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Lead and Ethanol Coexposure: Implications on the Dopaminergic System and Associated Behavioral Functions

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GUPTA, V. AND K. D. GILL. *Lead and ethanol coexposure: Implications on the dopaminergic system and associated behavioral functions.* PHARMACOL BIOCHEM BEHAV **66**(3) 465–474, 2000.—The present investigation involves ethanol's effects on the lead-induced alterations in the dopaminergic system. Ethanol, at a dose of 3 g/kg body weight for 8 weeks, resulted in a marked increase in the accumulation of lead in the blood and brain of animals receiving 50 mg lead/kg body weight. Levels of dopamine were found to decrease significantly, and were accompanied with increased norepinephrine levels in lead and ethanol coexposed animals. Uptake of tyrosine as well as the activities of tyrosine hydroxylase and monoamine oxidase were seen to increase significantly in lead as well as ethanol-treated animals, and these were increased to a greater extent when animals were administered lead and ethanol simultaneously. Dopamine receptor binding studies revealed a significant elevation in the number of binding sites in lead and ethanol-coexposed animals. The altered dopaminergic functions were reflected by the neurobehavioral deficits in terms of motor incoordination, aggressiveness, and hyperactivity of animals exposed to lead, the effect being more pronounced in lead- and ethanol-coexposed animals. In brief, results of this study suggests that ethanol potentiates lead-induced cellular damage at the neurochemical and neurobehavioral level. © 2000 Elsevier Science Inc.

Lead Ethanol Rat Brain Dopaminergic system Behavior

LEAD is a nonessential toxic heavy metal widely distributed in the environment. The toxic effects of lead demand particular attention because of its characteristically cumulative feature associated with slow turnover and a long biological half-life (56). Although exposure to lead had declined, chronic lead toxicity still remains a major public health problem afflicting millions of children and adults (62). The population in general is exposed to lead from both environmental and occupational sources. More recently, the indiscriminate use of lead in paints and gasoline had been a principal source of exposure to this heavy metal (14). The primary site of action of lead is the central nervous system. Neurotoxicity of lead is associated with several neurochemical, neuropathological, and neurobehavioral changes. The symptoms may include severe neurological deficits such as ataxia, headache, convulsions, and memory disorders (42).

The neurotoxic effects of lead have been found to be controlled by a number of nutritional, physiologic, and environmental factors (25). Ethanol, being one such factor, might affect the neurotoxicity of lead by regulating its absorption and distribution. Ethanol represents the second most widely abused drug in the world after caffeine, and millions of people consume it daily, despite a multitude of problems associated with it (49). Higher blood levels have been reported in alcoholic industrial workers exposed to lead compared to nonalcoholic workers (12,17). However, despite speculated importance of such factors such as alcohol in influencing the lead neurotoxicity, there is a paucity of information regarding the possible biochemical mechanism by which ethanol might be affecting the state of neuronal function in lead-exposed individuals.

Evidence has accumulated from both experimental and human studies suggesting that the behavioral abnormalities normally manifested in lead-exposed patients are primarily due to the toxic effects of lead on the dopaminergic transmission. Brain dopaminergic system has been shown to be in-

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volved in alcohol intoxication (6,43). Much less information is available regarding the possible effects of ethanol on the neurotoxicity of lead. This, however, warrants special attention in view of the tremendous increase in the worldwide production and consumption of alcohol and also the environmental lead pollution, and as a result, there is every likelihood of the coexposure of industrial workers as well as the general population to these two neurotoxins, i.e., ethanol and lead.

Behavior is regarded as the net result of sensory, motor, and cognitive functions in the nervous system, thereby making it a potentially sensitive end point of xenobiotic-induced neurotoxicity. The behavioral abnormalities, particularly those associated with the motor functions, normally manifested in lead-exposed patients have been attributed to the toxic effects of lead on dopaminergic transmission (13,60). Thus, it is of paramount importance to elucidate the biochemical mechanism by which lead and ethanol coexposure may affect this neurotransmitter system. The present study, therefore, involves a detailed investigation of the dopaminergic system and subsequent neurobehavioral deficits produced, if any, following coexposure to lead and ethanol.

METHOD

Animals

Male albino rats (Wistar strain) approximately 3 months old, weighing between 80–100 g, were obtained from the animal house of the Institute. Animals were housed in polypropylene cages and were fed standard rat pellet diet (Hindustan Lever Ltd., Bombay, India) and water ad lib.

