

GLYCINE MODULATES HEPATIC LIPID ACCUMULATION IN ALCOHOL-INDUCED LIVER INJURY

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We studied the effect of administering glycine, a non-essential amino acid, on serum and tissue lipids in experimental hepatotoxic Wistar rats. All the rats were fed standard pellet diet. Hepatotoxicity was induced by administering ethanol (7.9 g kg⁻¹) for 30 days by intragastric intubation. Control rats were given isocaloric glucose solution. Glycine was subsequently administered at a dose of 0.6 g kg⁻¹ every day by intragastric intubation for the next 30 days. Average body weight gain at the end of the total experimental period of 60 days was significantly lower in rats supplemented with alcohol, but improved on glycine treatment. Feeding alcohol significantly elevated the levels of cholesterol, phospholipids, free fatty acids and triglycerides in the serum, liver and brain as compared with those of the control rats. Subsequent glycine supplementation to alcohol-fed rats significantly lowered the serum and tissue lipid levels to near those of the control rats. 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 edema, which was significantly lowered on treatment with glycine. In conclusion, this study shows that oral administration of glycine to alcohol-supplemented rats markedly reduced the accumulation of cholesterol, phospholipids, free fatty acids and triglycerides in the circulation, liver and brain, which was associated with a reversal of steatosis in the liver and edema in the brain.

Key words: *cholesterol, ethanol, free fatty acids, glycine, lipids, phospholipids, triglycerides*

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INTRODUCTION

Alcohol is the most frequently abused psychoactive drug throughout the world and has been known in all civilizations since ancient times [16, 32]. Alcoholism is associated with numerous degenerative and inflammatory disorders affecting many organs including the liver, brain, kidney, heart, nerves, skeletal muscles and pancreas [6]. Steatosis, inflammation, necrosis, fibrosis and finally cirrhosis characterize the progression of alcoholic liver disease. When severe hepatitis occurs, death is the outcome [8].

Ethanol is known to have a profound effect on the metabolism of lipids and lipoproteins. Accumulation of lipids in the hepatocytes is the most striking initial manifestation of alcohol-induced liver injury [18]. In chronic lipid accumulation, the liver cells become fibrotic leading to impaired liver function. Enhanced lipid peroxidation has also been reported in ethanol-induced hyperlipidemia [1, 19].

Glycine helps convert many potentially harmful substances including toxic phenolic materials, such as benzoic acid (sodium benzoate) into harmless forms. It is important in the control of gluconeogenesis in the liver, and thus the blood sugar homeostasis. Glycine serves as a basic nitrogen source for the production of many amino acids and is useful in the synthesis of hemoglobin, glutathione, DNA and RNA. In addition, glycine has been found to be important as a part of the brain neurotransmission pathway. It is recognized to be a neuroinhibitory neurotransmitter along with GABA [23].

Glycine is also known to protect kidney proximal tubules [21] and hepatocytes [25] against hypoxia. Feeding glycine totally prevented mortality and markedly reduced lipopolysaccharide (LPS)-induced elevation of serum transaminase levels, hepatic necrosis and lung injury in rats. The elevation in serum tumor necrosis factor- α due to LPS was also blunted and delayed significantly [14]. In an *in vivo* study of ethanol-induced liver injury, glycine minimized liver damage and also decreased ethanol concentration in the stomach [13].

An effective, economical and simple treatment for reversal of liver injury when patients stop alcohol consumption could have a significant clinical impact. This study was designed to explore a possible new strategy to improve recovery from early alcoholic liver injury in the rat. Glycine, a non-toxic amino acid, might be a useful tool when used in

case of the prognosis of liver diseases. In the present study, we have evaluated the effect of oral administration of glycine on serum and tissue lipids in rats with alcoholic liver injury.

