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Effect of glycine on oxidative stress in rats with alcohol induced liver injury

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We studied the effect of administering glycine on tissue lipid peroxidation and enzymic and non-enzymic antioxidants in experimental hepatotoxic Wistar rats. Hepatotoxicity was induced by administering ethanol for 30 days by intragastric intubation. Glycine administered at a dose of 0.6 g kg^{-1} body weight for 30 days significantly inhibited the severe oxidative stress as evidenced by the decreased levels of liver and brain thiobarbituric acid reactive substances (TBARS) and hydroperoxides compared to control. The activities of enzymic and non-enzymic antioxidants such as reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in the liver and brain were significantly elevated on glycine supplementation as compared to the untreated alcohol fed rats. The levels of serum vitamin E and vitamin C were also increased to near normal levels on glycine treatment. Microscopic examination of alcohol treated rat liver showed inflammatory cell infiltrates and fatty changes, which were alleviated on treatment with glycine. Alcohol treated rat brain demonstrated oedma, which was significantly lowered on treatment with glycine. Thus our study shows that administering glycine to alcohol supplemented rats, markedly reduced the oxidative stress and elevated the enzymic and non-enzymic antioxidants in the liver and brain, which a was associated with a reversal of hepatic steatosis and cerebral oedma.

1. Introduction

Free radicals and associated oxidative stress have been implicated in eliciting pathological changes including atherosclerosis (Parathasarathy 1989), chronic alcohol toxicity (Dicker 1988), muscular dystrophy (Murphy 1989), rheumatoid arthritis (Chapmen 1989), ageing (Bunker 1992) and neurological disorders (Starke-Reed 1989). Alcoholic liver injury appears to be generated by the metabolism of ethanol and the toxic effects of acetaldehyde (Ishak 1991). Ethanol is also known to induce hyperlipidemia leading to enhanced lipid peroxidation (Baraona 1979). Increased lipid peroxidation has long been known to cause functional degeneration in various tissues (Suja 1997).

Cells have many different ways to protect themselves against the constant threat of free radicals produced during the peroxidation of lipids. There are a number of enzymic and non-enzymic antioxidants that act to minimize the detrimental effects of free radicals (Yagi 1991). Four enzymes, which often function in concert, are crucial to the intracellular detoxification of endogenous free radicals. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) convert radicals and peroxides to innocuous reduced forms, often with the concomitant oxidation of reduced glutathione (GSH) to its oxidized form (Das 1980). Other major non-enzymic antioxidant compounds that have received considerable attention are vitamin E and vitamin C (Gutteridge 1995).

Glycine is a dietary non-essential amino acid that can be readily synthesized from common metabolic intermediates in all organisms. Glycine is involved in many reactions such as gluconeogenesis, purine, haem and chlorophyll synthesis and bile acid conjugation (Gehan et al. 1996). Glycine lowers the rate of gastric emptying of ethanol resulting in the suppression of its absorption from the gastrointestinal tract (Akao 1995). In an *in vivo* study of ethanol induced liver injury using the Tsukamoto-French model with a design where alcohol and glycine were given together, glycine lowered ethanol concentration in the stomach and minimized liver damage (Iimuro 1996). Glycine derivatives are also known to decrease considerably the activation of lipid peroxidation in stress, reduce the duration of the alarm stage of stress, reaction and limit stress damage to the heart (Malyshev et al. 1996). Glycine is said to activate chloride channels in Kupffer cells, which hyperpolarizes the cell membrane and blunts intracellular Ca^{2+} concentration, similar to its action in the neurons and also decreases the levels of superoxide ions from neutrophils via glycine gated chloride channels (Wheeler et al. 2000). Glycine prevents hepatic cancer and certain melanomas *in vivo* by inhibiting endothelial cell proliferation and angiogenesis (Ikejima 1996). In addition, glycine given orally to schizophrenic patients to facilitate glutamatergic transmission at the level of *N*-methyl-D-aspartate receptor complex improved their muscle stiffness and extrapyramidal symptoms (Rosse et al. 1989).

