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OXIDATIVE STRESS IS THE PRIMARY EVENT: EFFECTS OF ETHANOL CONSUMPTION IN BRAIN

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ABSTRACT

Damaging effects of reactive oxygen species on living systems are well documented. They include oxidative attack on vital cell constituents. Chronic ethanol administration is able to induce an oxidative stress in the central nervous system. In the present study, 16-18 week-old male albino rats of Wistar strain were exposed to different concentration of ethanol for 4 weeks. This exposure showed profound effect on body weight. Ascorbic acid level; and activities of alkaline phosphatase and aspartate transaminase in the brain are dependent on the concentration of ethanol exposure. Chronic ethanol ingestion elicits statistically significant increase in thiobarbituric acid reactive substances level and decrease in gluatathione level in the brain. It reduces superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities in a dose dependent manner. However, histological examination could not reveal any pathophysiological changes. Therefore, we conclude that biochemical alterations and oxidative stress related parameters respond early in alcoholism than the histopathological changes in brain.

KEY WORDS

Ethanol, Brain, Oxidative stress, Glutathione, Transferase, Phosphatase.

INTRODUCTION

Ethanol is the most psychoactive substance used after caffeine. Chronic alcoholism is a major public health problem and causes multiorgan diseases and toxicity. Although the liver metabolizes the majority of ethanol ingested, it has intoxicating effects in the brain diseases (1). Chronic alcohol intake is associated with several degenerative and inflammatory processes in the central nervous system (CNS) (2). Evidence is accumulating that intermediates of oxygen reduction may be associated with the development of alcoholic disease (1).

The brain is deficient in oxidative defense mechanisms and hence is at great risk of damage mediated by reactive oxygen species (ROS) resulting in molecular and cellular dysfunction (3). The central nervous system (CNS) is vulnerable to free radical damage because of brain's high oxygen consumption, its abundant lipid content, and the relative paucity of antioxidant enzymes as compared with other tissues (4). Moreover, brain has a high ratio of membrane surface area of cytoplasmic ratio, extended axonal morphology prone to injury, and neuronal cells are non-replicating. ROS can increase the permeability of blood brain barrier, after tubulin formation, and inhibit the mitochondrial respiration. If unchecked it can lead to a geometrically progressing lipid peroxidation (5). Free radicals generated in the brain are also reported to influence gene expression, subsequently effecting apoptosis and neuronal death (6). The central nervous system is vulnerable to oxidative stress, especially when a toxicant can modify the physiological balance between anti- and pro-oxidant mechanisms (7).

The data generated on the neurological conditions is largely based on the experimental models. It is often not well established if an increase in free radicals initiates the disease process or is the sequel of the pathophysiological changes. That the free radical status is intimately linked to the degenerative processes in most neurological diseases, and

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its attenuation, so as to retard the vicious cycle of cell degeneration is a matter of great interest. In brain, an array of cellular defense systems exists to counterbalance the ROS. These include enzymatic and nonenzymatic antioxidants that lower the concentration of free radical species and repair oxidative cellular damage. Therefore, in the present study we investigated a dose dependent effect of ethanol on various oxidative stress and toxicity related biochemical parameters in brain, and results were analyzed with histological findings.

MATERIALS AND METHODS

16-18 week-old male albino rats of Wistar strain weighing 200-220g were used. The animals were housed in plastic cages inside a well-ventilated room. The room temperature was maintained at 25±2°C with a 12-h light/dark cycle. All rats had free access of standard diet (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

At the end of the experimental period, the animals were sacrificed by applying intra-peritoneal thiopentone (thiosol/ Na^+). The brain was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber. Various oxidative stress and toxicity related biochemical parameters were estimated. The Animal Ethics Committee of the Institution approved the procedures.

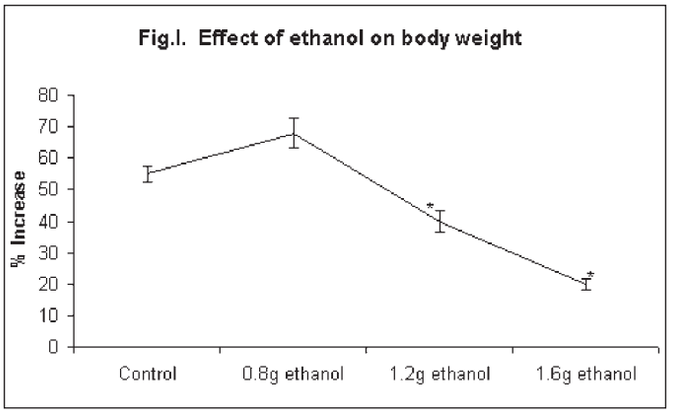
Brain was homogenized in 0.25M sucrose solution, diluted with 0.9% saline, and these diluted samples were then used for the estimation of tissue protein by the method of Lowry *et al* (9). Phosphatase [alkaline phosphatase (ALP), EC 3.1.3.1; acid phosphatase (ACP), EC 3.1.3.2 activities were measured using p-nitrophenyl phosphate as substrate (10). Activities of aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) of brain were measured by the method of Bergmeyer and Bernt (11).