MATERIALS and METHODS

Ammonium molybdate, sodium metaperiodate, acetylacetone and 1-amino-2-naphthol-4-sulfonic acid (ANSA) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Ethanol was obtained from Nellikuppam, Cuddalore District, South India. Glycine was obtained from S.D. Fine Chemicals Ltd., Mumbai, India. All other chemicals and solvents were of analytical grade and purchased from Central Drug House, Mumbai, India.

Sixty healthy, male adult Wistar rats (150–170 g) were procured from the Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University. They were housed in plastic cages with filter tops under controlled conditions of a 12 h light/12 h dark cycle, 50% humidity and 28°C. They all received standard pellet diet (Lipton Lever Ltd., Mumbai, India) and water *ad libitum*. The animals were maintained as per the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on animal care and use (Register Number: 160/1999/CPCSEA) [24].

The animals were divided into four groups of fifteen rats each. Groups 1 and 2 received a normal diet of standard pellets and isocaloric glucose from a 40% glucose solution. Liver cell damage was induced in rats of groups 3 and 4 by administering 20% ethanol (2.5 ml in the morning and 2.5 ml in the afternoon), equivalent to 7.9 g kg⁻¹ as an aqueous solution [28, 36] by intragastric intubation for 30 days. At the end of this period, the animals were treated as follows for the next 30 days.

Group 1. Experimental control rats continued to receive standard pellet diet and isocaloric glucose from a 40% glucose solution daily by intragastric intubation.

Group 2. Control rats continued to receive standard pellet diet and isocaloric glucose from a 40% glucose solution and in addition glycine (0.6 g kg⁻¹ in water) by intragastric intubation daily.

Group 3. Rats continued to receive standard pellet diet and 20% ethanol.

Group 4. Rats continued to receive standard pellet diet and 20% ethanol and in addition glycine

(0.6 g kg⁻¹ in water) by intragastric intubation daily [37].

The total duration of the experiment was 60 days, at the end of which part of the animals were fasted overnight, anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg kg⁻¹) and sacrificed by decapitation. Blood collected from the carotid artery was allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm and used for the estimations of phospholipids, triglycerides, free fatty acids and cholesterol. Liver and brain were cleared of adhering fat, weighed accurately and used for lipid extraction. Lipids were extracted from tissues as described previously by Folch et al. [10]. Total cholesterol was estimated by the method of Sackett [33]. Phospholipids, free fatty acids and triglycerides were determined by the method of Zilversmit and Davis [44], Falholt et al. [7] and Foster and Dunn [11], respectively.

Histological analysis

The remaining animals were subjected to whole-body perfusion using normal saline and 10% formalin under light ether anesthesia. Brain and liver

were removed and stored immediately in 10% formalin. The tissues were subsequently embedded in paraffin, thinly sectioned using a microtome (5 µm), stained with hematoxylin and eosin (H&E) and mounted in neutral distyrene-dibutyl phthalate-xylene (DPX) medium and examined by light microscopy [3].

Statistical analysis

All the grouped data were evaluated statistically and the significance of changes caused by the treatment was determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) [2] by using SPSS 9.05 for Windows. The results are expressed as means ± SD of ten rats from each group. The level of statistical significance was set at $p < 0.05$.

RESULTS

Table 1 shows the average weight gained by the rats during the total experimental period of 60 days. The final body weights of alcohol-treated rats (Group 3) were significantly lower than those of the control animals (Group 1). Treatment with gly-

Table 1. Average weight gain by the animals during the experimental period of 8 weeks

Groups	Initial total body weight (g)	Final total body weight (g)	Liver weight (g)	Liver weight/total body weight (%)
Control	135 ± 10.80	222 ± 8.53	3.30 ± 0.33	3.70
Control + Glycine	132 ± 8.31	205 ± 10.80	3.47 ± 0.39	4.70
Alcohol	140 ± 10.60	162 ± 8.53 [#]	4.61 ± 0.76 [#]	27.11
Alcohol + Glycine	137 ± 10.57	190 ± 10.82	3.47 ± 0.38	6.00
F-ratio	1.14	21.46*	3.81*	