Table 1: Effect of glycine on tissue TBARS and hydroperoxides of the control and experimental animals

Group	TBARS (mM/100 g tissue)		Hydroperoxides (mM/100 g tissue)	
	Liver	Brain	Liver	Brain
Control	27.58 ± 1.15^{a}	26.59 ± 1.40^{a}	63.28 ± 5.88^{a}	43.26 ± 3.18^{a}
Control + treatment	26.54 ± 1.04^{a}	26.72 ± 1.02^{a}	62.86 ± 4.82^{a}	42.64 ± 4.06^{a}
Alcohol	58.92 ± 4.76^{b}	67.94 ± 6.05^{b}	82.58 ± 6.51^{b}	69.12 ± 4.62^{b}
Alcohol + treatment	32.04 ± 3.37^{a}	25.96 ± 1.00^{a}	58.54 ± 2.27^{a}	50.42 ± 5.10^{a}
F-ratio	$30.44^{\#}$	$249.50^{\#}$	$10.28^{\#}$	$11.34^{\#}$

Values are mean ± SD of ten rats from each group
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)
# $p < 0.05$ (ANOVA)

Table 2: Effect of glycine on tissue GSH and GPx of the control and experimental animals

Group	GSH (mM/mg protein)		GPx (μg of GSH utilized /min/mg protein)	
	Liver	Brain	Liver	Brain
Control	17.48 ± 1.46^{a}	36.36 ± 1.83^{a}	13.80 ± 1.27^{a}	7.80 ± 0.53^{a}
Control + treatment	18.28 ± 1.68^{a}	38.84 ± 2.87^{a}	14.86 ± 1.34^{a}	7.25 ± 0.57^{a}
Alcohol	10.86 ± 1.43^{b}	13.26 ± 1.33^{b}	5.86 ± 0.53^{b}	2.84 ± 0.24^{b}
Alcohol + treatment	15.86 ± 1.56^{a}	31.18 ± 3.18^{a}	12.92 ± 0.86^{a}	7.12 ± 0.19^{a}
F-ratio	$14.24^{\#}$	$39.71^{\#}$	$63.67^{\#}$	$102.08^{\#}$

Values are mean ± SD of ten rats from each group
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)
# $p < 0.05$ (ANOVA)

On the basis of the ever-increasing list of the advantageous role of glycine we planned the present study. Our aim was to elucidate the antiperoxidative property of glycine on ethanol induced oxidative damage and its efficacy to inhibit lipid peroxidation in a rat model.

2. Investigations and results

We studied the effect of glycine on tissue lipid peroxidation and enzymic and non-enzymic antioxidant in experimental hepatotoxic Wistar rats. Hepatotoxicity was induced by administering ethanol (7.9 g kg^{-1} body weight) for 30 days by intragastric intubation. Simultaneously control rats were given isocaloric glucose solution. Glycine was subsequently administered at a dose of 0.6 g kg^{-1} body weight every day by intragastric intubation to both control and alcohol fed rats for the next 30 days.

Table 1 shows the concentration of tissue thiobarbituric acid reactive substances (TBARS) and hydroperoxides of control and experimental animals. TBARS and hydroperoxide levels in the liver and brain of rats receiving alcohol (group 3) were significantly higher than those of the control rats (group 1). Treatment with glycine to control rats (group 2) did not alter the TBARS and hydroperoxide concentration significantly, whereas TBARS and hydroperoxides level were lowered significantly both in the liver and brain on treatment with glycine to rats on alcohol supplementation (group 4).

As shown in Table 2 the concentration of GSH and GPx were significantly lower in liver and brain of rats receiving alcohol (group 3) than in control rats (group 1). GSH and GPx values did not alter significantly on treatment with glycine to control rats (group 2) whereas administration of glycine to alcohol treated rats (group 4) significantly elevated their activities as compared with those that on alcohol treatment alone.

Table 3 shows the activities of SOD and CAT in the tissues of control and experimental animals. SOD and CAT activities in the liver and brain of rats on alcohol supplementation (group 3) were significantly lower than the control rats (group 1). Treatment with glycine to control rats (group 2) did not alter significantly the SOD and CAT activities as compared to control rats (group 1) whereas administration of glycine to alcohol treated rats

Table 3: Effect of glycine on tissue SOD and CAT of the control and experimental animals

