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EFFECT OF ETHANOL ON CADMIUM-INDUCED LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES IN RAT LIVER

GEETA SHARMA, RAVINDRA NATH and KIRAN DIP GILL*
Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India

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Abstract—We have investigated the effects of the intragastric administration of cadmium (10 mg/kg body weight) and ethanol (5.56 g/kg body weight) alone as well as in combination on hepatic lipid peroxidation, the antioxidant defense system, and the morphology of liver in rats. Cadmium given in combination with ethanol led to a marked increase in cadmium accumulation in liver compared to the level in rats treated only with cadmium. Further, cadmium and ethanol coexposure produced a more pronounced elevation in lipid peroxidation (L-px), which was associated with a significantly greater inhibition of antioxidant enzymes, glutathione peroxidase (GSH-px; EC 1.11.1.9), glutathione reductase (GR; EC 1.6.4.2) and superoxide dismutase (SOD; EC 1.15.1.1), than cadmium treatment alone. The levels of glutathione (GSH) and total thiols (TSH) also decreased significantly after cadmium and ethanol coexposure. On histopathological examination, it was observed that the livers of rats coexposed to cadmium and ethanol showed a marked degeneration of hepatocytes which was not seen in rats treated only with cadmium.

Cadmium has been recognized as an environmental pollutant for several years [1], and its toxic effects have been the subject of numerous studies. The toxicity of heavy metals is reported to be influenced by a wide range of nutritional and physiological factors [2]. Cadmium is no exception in this regard, and it has been demonstrated that the accumulation of cadmium in tissues and the development of pathological lesions attributable to its presence are determined not only by the intake of this metal but also by that of several essential metals and nutritional factors [3]. Ethanol is one such factor whose influence on cadmium toxicity has not been widely reported.

Ethanol is known to enhance the carcinogenicity, mutagenicity [4] and hepatotoxicity of various chemicals [5]. Ethanol has also been demonstrated to enhance the absorption of lead in the body, and alcoholics have been reported to be more susceptible to lead intoxication [6, 7]. Although not many reports are available on the influence of ethanol on cadmium toxicity, a few studies have suggested that ethanol modifies cadmium-induced hepatotoxicity and nephrotoxicity by affecting hepatic and renal metabolism [8, 9] or by enhancing the absorption of cadmium by altering membrane permeability [7].

Lipid peroxidation (L-px) has now been identified as an important oxidative reaction underlying heavy metal toxicity. Several studies have confirmed the occurrence of lipid peroxidation in response to cadmium [10, 11] although it is believed by many investigators that this phenomenon may be a secondary event to cadmium exposure. The increase in lipid peroxidation can be attributed to alterations in the antioxidant defense system comprised of many enzymes and sulfhydryl groups which normally protect against free radical toxicity. The enhanced lipid peroxidation can also have a detrimental effect on cell membrane organization which is essential for cell function and viability. Ethanol can have a marked effect on all of these processes because of its ability to alter the permeability of cellular membranes [12] and by triggering the peroxidative reaction [13]. Very little information is available on the influence of ethanol on heavy metal-induced lipid peroxidation particularly with regard to cadmium. This study was undertaken to assess the hepatotoxicity of cadmium in ethanol-treated rats and the influence of ethanol on cadmium-induced lipid peroxidation and alterations in antioxidant enzymes. Finally, histopathological studies were carried out in an attempt to correlate these effects with observed biochemical changes.

* Corresponding author. Tel. 541032-39, Ext. 282.

MATERIALS AND METHODS

Animals and treatment

Male albino rats (Wistar strain), weighing 90 ± 10 g were obtained from the Institute Colony, were housed in polypropylene cages under hygienic conditions, and were fed rat pellet diet (Hindustan Lever Ltd., India) and water *ad lib.* The animals were divided into four groups of six to eight animals each.

Control. Animals were fed rat pellet diet and water *ad lib.* This group served as the control group.

Cadmium treated. Animals were given intragastrically 10 mg/kg body weight cadmium as cadmium chloride daily for 30 days and were fed rat pellet diet and water *ad lib.*

Ethanol treated. Animals were given 7 mL of 10% ethanol (v/v) intragastrically. Rat pellet diet was given *ad lib.*

Cadmium + ethanol treated. Cadmium as in group 2 plus ethanol as in group 3 were given.