Values are means ± SD of ten rats from each group. [#] Significant at $p < 0.05$ (DMRT). * $p < 0.05$ (ANOVA)

Table 2. Effect of glycine on cholesterol and free fatty acids in tissues of the control and experimental rats

Groups	Cholesterol (mg/100 g of tissue)		Free fatty acids (mg/100 g of tissue)	
	Liver	Brain	Liver	Brain
Control	286.35 ± 26.39	666.33 ± 55.49	641.00 ± 35.22	259.01 ± 21.79
Control + glycine	300.54 ± 28.80	687.00 ± 59.63	656.00 ± 52.65	274.63 ± 7.66
Alcohol	549.10 ± 47.74 [#]	854.96 ± 66.81 [#]	1383.00 ± 115.36 [#]	768.99 ± 53.20 [#]
Alcohol + glycine	362.16 ± 27.31	736.83 ± 63.82	736.00 ± 64.76	283.78 ± 22.05
F-ratio	34.54*	8.66*	120.71*	264.12*

Values are means ± SD of ten rats from each group. [#] Significant at $p < 0.05$ (DMRT). * $p < 0.05$ (ANOVA)

Table 3. Effect of glycine on phospholipids and triglycerides in tissues of the control and experimental rats

Groups	Phospholipids ($\mu\text{g/g}$ of tissue)		Triglycerides ($\text{mg}/100$ g of tissue)	
	Liver	Brain	Liver	Brain
Control	24.28 ± 2.80	18.03 ± 1.91	232.45 ± 20.47	251.00 ± 25.04
Control + glycine	25.79 ± 2.66	19.94 ± 0.91	253.94 ± 23.94	258.01 ± 21.18
Alcohol	$51.46 \pm 3.43^{\#}$	$37.55 \pm 2.09^{\#}$	$634.24 \pm 46.93^{\#}$	$706.38 \pm 70.14^{\#}$
Alcohol + glycine	25.48 ± 2.51	20.42 ± 1.80	255.82 ± 17.85	252.16 ± 16.39
F-ratio	80.42*	132.11*	206.56*	176.64*

Values are means \pm SD of ten rats from each group. $^{\#}$ Significant at $p < 0.05$ (DMRT). * $p < 0.05$ (ANOVA)

Table 4. Effect of glycine on phospholipids, triglycerides, free fatty acids and cholesterol in serum of the control and experimental rats

Groups	Phospholipids (mg/dl)	Triglycerides (mg/dl)	Free fatty acids (mg/dl)	Cholesterol (mg/dl)
Control	87.43 ± 4.91	42.09 ± 3.66	81.22 ± 7.28	93.34 ± 3.36
Control + glycine	89.54 ± 7.25	46.66 ± 1.01	87.74 ± 5.34	98.48 ± 3.93
Alcohol	$153.74 \pm 4.02^{\#}$	$85.68 \pm 2.96^{\#}$	$150.40 \pm 8.33^{\#}$	$160.58 \pm 8.89^{\#}$
Alcohol + glycine	93.69 ± 7.29	46.44 ± 3.51	89.39 ± 6.68	108.36 ± 9.11
F-ratio	80.42*	132.11*	206.56*	176.64*

Values are means \pm SD of ten rats from each group. $^{\#}$ Significantly at $p < 0.05$ (DMRT). * $p < 0.05$ (ANOVA)

cine along with alcohol (Group 4) improved the weight gain significantly. Control rats supplemented with glycine (Group 2) did not show any significant change in weight gain. The ratio of liver weight to the total body weight (final) showed a significant increase on alcohol treatment, and was lowered significantly on glycine supplementation.