Group	SOD (Units♣)		CAT (μM of H_2O_2 consumed/min/mg protein)	
	Liver	Brain	Liver	Brain
Control	5.82 ± 0.68^{a}	8.24 ± 0.20^{acd}	76.18 ± 3.70^{acd}	1.91 ± 0.43^{a}
Control + treatment	6.03 ± 0.48^{a}	8.82 ± 0.33^{ac}	84.38 ± 3.84^{ac}	1.98 ± 0.14^{a}
Alcohol	1.99 ± 0.12^{b}	5.24 ± 0.32^{b}	49.12 ± 3.68^{b}	1.54 ± 0.65^{b}
Alcohol + treatment	5.90 ± 0.23^{a}	7.56 ± 0.52^{ad}	73.06 ± 4.76^{ad}	1.82 ± 0.34^{a}
F-ratio	$5.44^{\#}$	$51.38^{\#}$	$36.78^{\#}$	$25.11^{\#}$

♣ – Enzyme concentration required to inhibit optical density at 560 nm of chromogen produced by 50% NBT reduction/min/mg protein
Values are mean ± SD of ten rats from each group
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)
# $p < 0.05$ (ANOVA)

Table 4: Effect of glycine on serum vitamin E and vitamin C levels of the control and experimental animals

Group	Vitamin C (mg/dl)	Vitamin E (mg/dl)
Control	2.08 ± 0.21^{a}	2.87 ± 0.26^{a}
Control + treatment	2.06 ± 0.20^{a}	2.82 ± 0.20^{a}
Alcohol	1.03 ± 0.06^{b}	1.38 ± 0.09^{b}
Alcohol + treatment	1.82 ± 0.18^{a}	2.54 ± 0.31^{a}
F-ratio	$59.90^{\#}$	$21.68^{\#}$

Values are mean ± SD of ten rats from each group
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)
# $p < 0.05$ (ANOVA)

(group 4) significantly elevated their activities as compared with those on alcohol treatment alone.

Vitamin E and vitamin C levels in the serum were significantly lower in rats receiving alcohol (group 3) than the control rats (group 1) as shown in Table 4. Significantly elevated levels of vitamin E and C were observed on treatment with glycine to rats on alcohol supplementation as compared with those of the control (group 1) and unsupplemented alcohol treated rats (group 2).

In the alcohol treated rat liver, fatty changes of both macro and microvasicular type and sinusoidal dilation were observed in all fields (Fig. 1).

The liver of alcohol treated rats which received 0.6 g kg^{-1} body weight of glycine showed loss of individual hepatocytes by degeneration and the space where the cell had originally been appeared empty, but there was no evidence of fatty change (Fig. 2). The liver of control rats, which received 0.6 g kg^{-1} body weight of glycine, showed only focal areas of fatty changes. But not to the extent seen in the liver of rats treated with alcohol only (Fig. 3). Control liver demonstrated normal liver morphology (Fig. 4).

The brain tissue in alcohol treated rats showed oedma, which was not evident in rats treated with glycine (Figs. 5, 6). Brain tissue of control rats treated with glycine revealed a normal pattern (Figs. 7, 8).

3. Discussion

Increasing experimental evidence suggests that oxygen derived radicals play a crucial role in ethanol toxicity (Reitz 1975). One of the consequences of free radical formation and oxidative stress is the enhancement of lipid peroxidation, a process leading to the oxidation of polyunsaturated fatty acids to lipid peroxides. Production of free radicals and progression of peroxidation are however

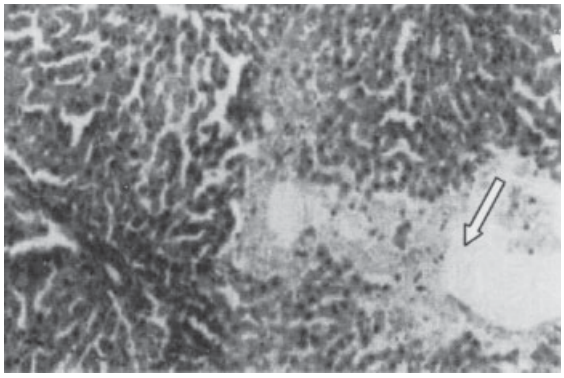

Fig. 2: Liver of alcoholic rat treated with glycine: Arrow indicates hepatocyte drop out, (⟹) fatty changes were markedly reduced. H & E × 10

inhibited in cells by the presence of antioxidants. Ineffective scavenging of free radicals due to depletion of antioxidants play a crucial role in cell injury (Pryor 1973). In our study, significantly elevated levels of TBARS and hydroperoxides in the liver and brain were seen in rats under alcohol treatment. These results are in agreement with the observations of previous researchers (Rajakrishnan 1996). In our initial studies we have also observed elevated RBC membrane lipid peroxidation on chronic alcohol supplementation (Senthilkumar 2003). Nutrients that neutralize alcohol by-products and protect cells against the damaging effects of alcohol include vitamin E and C, cysteine, glycine, glutathione and thiol compounds. On supplementing glycine to alcohol treated rats we observed significantly decreased levels of TBARS and hydroperoxides.