A pair-fed group was maintained simultaneously for each of the treated groups, i.e. cadmium, ethanol, and cadmium + ethanol. Rats pair-fed to the cadmium-treated group received diet restricted in quantity to that consumed by the cadmium-treated rats. In rats pair-fed to ethanol and cadmium + ethanol-treated animals, sucrose was substituted for ethanol isocalorically. The diet in each of these pair-fed groups was also restricted to that consumed by the respective treated groups.

After 30 days of treatment animals were fasted overnight, anesthetized, and killed by decapitation. Livers were removed, rinsed in ice-cold saline, and weighed. Ten percent (w/v) homogenate was prepared in ice-cold 0.15 M KCl, using a mechanically driven teflon pestle in a Potter–Elvejhem type homogenizer.

Chemicals

Cadmium chloride, nicotinamide adenine dinucleotide phosphate (reduced), nicotinamide adenine dinucleotide (reduced), glutathione reductase, oxidized and reduced glutathione, nitroblue tetrazolium, phenazine methosulfate, 5,5′-dithiobis(2-nitrobenzoic acid) and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); thiobarbituric acid, sodium azide and ethylenediaminetetraacetic acid were from BDH Chemicals Ltd., Poole, U.K. Tris(hydroxymethyl) amino methane was from Fluka A.G. Buchs, Switzerland; ethanol was from E. Merck, Munich, Germany; and Folin and Ciocalteua phenol reagent were from Sisco Research Laboratories, Bombay, India.

Analytical procedures

Metal analysis. Livers were analyzed for cadmium content by the method of Evenson and Anderson [14]. The appropriate amount of tissue was weighed, digested in a HNO_3:$HClO_4$ (5:1) mixture, and analyzed for cadmium on an atomic absorption spectrophotometer Perkin–Elmer model 4000. The results are expressed as nmol/g tissue weight.

Lipid peroxidation. L-px in the liver homogenate was ascertained by the formation of malondialdehyde (MDA) and measured by the thiobarbituric acid method as described by Wills [15]. The amount of MDA formed was calculated using the molar extincition coefficient of MDA-TBA chromophore (1.56×10^5), and results are expressed as nmol of MDA/mg protein.

Enzyme assays

Glutathione peroxidase (GSH-px; EC 1.11.1.9). The activity of selenoenzyme GSH-px was assayed by the method of Lawrence and Burk [16] wherein the oxidation of NADPH by H_2O_2 was followed at 340 nm. Results were calcuated using the molar extinction coefficient of NADPH at 340 nm which is 6.22×10^{-6} and expressed as nmol of NADPH oxidized/min/mg protein.

Glutathione reductase (GR; EC 1.6.4.2). GR was assayed by the method of Horn [17] by measuring the reduction of oxidized glutathione to reduced glutathione by NADPH at 340 nm. Results are expressed as nmol of NADPH oxidized/min/mg protein.

Superoxide dismutase (SOD; EC 1.15.1.1). SOD was quantitated by the method of Nishikimi *et al.* [18] in which the reduction of nitroblue tetrazolium to a blue formazan (absorption maximum at 560 nm) with NADH mediated by phenazine methosulfate was inhibited by addition of the enzyme from hepatic supernatants. Enzyme activity is expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the reaction by 50%.

Catalase (EC 1.11.1.6). Catalase was assayed by the method of Luck [19] wherein the breakdown of H_2O_2 by catalase is measured at 240 nm. Enzyme activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.71) at 240 nm, and results are expressed as μmol of H_2O_2 decomposed/min/mg protein.

Estimation of glutathione (GSH) and total thiols (TSH)

GSH and TSH were estimated by the method of Ellman [20]. Results are expressed as nmol of GSH or TSH/mg protein using the molar extinction coefficient of nitromercaptobenzoic acid at 412 nm (1300).

Protein estimation

Protein in the sample was estimated by the method of Lowry *et al.* [21] using bovine serum albumin as the standard.