Experimental Design

Animals were divided into four groups of six animals each, and given the following treatments.

Control group. Animals received equal volume of normal saline (2.5 ml/100 g body weight), intragastrically, throughout the course of the treatment.

Lead-treated group. Animals received lead, intragastrically as lead acetate at a dose of 50 mg/kg body weight/day for 8 weeks.

Ethanol-treated group. Animals received ethanol (15%) 3 g/kg body weight for 8 weeks, intragastrically.

Lead + ethanol-treated group. Animals received lead as in group II along with ethanol as in group III.

During the course of the treatment, body weight, dietary intake, and water intake of animals was recorded every day. General appearance of the animals as well as their activity in the home cage and the grooming behavior (16) of animals was monitored. After the completion of the treatment, animals were fasted overnight and sacrificed by cervical dislocation. The cerebrum region (including the cerebral cortex, striatum, midbrain, hypothalamus, and hippocampus) was dissected from the brain as explained by Glowinski and Iversen (24). Ethical clearance for killing of animals was duly obtained from the Institute's ethical committee.

Biochemical Procedures

Estimation of lead. Tissue (200–400 mg)/blood (0.5 ml) were digested by the wet acid digestion method of Evenson and Anderson (22), and lead levels were measured on a Direct Current Plasma Emission Spectrophotometer (Beckman, Spectrapan V).

Subcellular fractions: Preparation of synaptic plasma membranes. Synaptic plasma membranes (SPM) were prepared by the discontinuous sucrose density gradient centrifugation method of Jones and Matus (30).

Preparation of synaptosomes. Synaptosomes were obtained by the discontinuous sucrose gradient centrifugation method of Gray and Whittaker (27).

Tyrosine hydroxylase assay. Tyrosine hydroxylase (TH) was assayed by the method of McGeer and McGeer (39). Tissue homogenate (500–800 μg protein in 0.1 ml) was incubated with 0.1 ml of 0.28 M phosphate buffer (pH 6.2) and 0.1 ml of 150 μM tyrosine solution containing 0.1 μCi of ^{14}C L-tyrosine (specific activity 450 mCi/mmol). Blanks contained tissue homogenate heated to 80–90°C for 10–12 min. Incubations were carried out for 30 min at 37°C, and were stopped by the addition of 2 ml of a 1:1 mixture of 0.4 N perchloric acid and 0.2 N acetic acid containing 0.1 μg each of cold carrier DOPA, dopamine, and norepinephrine. Tubes were centrifuged and the supernatant combined with 1.25 ml of 2.0 M ethylene diamine tetraacetic acid. The precipitate was resuspended in 1.5 ml of 0.3 M KH_2PO_4, the mixture recentrifuged, and the supernatant added to the original supernatant plus EDTA. The pH of the mixture was then made 9–9.2 using 1 N NH_4OH and stirred with 300 mg of alumina. The alumina was washed twice with approximately 15 ml water. ^{14}C-Dopa formed was then eluted with 2 ml of 0.5 N acetic acid, and radioactivity determined after adding scintillation mixture. 5–15% of the ^{14}C-tyrosine added in the reaction mixture appeared as ^{14}C-Dopa.

Estimation of dopamine and norepinephrine. Catecholamines were estimated by the fluorimetric method as described by Cox and Perhach (15). The brain samples (280 mg) were homogenized in 10 volumes of cold, acidified butanol. The homogenates were centrifuged for 5 min at 800 × *g*. A 2.5-ml portion of the supernatant fluid was transferred to a glass stopped centrifuge tube containing 2.5 ml of distilled water and 0.5 ml of heptane. The tubes were shaken for 5 min and centrifuged. The aqueous phase (2.5 ml) were transferred to a screw-capped test tube containing 200 mg of alumina; 1.0 ml of 2 M CH_3COONa was added, and the tubes were gently shaken and centrifuged. The alumina was then washed with 2.0 ml of distilled water and 2.0 ml of 0.1 N acetic acid was added to the alumina. The tubes were shaken for 10 min, centrifuged, and 1.0 ml of aqueous phase was transferred to a small test tube for fluorescence assay of norepinephrine and dopamine as described by Ansell and Beeson (3). Internal standards (100–500 ng) were prepared by adding known amounts of each standard to a homogenate pool and running in parallel with the tissue samples. The pool without added standards served as a blank for the standards. Recovery of DA and NA ranged from 80–85% with coefficient of variation 5–15%. The sensitivity of the method was 50 ng for NA and 70 ng for DA.