Previous studies show that on chronic alcohol administration hepatic GSH levels were markedly decreased (Rajakrishnan 1996). Our results are in line with the above findings. Glycine supplementation to alcohol treated rats helped to restore the liver and brain GSH and GPx levels close to those of the control rats. In this regard, studies have shown that the carboxy terminal glycine moiety of GSH is known to protect the molecule against cleavage by intracellular γ-glutamyl cyclotransferase. Thus glycine intake prevents intracellular degradation of GSH, and if at all metabolized, it is done so only extracellularly (Ilutter 1997).

Our results also show decreased activities of SOD and CAT in the liver and brain of alcohol treated rats. Inhibition of CAT activity by ethanol may cause the accumulation of hydrogen peroxide or products of its decomposition. Loss of SOD and CAT activities also results in

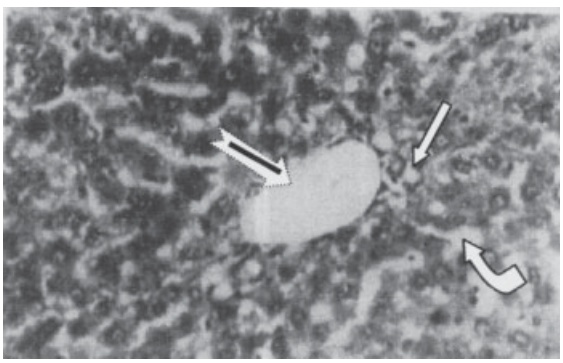

Fig. 1: Liver of alcoholic rat: Arrow indicates fatty changes of macrovesicular type (⟹), microvesicular type (⇝) and sinusoidal dilation (⟹). H & E × 10

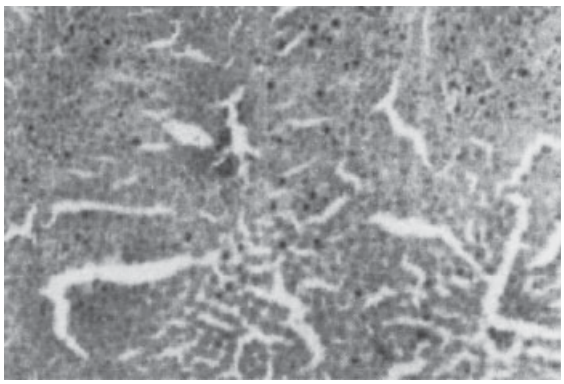

Fig. 3: Liver of control rat treated with glycine: H & E × 10

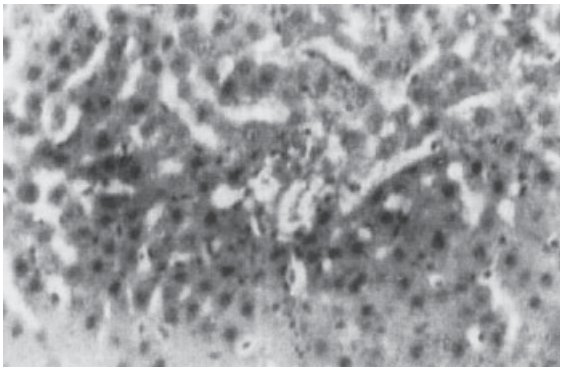

Fig. 4: Liver of control rat: H & E × 10

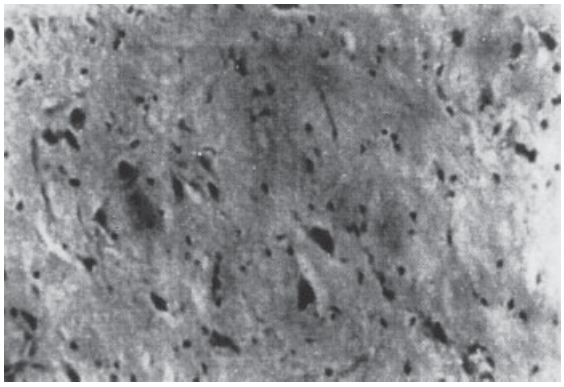

Fig. 6: Brain of alcoholic rat treated with glycine: Oedma is markedly reduced. H & E × 10