Ascorbic acid (12) and reduced glutathione (GSH) content (13) of the tissue were estimated. Lipid peroxidation was estimated using Trichloroacetic acid (TCA)- Thiobarbituric acid (TBA)- Hydrochloric acid (HCl) (14). Catalase (EC 1.11.1.6) activity was determined as described by Das and Vasudevan (8). Activities of glutathione reductase (GR, EC 1.6.4.2) (15), glutathione S-transferase (GST; EC 2.5.1.18) (16), glutathione peroxidase (GPx, EC 1.11.1.9), (17) and superoxide dismutase (SOD, EC 1.15.1.1) activity were also measured (18). Pieces of brain were fixed in formalin, routinely processed and embedded in paraffin wax. Sections were cut at 5mm thickness, stained with haematoxylin and eosin and examined by light microscopy.

Results were expressed as mean±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 $p<0.05$.

RESULTS & DISCUSSION

In the present study, dose dependent effects of ethanol on oxidative stress and toxicity related biochemical parameters in brain tissues were investigated. Figure I is showing effects of ethanol on rat body weight. Rats that were consuming high amount of ethanol showed a lower increase in body weight due essentially to fat mass reduction. Reduced adipose tissue may be the foremost cause of lower body weight (19). Alcoholics have reduced brain weight compared with controls and the degree of brain atrophy correlates with the rate and amount of alcohol consumed over a lifetime (20). The reduction in brain weight and volume is largely accounted for by a reduction in white matter (21). However, no significant change in rat brain weight was observed in this study (Figure II).



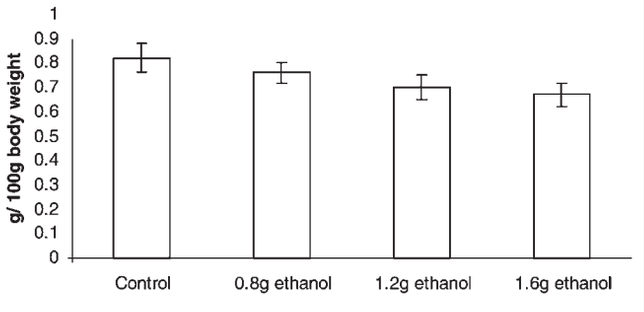
Values are mean±SEM of 6 rats in each group.
*Indicates $p< 0.05$ when compared to control group.

Table 1 : Effect of ethanol on protein content, and activities of acid phosphatase, alkaline phosphatase, aspartate transaminase, alanine transaminase in brain homogenate

Treatment	Protein	ACP	ALP	AST	ALT
	mg/ 100g tissue	U/100mg wet tissue		μg of pyruvate produced/ h/ mg tissue	
Control	9.1 ± 0.73	85.26 ± 4.26	65.21 ± 2.38	8.72 ± 0.34	4.06 ± 0.25
0.8g Ethanol	8.86 ± 0.57	87.12 ± 4.35	59.43± 2.96	9.08 ± 0.45	4.19 ± 0.29
1.2g Ethanol	8.46 ± 0.43	92.93 ± 5.54	52.16 ± 3.63*	9.87 ± 0.59	4.37 ± 0.23
1.6g Ethanol	8.14 ± 0.43	97.46 ± 5.82	44.70 ± 3.52*	10.32 ± 0.43*	4.48 ± 0.17

Values are mean±SEM of 6 rats in each group. *Indicates p< 0.05 when compared to normal healthy control.

Fig.II. Effect of ethanol on brain weight



Values are mean±SEM of 6 rats in each group.

Ethanol treatment in the present study caused insignificant depletion in protein content of brain (Table 1). The aspartate and alanine aminotransferases are important enzymes of brain; their activities are related with the maintenance of amino acid homeostasis and might be an indicator of mitochondrial injury (22). When rats were exposed to1.6 g ethanol/ kg body weight/ per day for 4 weeks, 10.5% increase in ALT level and 18.3% increase in AST level were observed; though significant increase observed in AST level (Table 1). Sedman *et al* (23) concluded that ethanol caused a significant increase in protein turnover in all sections of brain. Amino acid incorporation into brain protein has been shown to reduce upon ethanol administration (24).

