casein), 71% carbohydrate (equal parts of arrowroot and starch), 7% fat (groundnut oil), 4% salt mixture and adequate vitamins mixture (8). Food and water were given *ad libitum*.

The rats were divided into the following groups each

containing 6 rats.

Group I: Control rats- which were fed normal diet and water.

Group II: Ethanol treated rats (0.8g ethanol/ kg body weight/ day for 4 weeks).

Group III: Ethanol treated rats (1.2g ethanol/ kg body weight/ day for 4 weeks)

Group IV: Ethanol treated rats (1.6g ethanol/ kg body weight/ day for 4 weeks)

Group V: Ethanol treated rats (2g ethanol/ kg body weight/ day for 4 weeks)

Ethanol was diluted with distilled water to get desired concentration and fed orally. The animals were weighed weekly and their general condition and behavior were recorded including their daily intake of liquid.

Experimental Procedure

At the end of the experimental period, the animals were sacrificed by cervical dislocation. The liver was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber. Various oxidative stress related parameters were estimated. The Animal Ethics Committee of the Institution approved the procedures.

Biochemical Methods

Liver was homogenized in 0.25M sucrose solution, diluted with 0.9% saline, and these diluted samples were used for the estimation of tissue protein by the method of Lowry *et al* (9). Ascorbic acid content was estimated using thiourea reagent and 2,4-dinitrophenylhydrazine (10). Lipid peroxidation was estimated using Trichloroacetic acid (TCA)-Thiobarbituric acid (TBA)- Hydrochloric acid (HCl) at 535 nm (11). The reduced glutathione (GSH) content of the tissue in 0.1M phosphate buffer (pH 7.4) was measured following the method of Ellman (12).

Fig. 1. Percentage change in body weight in different groups of rats exposed to different concentration of ethanol.

Catalase (EC 1.11.1.6) activity was determined by the method of Beers and Sizer (13) at 240 nm. Glutathione reductase (GR, EC 1.6.4.2) activity was determined at 340 nm by the method of Goldberg and Spooner (14). Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (15). Glutathione peroxidase (GPx, EC 1.11.1.9) activity was measured as described by Paglia and Valentine (16). The superoxide dismutase (EC 1.15.1.1) activity was measured by the inhibition of auto oxidation of pyrogallol at 420 nm (17). Estimation of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was done following the method of Beutler (18).

Results were expressed as mean $\pm$ SEM (standard error). Statistical significance was determined by Student's 't' test for unpaired data. The values of significance were evaluated with 'p'values. The differences were considered significant at $p < 0.05$.

RESULT

Changes in body weight of animals during the experiment are presented in Figure 1 as percentage change in relation to initial body weight. Animals in group II (0.8g ethanol /kg body weight/ day) showed highest increase in body weight (69%), which is even greater than the group I animals (54%). In groups III, IV and V, the alcohol-exposed animals showed gradually lowering levels in body weight (39%, 23% and 18% respectively). The relative weights of liver of different groups of rats are presented in Figure 2. In the groups II and III, the liver weights (g/100g body weight) were found to be almost same as that of group I. Whereas the liver weights of animals in groups IV and V were found to be reduced.

Significant decrease in ascorbic acid content of liver was found in the group consuming 2g ethanol per kg body weight per day (group V) compared to control rats (Group I). Thiobarbituric acid reactive substances (TBARS) level of liver was increased in the groups consuming higher concentration of ethanol (i.e. Groups III, IV and V) in comparison to group I. While the GSH

Fig. 2. Relative weights of liver of different groups of rats exposed to different concentration of ethanol

content of liver was found to be decreased significantly in groups III to V, when compared with the control group I (Table I).

In ethanol treated groups, the superoxide dismutase activity was found to be significantly higher in comparison to group I. Interestingly, the activity of catalase was increased in lower concentration (group II and III) of ethanol, but in higher concentration (group IV and V), it was significantly decreased compared to the group I. Whereas the glucose-6-phosphate dehydrogenase activity was found to be unaltered in all the ethanol treated groups (Table II).

Ethanol exposure caused significant decreases in the glutathione reductase and glutathione peroxidase activities of liver. While glutathione S-transferase activities of liver of all the ethanol exposed groups were found to be significantly increased compared to control group (Table III).

DISCUSSION

In the present study, rats treated with ethanol in lower concentration showed gain in body and liver weight compared to those of control group (Figs. 1 and 2). While rats treated with higher concentration of alcohol

Table I. Effect of different dose of alcohol on ascorbic acid content, thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) levels in liver homogenate

Group	Ascorbic acid (mg/ g tissue)	TBARS ^a	GSH (μg/ mg tissue)
Group I	2.87 ± 0.18	0.652 ± 0.015	3.82 ± 0.23
Group II	2.82 ± 0.24	0.698 ± 0.021	3.23 ± 0.34
Group III	2.69 ± 0.62	0.783 ± 0.013*	2.65 ± 0.13*
Group IV	2.53 ± 0.43	0.872 ± 0.023*	2.26 ± 0.18*
Group V	2.32 ± 0.16*	0.883 ± 0.034*	2.12 ± 0.21*

a: μmol MDA formed /min/ 100 mg tissue.
Values are mean ± SEM of 6 rats in each group. * Indicates p < 0.05 when compared with normal healthy control.