oxygen intolerance and triggers a number of deleterious reactions (Fridovich 1989). It is clear that cytotoxicity of molecular oxygen is determined by the delicate balance between rates of the partially reduced oxygen species and the rates of their removal by the different defense mechanisms, any shift in this balance can lead to cellular damage (Paul 1989). The observed restoration of the SOD and CAT activities on glycine supplementation may be due to a direct stimulatory effect of glycine on SOD and CAT. The decrease in the tissue lipid peroxidation on glycine treatment can also be correlated with the elevated SOD and CAT activities.

One of the most effective natural, primary antioxidant is α-tocopherol. This fat-soluble vitamin is important because the most significant free radical damage to the body is damage to the cell membrane and to low density lipoproteins, which are composed of lipids (McCay 1985). Decreased concentration of vitamin E observed on alcohol supplementation may be due to its increased utilization in scavenging the oxygen radicals or decreased vitamin C concentration because there is a well established relationship between vitamins E and C (Sevanian 1985). Vitamin C is a cofactor in many biological processes including the conversion of dopamine to noradrenaline, in the hydroxylation steps, in the synthesis of adrenal steroid hormones, in resistance to infection and in cellular respiration. Vitamin C may act as a free oxygen radical scavenger. Both vitamin E and C are highly efficient at mopping up free radicals and sometimes even mutually co-operate in doing so (McCay 1985).

In our study, the concentration of vitamin C and vitamin E in alcohol supplemented animals was significantly lower than in control animals. In this context, vitamin C levels are known to be reduced in the brain, liver and kidney of rats with acute ethanol poisoning (Sevanian 1985). Glycine feeding to alcohol supplemented animals elevated the vitamin E and vitamin C levels. The increase in vitamin E and vitamin C levels on glycine supplementation may be the cause for the decreased oxidative stress. We observed no marked changes when glycine was fed alone.

Significant pathomorphological alterations in the liver and brain were observed in alcohol treated rats. These changes can alter the properties of a cell. The microscopic changes observed in the liver of alcohol treated rats were predominant in the centrilobular region. Hepatic damage observed may be partially attributed to cytochrome-P_{450} generated metabolic cytochrome-P_{450} dependant enzyme activities in liver that tend to be present at their greatest concentration near the central vein, and lowest near the peripheral sites (Pieffer 1979). Supplementing glycine to alcohol treated rats reduced the fatty change and improved the histomorphology of the liver.

Microdysplasia and spongioform changes have been demonstrated in the hypothalamic and thalamic regions of the brain of alcohol treated rats (Sarker 1995). These are indicative of local brain development disorders. In the present study, we observed oedma in the brain of alcohol treated rats, which was reversed on treatment with glycine.

The findings of the present study show that increased oxidative stress associated with decreased antioxidant levels in alcohol fed rats are mitigated by glycine administration. The observed data are highly significant which demonstrates the potential efficacy of glycine in protecting the tissues from peroxidative damage.