Table 2 highlights the concentration of cholesterol and free fatty acids in the liver and brain of control and experimental animals. Cholesterol and free fatty acid levels were significantly higher in the liver and brain of alcohol-treated animals (Group 3) as compared with those of the control group (Group 1). Treatment with glycine (0.6 g kg^{-1}) (Group 4) of alcohol-supplemented rats reduced the levels of cholesterol and free fatty acids significantly as compared with the untreated alcohol-supplemented rats. Control rats supplemented with glycine (Group 2) did not show any significant change in the concentrations of free fatty acids and cholesterol in the liver and brain.

Table 3 shows the concentration of phospholipids and triglycerides in the liver and brain of control and experimental animals. The concentra-

tions of phospholipids and triglycerides were significantly higher in alcohol-treated animals (Group 3) as compared with those in control rats (Group 1). Treatment with glycine (0.6 g kg^{-1}) of alcohol-supplemented rats (Group 4), significantly lowered the levels of liver and brain triglycerides and phospholipids as compared with those of the alcohol-treated rats which did not receive glycine (Group 3). Control rats supplemented with glycine (Group 2) did not show any significant change in the concentrations of triglycerides and phospholipids in the liver and brain.

Table 4 shows the concentration of serum phospholipids, triglycerides, cholesterol and free fatty acids of control and experimental animals. The concentrations of phospholipids, triglycerides, cholesterol and free fatty acids were significantly higher in alcohol-treated animals (Group 3) as compared with those in control rats (Group 1). Treatment with glycine (0.6 g kg^{-1}) to alcohol-supplemented rats (Group 4), significantly lowered the levels of serum triglycerides, phospholipids, cholesterol and free fatty acids as compared with those in the alcohol-treated rats which did not re-

ceive glycine (Group 3). Control rats supplemented with glycine (Group 2) did not show any significant change in the concentrations of triglycerides, phospholipids, cholesterol and free fatty acids in the serum.

Histopathological findings

In the liver of alcohol-treated rats, fatty changes of both macro- and microvesicular type and sinusoidal dilation were observed in all fields (Fig. 3).