Histopathological studies

To study the effects of cadmium, ethanol and their coexposure on hepatic morphology, livers were fixed in buffered formalin; paraffin sections were made, stained with hematoxylin and eosin, and observed under a light microscope.

Statistical analysis

Statistical analysis was carried out using the one-way analysis of variance (ANOVA). Significance was calculated using pre-planned orthogonal contrasts comparing two groups. F values having $P < 0.05$ were considered significant.

RESULTS

Cadmium levels

Table 1 shows the effect of cadmium, ethanol and their coexposure on cadmium levels in rat liver.

All cadmium-treated rats showed a significant accumulation of cadmium in liver compared to control rats. However, this accumulation increased to almost 2.5 times in cadmium and ethanol-coexposed rats. A slight increase in cadmium levels was also observed in livers of rats treated only with ethanol.

Lipid peroxidation and antioxidant enzymes

As shown from the data in Table 1, cadmium treatment led to a 1.41-fold increase in lipid peroxidation measured in terms of MDA formation. There was a further increase in lipid peroxidation which was significant in rats coexposed to cadmium and ethanol, relative to controls as well as to only cadmium treated groups. Lipid peroxidation was

Table 1. Effects of cadmium, ethanol and their coexposure on hepatic cadmium levels and lipid peroxidation in rats

Treatment	Cadmium (nmol/g tissue weight)	Lipid peroxidation (nmol MDA/mg protein)
Control	0.03 ± 0.02	1.37 ± 0.07
Cadmium	57.42 ± 5.88*	1.93 ± 0.15*
Ethanol	0.46 ± 0.09*	1.84 ± 0.36*
Cadmium + ethanol	147 ± 20.56*†	3.23 ± 0.12*†

Control rats (N = 6) received diet and water *ad lib*. Cadmium-treated rats (N = 8) received 10 mg cadmium/kg body weight. The ethanol-treated group (N = 8) received 5.56 g ethanol/kg body weight. The cadmium + ethanol-treated group (N = 8) received cadmium (10 mg/kg body weight) and ethanol (5.56 g/kg body weight). Values are means ± SD.

* Significantly different from the control group ($P < 0.05$).

† Significantly different from the cadmium-treated group ($P < 0.05$).

Table 2. Effects of cadmium, ethanol and their coexposure on hepatic antioxidant enzymes in rats

Treatment	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/min/mg protein)	Superoxide dismutase (units/mg protein)	Catalase (μmol H_2O_2 decomposed/min/mg protein)
Control	335.41 ± 38.80	118.14 ± 4.92	20.99 ± 1.22	155.88 ± 6.90
Cadmium	218.40 ± 7.70*	78.76 ± 6.80*	16.65 ± 0.99*	118.92 ± 5.60*
Ethanol	270.23 ± 20.70*	122.81 ± 17.21	20.04 ± 1.71	144.38 ± 12.42
Cadmium + ethanol	191.80 ± 23.31*†	56.42 ± 4.53*†	15.81 ± 0.75*	99.27 ± 4.76*†

Control rats (N = 6) received diet and water *ad lib*. Cadmium-treated rats (N = 8) received 10 mg cadmium/kg body weight. The ethanol-treated group (N = 8) received 5.56 g ethanol/kg body weight. The cadmium + ethanol-treated group (N = 8) received cadmium (10 mg/kg body weight) and ethanol (5.56 g/kg body weight). Values are means ± SD.

* Significantly different from the control group ($P < 0.05$).

† Significantly different from the cadmium-treated group ($P < 0.05$).

enhanced 2.37-fold in cadmium and ethanol-coexposed rats as compared to control rats. Rats exposed only to ethanol also demonstrated a significant increase in lipid peroxidation as compared to controls.

This increase in lipid peroxidation was associated with marked alterations in various antioxidant enzymes. Table 2 depicts the effects of cadmium, ethanol and their coexposure on various antioxidants. A significant decrease (35%) in the activity of GSH-px was observed in cadmium-treated rats compared to controls. This decrease was further aggravated to 43% when the rats were coexposed to cadmium and ethanol. The decrease in the cadmium and ethanol-coexposed group was significant relative to the cadmium-treated as well as the control group. A significant decrease (20%) in the activity of GSH-px also was observed in ethanol-treated animals. The results indicate that both cadmium and ethanol treatment had a detrimental effect on the activity of GSH-px which was augmented by their coexposure. A twenty percent decrease in the activity of SOD was observed in the cadmium-treated group. However, no further decrease in the activity of SOD was observed with cadmium and ethanol coexposure, although the activity was significantly less (27%) than in the control group. No change in SOD activity was observed in ethanol-treated rats.