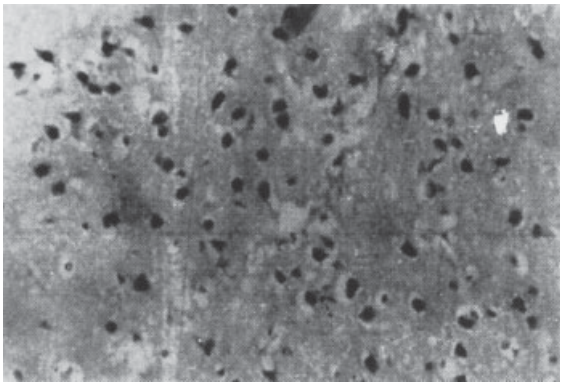

Fig. 5: Brain of alcoholic rat: Shows oedma: H & E × 10

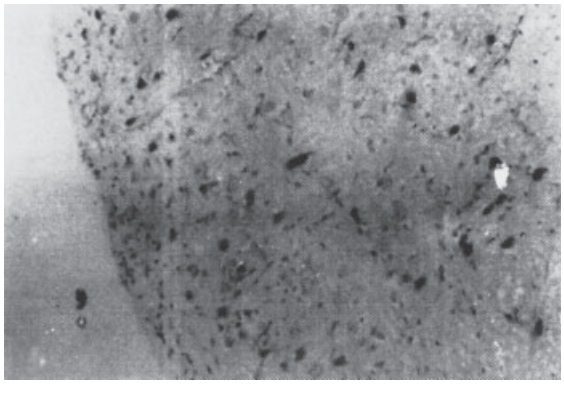

Fig. 7: Brain of control rat: H & E × 10

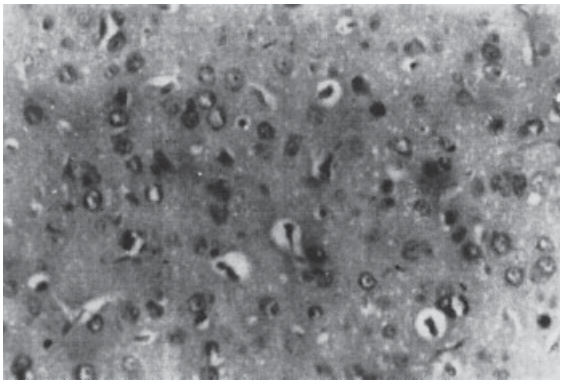

Fig. 8: Brain of control rat treated with glycine: H & E × 10

4. Experimental

Male albino rats weighing 150 to 170 g were procured from the Department of Experimental Medicine, Raja Muthiah Medical College and Hospital, Annamalainagar and were maintained in polypropylene cages in a controlled environment (22–24 °C) under a 12 h light-dark cycle. Standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water were provided *ad libitum*. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institute Guide 1985).

1,1′,3,3′-tetra ethoxy propane, reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADP), phenazine metho sulphate (PMS), nitroblue tetrazolium (NBT) and 5,5′ dithio-bis-2-nitro benzoic acid (DTNB) were purchased from Sigma Chemical Co, St. Louis, MO, USA. Ethanol was obtained from Nellikuppam, Cuddalore District, South India. Glycine was purchased from S.D. Fine Chemicals Ltd., Mumbai, India. Other chemicals used were of analytical grade and were obteined from Central Drug House, New Delhi, India.

The animals were divided into four groups of ten rats each and all were fed the standard pellet diet. Groups 1 and 2 received standard pellet diet and isocaloric glucose from a 40% glucose solution. Liver cell damage was induced in rats of groups 3 and 4 by giving 20% ethanol, 5 ml each (2.5 ml in the forenoon and 2.5 ml in the afternoon) i.e., equivalent to 7.9 g kg^{-1} body weight as an aqueous solution using and intragastric tube daily for 30 days (Senthilkumar 2002). At the end of this period the animals were treated as follows for the next 30 days.

Group 1: Control rats continued to receive isocaloric glucose from a 40% glucose solution daily by intragastric intubation.

Group 2: Rats continued to receive isocaloric glucose from a 40% glucose solution daily and they also received glycine at a dose of 0.6 g kg^{-1} body weight in distilled water by intragastric intubation (Ming et al. 1998).

Group 3: Rats continued to receive 20% ethanol.

Group 4: Rats continued to receive 20% ethanol plus 0.6 g kg^{-1} body weight of glycine in distilled water by intragastric intubation (Senthilkumar 2003).

The total duration of the experiment was 60 days, at the end of which the animals were anaesthetized using light ether and killed by cervical decapitation. Blood was collected and processed for the determination of vitamin E and vitamin C. Liver and brain were cleared of adhering fat weighed accurately and used for the preparation of homogenates.

Vitamin E and vitamin C was estimated by the method of Baker et al. (1980) and Roe and Keuther (1943), respectively. The concentration of TBARS was estimated in the tissues by the method of Okhawa et al. (1979). Hydroperoxides in tissues were determined by the method of Jiang et al. (1992). SOD (EC. 1.15.1.1) activity in the tissues was assayed by the method of Kakkar et al. (1984). CAT (EC. 1.11.1.6) activity was assayed by the method of Sinha (1972). GSH was determined by the method of Ellman (1959). GPx (EC. 1.11.1.9) was determined by the method of Rotruck et al. (1973). Tissue proteins were estimated by the method of Lowry et al. (1951).

All the grouped data were evaluated statistically and the significance of changes caused by the treatment was determined using ANOVA followed by Duncan's Multiple Range Test (DMRT) (Duncan 1957). Results are presented as means ± SD of ten rats from each group. The statistical significance was set at $p < 0.05$.

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