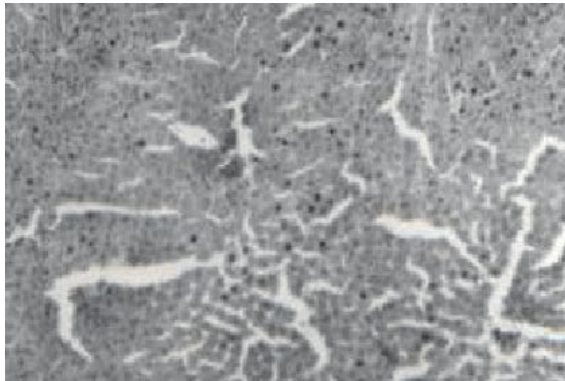


Fig. 1. Liver of control rat: H & E $\times 10$

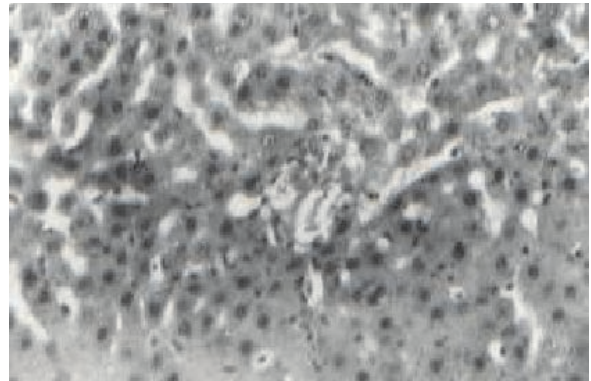


Fig. 2. Liver of control rat treated with glycine: H & E $\times 10$

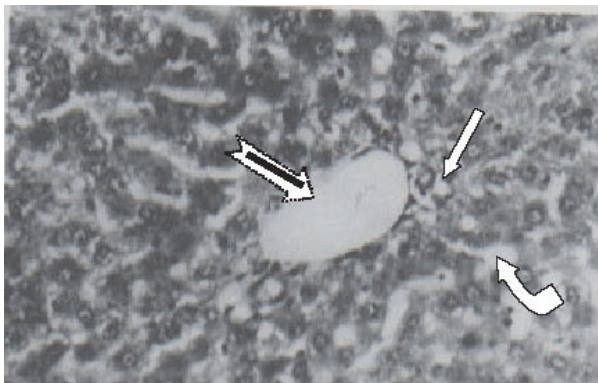


Fig. 3. Liver of alcoholic rat: arrow indicates fatty changes of macrovesicular type (\Rightarrow), microvesicular type (\curvearrowright) and sinusoidal dilation ($\Rightarrow\Rightarrow$). H & E $\times 10$

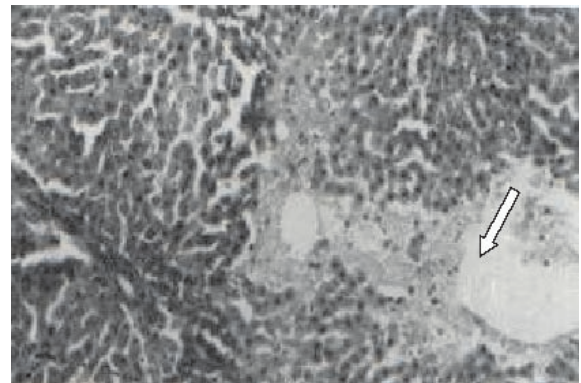


Fig. 4. Liver of alcoholic rat treated with glycine: arrow indicates hepatocyte drop out, (\Rightarrow) fatty changes were markedly reduced. H & E $\times 10$