Similarly, a significant decrease (24%) in the activity of catalase was observed in cadmium-treated rats. Upon cadmium and ethanol coexposure, there was a further decline in the activity of catalase which was significant compared to controls as well as to cadmium-treated rats. A decrease of 37% as compared to control rats was observed in cadmium and ethanol-coexposed rats. Ethanol treatment alone had no significant effect on catalase activity in rat liver.

The results presented in Table 2 also indicate a significant decrease (33%) in the activity of glutathione reductase in rat liver after cadmium treatment. This activity decreased further to 52% as compared to controls in cadmium and ethanol-coexposed rats. The results thus suggest that cadmium decreases the activity of GR and ethanol accentuates the inhibitory effect of cadmium.

Glutathione and total thiol levels

The data in Table 3 demonstrate a marked decrease in glutathione levels in cadmium-treated rats. The levels of reduced glutathione were 34% less than those of the control group. Cadmium and ethanol coexposure produced a further decrease (46%) in GSH levels as compared to controls as well as cadmium-treated rats. Ethanol treatment alone had no effect on glutathione levels. A similar trend

Table 3. Effects of cadmium, ethanol and their coexposure on hepatic glutathione and total thiol levels

	Glutathione	Total thiols
	(nmol/mg protein)	
Control	32.15 ± 0.51	76.49 ± 2.40
Cadmium	21.38 ± 1.76*	54.41 ± 2.41*
Ethanol	33.22 ± 0.35	80.56 ± 4.23
Cadmium + ethanol	17.25 ± 1.30*†	41.42 ± 2.26*†

Control rats (N = 6) received diet and water *ad lib*. Cadmium-treated rats (N = 8) received 10 mg cadmium/kg body weight. Ethanol-treated rats (N = 8) received 5.56 g ethanol/kg body weight. The cadmium + ethanol-treated group (N = 8) received cadmium (10 mg/kg body weight) and ethanol (5.56 g/kg body weight). Values are means ± SD.

* Significantly different from the control group ($P < 0.05$).

† Significantly different from the cadmium-treated group ($P < 0.05$).

was observed in total thiol levels, wherein a 29% decrease was observed in cadmium-treated rats. Upon cadmium and ethanol coexposure there was a further decrease in total thiol levels as compared to both the control group and the cadmium-treated group.

Histopathological observations

Figure 1 shows cross-sections of livers stained with hematoxylin and eosin (H & E) as observed under light microscopy. Histopathology of livers from control group animals showed normal lobular architecture, normal hepatocytes without any degenerative changes, and occasional inflammatory cells in the portal tract, well within normal limits. Livers from the group of animals treated with cadmium alone showed normal morphology which was indistinguishable from that of the control group. Hepatocytes from ethanol-treated animals showed a moderate degree of macro- and microvesicular fatty changes (steatosis). In addition, mild degenerative changes were also observed. In the cadmium and ethanol-coexposed group, marked degenerative changes in periportal hepatocytes were observed, associated with focal and spotty necrosis and occasional areas of reticular collapse. In addition, an increase in the portal inflammatory infiltration (portal triaditis) was also observed in the livers of rats coexposed to cadmium and ethanol.

Since the results obtained in the case of the pair-fed animals were not significantly different from those of the animals of the *ad lib*. control group, the data have been presented and discussed taking into consideration only the control group.

DISCUSSION

Cadmium from the environment enters the body through the lungs and intestines. This cadmium is then transported into the blood and accumulates in the liver where it induces the synthesis of metallothionein (MT), a cytosolic protein to which cadmium binds. The data show that a significant amount of cadmium is deposited in the liver of cadmium-treated rats. This accumulation of cadmium increased significantly when animals were coexposed to cadmium and ethanol. It is now generally accepted that ethanol can induce *in vivo* changes in membrane lipid composition and fluidity [13] which may be the causative factor for increased cadmium uptake. This can be clearly seen from our data (Table 1) in which animals exposed to only ethanol had a significantly greater deposition of cadmium in the liver as compared to the controls. This increase in uptake from the environment or food and drinking water may be due to increased permeability of membranes leading to more absorption of cadmium in the presence of ethanol.

Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated fatty acids which in the membranes is considered to be a basic mechanism of toxicity for environmental pollutants. Peroxidation of membrane lipids has been reported to be associated with heavy metal toxicity [22]. Our results indicate a significant increase in L-px in the liver of cadmium-treated rats and are in accord with those of Gabor *et al.* [10]. The question of whether or not ethanol induces L-px has been a subject of controversy. Studies on chronic ethanol consumption in rats have provided evidence in favor of [23] and against [24] the involvement of lipid peroxidation. However, our results exhibited a significant increase in L-px in the liver of rats treated only with ethanol. Exposure to ethanol can increase lipid peroxidation by either enhancing the production of free radicals or inhibiting the endogenous protectants involved in the defense against active oxygen species [13]. Lipid peroxidation was further increased significantly when the rats were coexposed to cadmium and ethanol. This could be due to either the effect of both ethanol and cadmium on L-px or to significantly higher uptake of cadmium in the presence of ethanol.

These marked changes in L-px are contributed to by the effect of both cadmium and ethanol on the antioxidant defense system. The data presented in Tables 2 and 3 show that both cadmium and ethanol individually as well as in combination exert their specific detrimental effects on different antioxidant enzymes.

GSH-px is a selenium-containing antioxidant enzyme which catalyzes the detoxification of lipid peroxides and hydrogen peroxide. Our results show a reduction in the activity of GSH-px in the liver of cadmium-treated rats. Flagel *et al.* [25] observed that cadmium interferes with the absorption of selenium, and that cadmium treatment results in