Intracellular acid phosphatase (ACP) is largely confined to lysosomes, which primarily respond to cellular injury (25). Although ACP activity is observed to some extent in white matter in the brain, but it is found to be concentrated in the gray matter (26). Acid phosphatase activity insignificantly increased in the present study (Table 1). Alkaline phosphatase (ALP) is a membrane-associated enzyme, which predominantly concentrated in the vascular endothelium in the brain. However, in the present investigation, higher concentration (1.2g and 1.6 g ethanol/ kg body weight/ per day for 4 weeks) ethanol exposure contributed significant change in ALP activity (Table 1).

Effects of ethanol showed increased oxidative stress by decreasing the glutathione (GSH) level, ascorbate level and increasing thiobarbituric acid reactive substance (TBARS) in brain (Table 2). Under in *vivo* conditions, GSH acts as an antioxidant. The decrease in GSH levels represents increased utilization due to oxidative stress (27). Svensson et al (28) showed that ethanol can alter the brain extracellular ascorbic acid levels and that this effect seems to be attributed to ethanol itself. Carney et al (29) suggested that the elevated level of TBARS might be due to relatively high concentration of easily peroxidizable fatty acids in the brain. In addition, it is known that certain regions of the brain are highly enriched in iron, a metal that, in its free form, is catalytically involved in production of damaging oxygen free radical species (30). In this process,

Table 2 : Effect of ethanol on ascorbic acid, thiobarbituric acid reactive substances (TBRS), reduced glutathione content in brain homogenate

Treatment	Ascorbic acid (pmol/ μg tissue)	TBARS (μmol MDA formed/min/g tissue)	GSH (mg/ 100g tissue)
Control	58.24 ± 2.38	1.26 ± 0.09	35.19 ± 2.18
0.8g Ethanol	52.34 ± 3.16	1.76± 0.12*	29.87 ± 2.03
1.2g Ethanol	47.62 ± 2.56	2.12± 0.14*	21.46 ± 1.26*
1.6g Ethanol	46.13 ± 2.78*	2.61± 0.23*	15.20 ± 1.16*

Values are mean±SEM of 6 rats in each group. *Indicates p< 0.05 when compared to normal healthy control.

Table 3 : Effect of ethanol on glutathione peroxidase (GPx), glutathione s-transferase (GST) and glutathione reductase (GR) in brain homogenate

Treatment	GPx (U^a/ mg protein)	GST (U^b/ mg protein)	GR (U^a/ g tissue)
Control	3.41 ± 0.24	9.18 ± 0.49	2.53 ± 0.17
0.8g Ethanol	3.26 ± 0.17	8.69 ± 0.52	2.24 ± 0.19
1.2g Ethanol	2.46 ± 0.12*	6.56 ± 0.45*	1.62 ± 0.16*
1.6g Ethanol	2.01 ± 0.11*	4.43 ± 0.27*	0.87 ± 0.08*

a: *: nmol NADPH breakdown/ min,* b: m*mole CDNB conjugate formed/ min*
Values are mean±SEM of 6 rats in each group. *Indicates p< 0.05 when compared to normal healthy control.

the ferric iron is reduced by superoxide, with subsequent oxidation of ferrous iron by H_2O_2 forming hydroxyl radical:

$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$

Glutathione peroxidase activity was reduced by 30.7% and 40.9% respectively, and glutathione reductase activity was reduced by 35.9% and 65.6% respectively compared to control group when rats were exposed to 1.2g and 1.6 g ethanol/ kg body weight/ per day for 4 weeks (Table 3). Decreased glutathione reductase activities associated with thiol depletion are important factors sustaining a pathogenic role in alcohol-related pathologies (1, 31). Glutathione S-transferases (GST) mainly detoxifies electrophilic compounds (32). GST level in brain was reduced by 28.5% and 51.7% respectively when rats were exposed to 1.2g and 1.6 g ethanol/ kg body weight/ per day for 4 weeks (Table 3). Davenport et al (33) concluded that GST and GSH are sensitive and potentially relevant indicators of neurotoxicity. Yu (34) inferred that the depletion of GSH content might also lower the GST activity.