Table II. Effect of different dose of alcohol on catalase, superoxide dismutase, and glucose 6-phosphate dehydrogenase activity in liver homogenate

Group	Catalase ^a	Superoxide dismutase ^b	Glucose 6-phosphate dehydrogenase ^c
Group I	38.76 ± 0.66	6.33 ± 0.15	8.42 ± 0.23
Group II	43.24 ± 0.78 *	7.13 ± 0.34*	8.34 ± 0.46
Group III	40.67 ± 1.13	8.34 ± 0.45*	8.31 ± 0.32
Group IV	32.63 ± 0.83*	9.07 ± 0.21*	8.29 ± 0.36
Group V	31.24 ± 0.72 *	9.80 ± 0.47*	8.30 ± 0.24

a: μ mole H₂O₂ decomposed / min/ mg protein; b : U/ mg protein; c: nmol /mg protein.
Values are mean ± SEM of 6 rats in each group. * Indicates p < 0.05 when compared with normal healthy control.

Table III. Effect of different dose of alcohol on glutathione reductase, glutathione peroxidase, and glutathione s-transferase activity in liver homogenate

Group	Glutathione reductase ^a	Glutathione peroxidase ^b	Glutathione S-transferase ^c
Group I	53.86 ± 1.34	80.46 ± 2.48	6.94 ± 0.11
Group II	43.24 ± 0.46*	74.43 ± 1.21*	8.21 ± 0.38*
Group III	34.45 ± 1.69*	68.73 ± 2.46*	10.94 ± 0.27*
Group IV	27.76 ± 0.84*	59.24 ± 1.58*	13.93 ± 0.31*
Group V	24.45 ± 2.23*	53.93 ± 0.69*	14.59 ± 0.46*

a: nmol NADPH breakdown/ min/ mg protein; b : nmol NADPH breakdown/ min/ mg protein; c : μ mole CDNB conjugate formed / min/ mg protein.
Values are mean ± SEM of 6 rats in each group. * Indicates p < 0.05 when compared with normal healthy control.

showed lesser gain in body and liver weight (Figs. 1 and 2). Rats which were consuming high amount of alcohol showed a lower body and liver weight due essentially to fat mass reduction. Reduced adipose tissue may be the foremost cause of lower body weight. As ethanol can supplement a large part of energy requirement therefore nutritional disorders may occur in these groups of rats.

Ethanol-induced toxicity is protected by ascorbic acid. But in the present study, high concentration of ethanol administration decreased ascorbate level in the liver of rats (Table I). The thiobarbituric acid reactive substances (TBARS) level of liver was increased, while the reduced glutathione (GSH) content of liver was decreased with increasing concentration of ethanol exposure. These may be due to enhancement in the production of oxygen-reactive species and/ or due to reduction in the level of endogenous antioxidants (4, 5, 6, 7, 19).

The amount of SOD is organ specific and it is abundant in hepatic tissue. Three types of SOD have been purified; CuZn-SOD, Mn-SOD (20) and extra cellular SOD (EC-SOD) (21). CuZn-SOD consists of two protein subunit each unit has an active site containing one Cu ion and one Zn ion (20). Cu ion serves as an active redox site and Zn ions maintain the protein structure (22). CuZn-SOD is found abundantly in cytosol. Mn-SOD is located in the mitochondrial matrix. It has four subunits with Mn in each subunit. EC-SOD is a tetrameric glycoprotein, which contains Cu and Zn. The presence of SOD in various compartments of our body enables SOD to dismutate superoxide radicals immediately. In the present work, superoxide dismutase activities increased on alcohol exposure. Over expression of SOD results in increased dismutation of superoxide to H_2O_2 . Evidence is accumulating that intermediates of oxygen reduction may in fact be associated with the development of alcoholic liver disease. Ethanol significantly increased superoxide dismutase (SOD) activity in liver (6). It may be due to increased Mn-SOD gene expression (23), and /or the increased activities of Cu, Zn-SOD in the liver (19).

Although Oh *et al* (7), reported significant decrease in glucose-6-phosphate dehydrogenase activity in ethanol treated groups compared to control, but in the present study, glucose-6-phosphate dehydrogenase activity was found to be unaltered in either of the groups tested. Catalase activity increased in lower concentration of alcohol exposure, but in higher concentration of alcohol exposure catalase activity decreased compared to control groups (Table II). This finding suggested that catalase activity in the liver is changeable (6, 19).

Ethanol exposure caused significant decrease in glutathione reductase (GR) and glutathione peroxidase

(GPx) activity and showed increase in glutathione S-transferase (GST) activity (Table III). A significant decrease in hepatic GR activity and glutathione level after ethanol treatment is indicative of impaired reduction of oxidized glutathione to reduced form (4). Decrease in glutathione peroxidase activity may be due to either free radical dependent inactivation of enzyme or depletion of its co-substrates i.e. GSH and NADPH in ethanol treated rats (5). Increase in glutathione s-transferase suggests its activation due to oxidative stress (25).

Our study revealed that higher doses of ethanol (1.6 g and 2g/ kg body weight/ day) caused deleterious effects on rats producing oxidative stress in the liver as a result of the generation of superoxide radicals by ethanol metabolism. Intermediates of oxygen reduction may in fact be associated with the development of alcoholic liver disease. Free radical mediated damage is aggravated by a reduction in cytoprotective enzymes and other antioxidants. In addition, other factors contribute to the reduced levels of natural antioxidants are nutritional deficiencies and malabsorption. Ethanol ingestion perturbs the antioxidant system in a dose and time dependent manner. Our future studies will focus on whether these effects will be reversed by supplementation of antioxidants or from abstinence from alcohol.

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