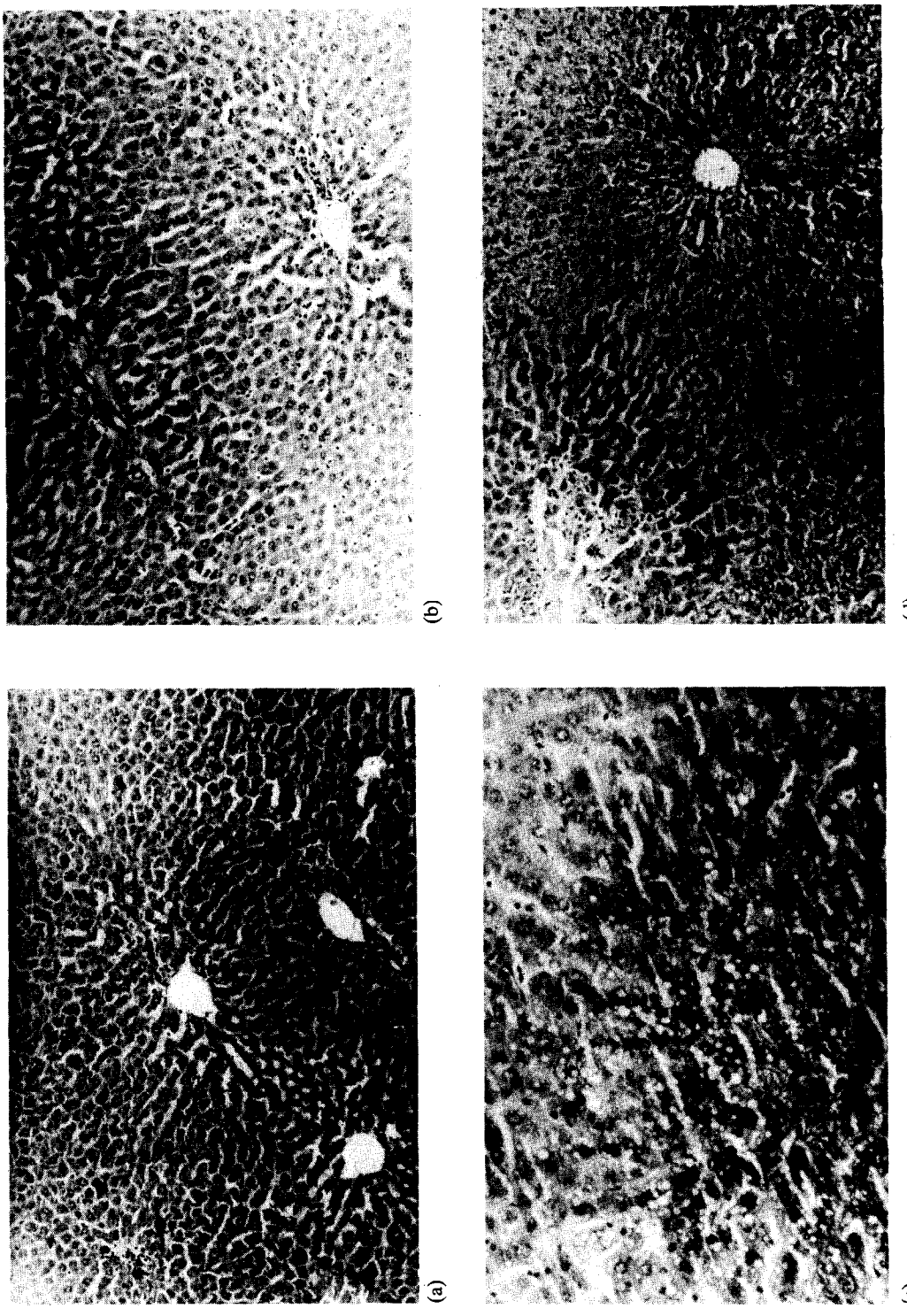

Fig. 1. (a) Section of liver showing normal portal tract, central vein and parenchymal cells from control rats. (b) Liver of cadmium-treated rats showing normal morphology. (c) Liver of ethanol-treated rats showing macrovesicular steatosis. (d) Liver of cadmium and ethanol-coexposed rats showing marked degenerative changes in periportal hepatocytes. Cross-sections of liver were stained with hematoxylin and eosin; magnification, 250×.

decreased selenium levels. Furthermore, it has been shown that selenium treatment to rats exposed to cadmium overcomes the inhibition of GSH-px [26]. Since selenium is required as an essential cofactor for GSH-px, its deficiency may lead to a decrease in GSH-px activity. A significant decrease in the activity of GSH-px in the group treated with only ethanol was observed. This decline can be attributed to the decreased levels of selenium due to ethanol exposure [27]. A significant decrease in GSH-px activity in the cadmium and ethanol-coexposed group as compared to the only cadmium or only ethanol group might be due to the effects of both of these toxicants on GSH-px.

SOD is a copper- and zinc-containing enzyme responsible for catalytic dismutation of highly reactive and potentially toxic superoxide radicals (O_2^-) to H_2O_2 [28]. In the present study cadmium administration was found to inhibit this enzyme significantly in the liver. A similar decrease in the activity of SOD has been reported in erythrocytes and lung lavage taken from rats exposed to cadmium fumes [29]. It is possible that cadmium present in tissues interacts with copper and zinc in SOD molecules and produces inhibition of the enzyme activity. The possibility of such an interaction has been demonstrated by Bauer *et al.* [30] wherein ^{111}Cd replaced zinc in Cu-Zn SOD to form Cu ^{111}Cd-SOD. A similar effect was observed by Keen *et al.* [31] in the livers of alcoholic monkeys. In the cadmium and ethanol-coexposed group although the activity of SOD was less than in the control group this activity did not decline further upon coexposure to cadmium and ethanol.

Catalase is another iron-containing antioxidant enzyme with hematin as the prosthetic group. It is ubiquitously present in all aerobic cells containing a cytochrome system. Catalase is most abundant in the liver and is responsible for the catalytic decomposition of hydrogen peroxide to oxygen and water. The decrease in catalase activity on cadmium treatment may reflect decreased absorption of the essential element required for the activity of this enzyme. Cadmium has been shown to depress the iron levels in liver and kidney of rats [32]. Bremner [33] reported that the ability of cadmium to interfere with iron metabolism is due to a common transport system which is shared by cadmium and iron. Kench and Gubb [34] reported that the biosynthesis of catalase is impaired in cadmium-poisoned chicks. Furthermore, after cadmium and ethanol coexposure, the decrease in the activity of catalase was more pronounced compared to that in only cadmium-treated rats. This may be due to a higher accumulation of cadmium in this group, compared to the only cadmium-treated group, which can eventually inhibit not only iron transport but also the biosynthesis of catalase.