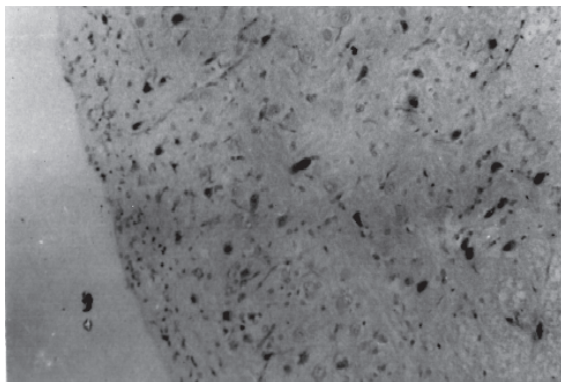


Fig. 5. Brain of control rat: H & E $\times 10$

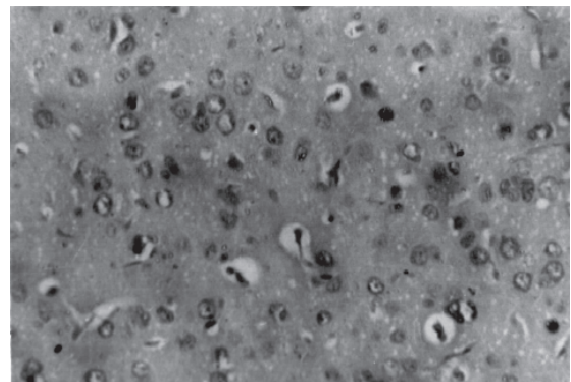


Fig. 6. Brain of control rat treated with glycine: H & E $\times 10$

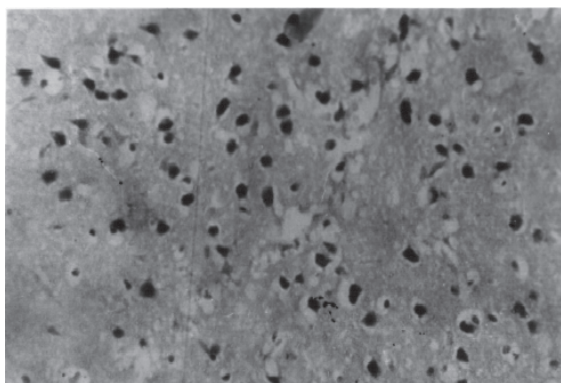


Fig. 7. Brain of alcoholic rat shows edema: H & E $\times 10$

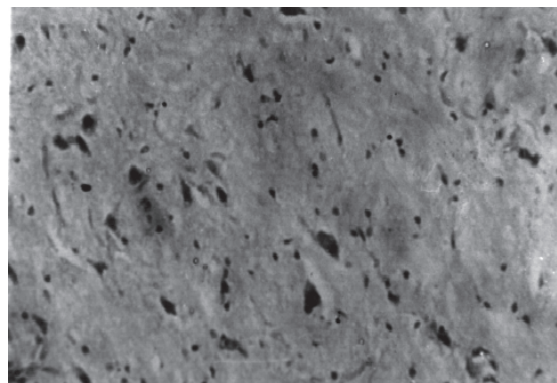


Fig. 8. Brain of alcoholic rat treated with glycine: edema is markedly reduced. H & E $\times 10$

The liver of alcohol-treated rats which received 0.6 g kg^{-1} 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. 4). The liver of control rats, which received 0.6 g kg^{-1} 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. 2). Control liver demonstrated normal liver morphology (Fig. 1).

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

DISCUSSION

Hyperlipidemia is an important associated complication of alcohol induced liver injury. Iimuro et al. [13] have shown that glycine can markedly reduce ethanol concentration in the stomach, urine, breath, blood and feces, and also the activities of the liver marker enzymes, such as aspartate transaminase and alanine transaminase (AST and ALT). Our present work shows that glycine prevents the accumulation of lipids in the liver, brain and blood on chronic ethanol intoxication.

Alcohol is rich in calories and devoid of nutrients, thus contributing to accumulation of fat in the liver. On the other hand, alcohol is known to reduce the absorption of other foodstuff and nutrients from intestines [20], which may be the cause for the decreased gain in the total body weight observed in our study. These results correlate with our previous findings [29]. Moreover, the ratio between liver

weight and the total body weight showed a 3-fold decrease in alcohol-fed rats supplemented with glycine than those of the unsupplemented alcohol-fed rats.

Alcohol consumption in quantities that lead to chronic alcoholism, unlike other drugs, has a profound effect on the intermediary metabolism of the liver [4]. Marked alterations in lipid metabolism have been reported on chronic ethanol feeding [5]. The exact mechanism by which ethanol causes fatty liver is complex. The main pathway for the hepatic oxidation of ethanol to acetaldehyde proceeds through aldehyde dehydrogenase, which is associated with the reduction of nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH) and produces a striking redox change associated with metabolic disorders. Reducing equivalents inhibit tricarboxylic acid cycle activity and fatty acid oxidation. Ethanol also inhibits lipoprotein export and increases fatty acid uptake [12]. Furthermore, interaction of ethanol with biological membranes including lipid and protein components is complex and can cause significant changes in membrane function [31]. Chronic ethanol administration is known to render the membranes resistant to the disordering effect of acute ethanol exposure in a variety of cells and subcellular organelles (e.g. brain, liver and erythrocytes) [40]. All these lead to a marked accumulation of fat in the liver during chronic alcohol consumption.

Our results show elevated concentrations of cholesterol and free fatty acids in the liver and brain of rats on alcohol consumption. Fatty acid accumulation in the tissues may be directly due to lipid breakdown and indirectly due to the oxidation of ethanol by the liver to acetate and its conversion

to fatty acids, which is a mean to remove excess hydrogen generated by ethanol. The increased free fatty acid levels may cause greater generation of reduced NADPH or NADH, which may result in the activation of NADPH-dependent microsomal peroxidation [30]. An increased accumulation of lipids in liver and other organs during alcohol ingestion has also been reported by Tsukada [38].