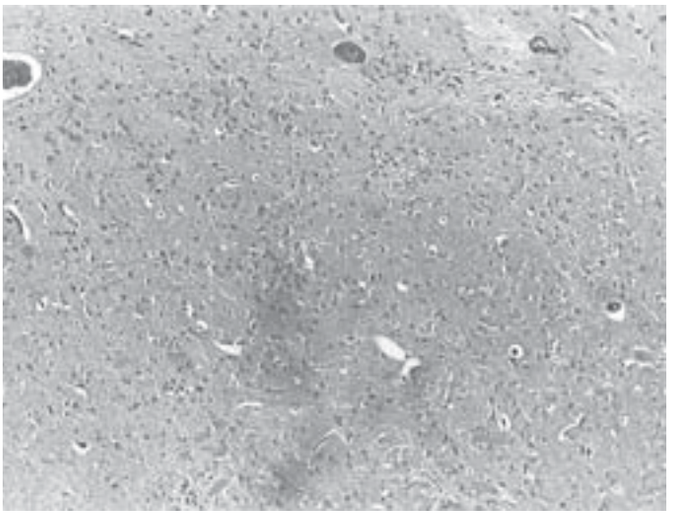
Ethanol can react with the $OH^{\bullet}$ radical to form the alpha-hydroxyethyl radical, which is considered to be less toxic. It also can stimulate H_2O_2 degradation through catalase activation (7). Significant decrease in catalase activity in brain was observed when rats were exposed to 1.2g and 1.6 g

Table 4 : Effect of ethanol on catalase and superoxide dismutase activity in brain homogenate

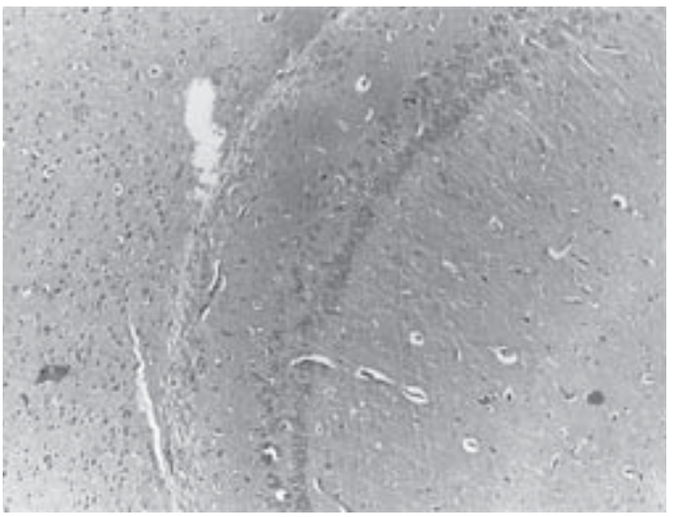
Treatment	Catalase (U^a/ mg protein)	SOD (U^b/ mg protein)
Control	3.12 ± 0.22	7.75 ± 0.39
0.8g Ethanol	2.84 ± 0.19	7.23 ± 0.42
1.2g Ethanol	2.23 ± 0.08*	6.28 ± 0.39
1.6g Ethanol	1.67 ± 0.14*	5.23 ± 0.41*

a: µ*mole H_2O_2 decomposed/ min; b: one unit of the enzyme is the amount of SOD capable of inhibiting 50% of the rate of pyrogallol exidation observed in the control.*
Values are mean±SEM of 6 rats in each group. *Indicates p< 0.05 when compared to normal healthy control.

Histological features of brain



Control group: H& E x100
Low power view of the brain showing normal neuron



1.6g ethanol/ kg bw/day for 4 weeks treated group:H & E x 100
Low power view of brain showing normal neurons in layers. No degenerative change is observed.

ethanol/ kg body weight/ per day for 4 weeks (Table 4). Aydin et al (35) showed that catalase activity significantly decrease only in brain tissues. However, high concentration of ethanol intake caused significant decrease in SOD level in brain.

Interestingly, histological examination of brain section showed normal neurons in layers without any degenerative change at high concentration (1.6 g ethanol/ kg body weight/ per day for 4 weeks) of ethanol exposure. This study suggested that 1.6 g ethanol/ kg body weight/ per day for 4 weeks can cause oxidative stress to the brain, but the same amount and duration of ethanol exposure was unable to produce degenerative changes. The changes in the antioxidant levels might be the adaptive response.

There are multiple mechanisms by which alcohol can damage the brain. Evidence has been accumulated indicating that chronic ethanol consumption leads to direct or indirect changes in the viability of central nervous system cells via oxidative stress. The outcome of the present study indicates that oxidative stress is the primary event compared to pathophysiological changes.

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