Our results show a decline in the activity of glutathione reductase in liver on cadmium exposure. GR is an enzyme which accounts for a very high GSH:GSSG ratio, or in other words it keeps glutathione in the reduced form, which is the biologically active form. It is postulated that cadmium affects the activity of GR by binding to the active sulfhydryl group in the form of a cysteine residue that undergoes reduction and oxidation during the catalytic cycle which reduces GSSG to GSH. The affinity of cadmium for sulfhydryl groups is well established. Cadmium may be inhibiting GR activity by blocking the sulfhydryl groups required for the catalytic cycle. The activity of this enzyme further declined significantly in cadmium and ethanol-coexposed rats as compared to only cadmium-treated rats, thus implying that the coexposure is more harmful to the protective ability of this enzyme.

The affinity of cadmium for sulfhydryl groups makes it an attractive mechanism for cellular resistance to cadmium toxicity. GSH is an important naturally occurring antioxidant, as it prevents free radical mediated L-px, by allowing the hydrogen of the -SH group to be abstracted instead of methylene hydrogen of unsaturated lipids [35]. Thus, the level of GSH in a tissue is considered a critical determinant of the threshold for tissue injury caused by toxic substances [36]. Our study shows a significant decrease in the levels of GSH and the total thiols after cadmium exposure. No change in the thiol levels was observed in only ethanol-treated rats, and the results are in accord with those of Aykac *et al.* [37]. A significant depression in the thiol levels was further observed in the cadmium and ethanol-coexposed group which may be due to increased cadmium uptake in this group. This observation can be of serious consequence as a decreased level of GSH upon cadmium exposure causes decreased degradation of lipid peroxides leading to their accumulation in the liver. This decrease in sulfhydryl groups upon cadmium and ethanol coexposure amplifies the toxicity of cadmium.

It can be postulated that a reduction in the activity of any one of these antioxidant factors may not be as serious as the concomitant decrease in the activity of all these factors. This decrease in the activity of the cell can further lead to uncontrolled free radical chain reaction and accumulation of peroxides, hydroperoxides and other free radical species. These have been reported to react with lipids, proteins and other cellular components and have damaging effects on the integrity of cellular membranes.

During histopathological examination it was observed that livers of cadmium-treated rats showed no significant change in the morphology of the liver from that of control rats. However, upon cadmium and ethanol coexposure, a substantial necrosis of periportal hepatocytes was observed which resulted in the collapse of the reticular framework (Fig. 1d). Since periportal hepatocytes constitute metabolically the most active cells, their degeneration can be detrimental to the normal physiological role of the liver cell. Increased lipid peroxidation is one factor that could be responsible for such a degenerative change. Jamall and Smith [26] also observed that morphological changes in the hearts of rats treated with cadmium were associated with increased lipid peroxidation. A significant increase in serum alanine transaminase levels was also observed in cadmium and ethanol-coexposed rats when compared to only cadmium-treated rats (data not shown), confirming the cell damage in livers of rats coexposed to cadmium and ethanol. The results thus suggest that the coexposure of rats to cadmium and ethanol even

at low doses of cadmium (which by themselves do not result in any morphological changes) can be much more toxic than the exposure of rats to cadmium alone.

The results reported here indicate that rats subjected to a combined exposure of cadmium and ethanol are more vulnerable to the hepatotoxic effects of cadmium. An insight into the biochemical events reveals that ethanol accentuates the cadmium-induced lipid peroxidation by potentiating the inhibition of endogenous antioxidants and by increasing the formation of free radicals. Ethanol achieves this by increasing the uptake and accumulation of cadmium and also by exerting its own deleterious effects. Morphological studies also support the biochemical findings that ethanol makes the rats more vulnerable to the toxic effects of cadmium.

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