Alcohol-treated rats on glycine supplementation showed significantly decreased levels of cholesterol and free fatty acids in the liver and brain. Glycine is known to decrease ethanol concentrations precipitously in urine, breath, peripheral blood, portal blood, feces and stomach contents. Hepatic sterosis and necrosis is also reduced significantly by glycine. Moreover, glycine dramatically increased the first pass elimination of ethanol *in vivo* but had no effect on alcohol metabolism in the perfused liver [43]. Bile acid formation may be augmented in the presence of glycine, leading to increased excretion of cholesterol. Moreover, glycine by virtue of its ability to lower the concentration of alcohol in circulation [22] and tissues can decrease the accumulation of cholesterol in the liver.

Phospholipids are vital components of biomembranes. They are primary targets of peroxidation and can be altered by ethanol [42]. Our studies show increased phospholipid levels in liver and brain of alcohol-supplemented rats. These elevated levels can result in the modification of composition, structure and stability of cell membranes, resulting in membrane dysfunction [15]. The high phospholipid content in liver and brain of alcohol-fed rats may be due to augmented synthesis or increased free fatty acid levels.

We observed near normal levels of phospholipids in the liver and brain of rats that received glycine. Glycine is known to reduce hepatocyte destruction concomitant with reduced phospholipid metabolism [34]. Theoretically, glycine, a small amphipathic amino acid, might change membrane structure and alter the availability of phospholipids as substrate for phospholipases [39]. There are reports showing that glycine prevents hypoxic cell injury by attenuating membrane phospholipid degradation and blocking free fatty acid release. Moreover, the ability of glycine to prevent membrane alterations is induced by exogenous phospholipase A₂ [41]. Thus, a possible role of glycine in protecting cell membranes is by preventing membrane phospholipid degradation and the inter-

action of glycine with exogenous phospholipase A₂.

Studies show that repeated treatment of rats with ethanol results in accumulation of triglycerides in the liver [17]. Oliva et al. have also found that alcohol supplementation caused four-fold increase in liver triacylglycerol and cholesterol ester levels [26]. Our results correlate with these findings. The increased triglyceride levels may be due to a number of factors, such as increased availability of free fatty acids and L-glycerophosphate in the liver of alcohol-fed rats, increased secretion of very low density lipoproteins (VLDL) by the liver, disturbed catabolism of VLDL and decreased removal of triglycerides from serum due to diminished lipoprotein activity [9]. Glycine treatment to alcohol-supplemented rats decreased the liver and brain triglyceride levels significantly, possibly because glycine stimulates enzymes of fatty acid oxidation and lowers the activity of lipogenic enzymes. In this context, previous researchers have shown that N-acetyl-L-cysteine decreased the level of liver triglycerides significantly in alcohol-supplemented rats [29].

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 in the present study may be partially attributed to cytochrome-P₄₅₀ generated metabolic cytochrome-P₄₅₀ dependant enzyme activities in liver that tend to be present at their greatest concentration near the central vein, and lowest near the peripheral sites [27]. Supplementing glycine to alcohol-treated rats reduced the fatty change and improved the histomorphology of the liver.

Microdysplasia and spongiform changes have been demonstrated in the hypothalamic and thalamic regions of the brain of alcohol-treated rats [35]. These are indicative of local brain development disorders. In the present study, we observed edema in the brain of alcohol-treated rats, which was reversed on treatment with glycine.

Control rats were also treated with glycine to examine the role of glycine *per se* under controlled conditions, and to evaluate statistically the extent of benefit it offers in alcohol-induced hyperlipidemia. The data did not show any significant effect

on serum or tissue lipids when glycine was administered.

Thus, our results demonstrate that supplementing glycine a non-essential amino acid to rats on chronic alcohol treatment significantly reduces the accumulation of lipids in the circulation, liver and brain observed on chronic alcohol feeding. These findings suggest a possible, effective and economic mode of managing alcoholic patients.

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