Dopamine-β-hydroxylase assay (DBH). The activity of DBH was assayed by following the method of Kato et al. (33), which involves the use of tyramine as the substrate. Tissue homogenate was prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.01% Triton X-100 in an ice bath. The standard assay system for DBH contained 0.2 M sodium acetate buffer (pH 5.0), 30 mM N-ethylmaleimide, 10 μM $CuSO_4$, 25,000 units of catalase, 1 mM pargyline hydrochloride and enzyme preparation (1 mg) in a reaction volume of 1.0 ml. The reaction mixture was vortexed thoroughly followed by the addition of 10 mM ascorbic acid, 10 mM sodium fumarate, and 20 mM tyramine hydrochloride. In the case of

blanks, in addition to the above reaction mixture 100 μM fusaric acid was also added. To another blank incubation mixture 2 nmol of octopamine was added, which served as an internal standard. The reaction mixtures were incubated at 37°C for 45 min. The reaction was stopped by adding 0.2 ml of 3 M trichloroacetic acid in an ice bath, and the mixture was immediately centrifuged at 2500 rpm for 10 min. The supernatant was immediately transferred to a glass column of Dowex 50W-X4 (H^+, 200–400 mesh). Octopamine was eluted with 3 N NH_4OH and chemically converted to *p*-hydroxybenzaldehyde by adding 10 μl of 2% $NaIO_4$ solution followed by 10 μl of 10% $Na_2S_2O_3$ solution. The solution was then acidified by adding 0.5 ml of 6 N HCl. *p*-Hydroxybenzaldehyde in the solution was extracted using ethyl ether by vigorous shaking. The ether phase was then extracted with 1.0 ml of 3 N NH_4OH by vigorous shaking. The absorbance of the NH_4OH layer was measured at 333 and 360 nm, and the difference in absorbance was used to calculate the activity of DBH, which is presented as pmol octopamine formed/min/mg protein. Recovery of octopamine added as internal standard to the incubation medium was 90% with sensitivity of the method being 1 nmol and coefficient of variation 10–15%.

Monoamine oxidase assay. The activity of enzyme monoamine oxidase (MAO) was measured by the method of McEwen and Cohen (38). The incubation medium consisted of 0.6 ml of tissue homogenate containing 2.5 mg protein, 0.75 ml of 0.2 M phosphate buffer, pH 7.2 and 0.15 ml of buffered 8 mM benzylamine. The reaction mixture was incubated at 37°C for 3 h, together with control reaction mixtures, without benzylamine. Following incubation, 0.15 ml of 8 mM benzylamine was added to each control mixture, and all tubes were treated with 0.15 ml of 60% perchloric acid and 1.5 ml of cyclohexane. The contents were emulsified, allowed to stand at room temperature for 15 min, and then after second emulsification, centrifuged for 10 min at 2000 rpm. The absorbance of the cyclohexane extracts from the complete reaction mixtures were measured at 242 nm. The enzyme activity has been expressed as nmol of benzaldehyde formed/min/mg protein. Recovery of benzaldehyde added as internal standard was 85% with sensitivity of the method 0.25 nmol benzaldehyde and coefficient of variation 5–15%.

Tyrosine uptake. Low- and high-affinity tyrosine uptake studies were carried out in the synaptosomes by the method of Diez-Guerra et al. (19). Low-affinity and high-affinity tyrosine uptake were measured in the presence of high (500 μM) and low (5 μM) concentrations of tyrosine. The synaptosomal suspension (equivalent to 0.1 mg of protein) was preincubated for 5 min at 37°C in modified Krebs-Henseleit medium (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM D-glucose, 20 mM HEPES-Tris, pH 7.4). The uptake was started by the addition of 80 μl of a solution containing ^{14}C tyrosine. After incubation with gentle agitation, uptake was terminated by diluting the incubation mixture with 3 ml of modified Krebs-Henseleit medium and filtering through moist Millipore filters (0.45-μm pore size). Filters were rinsed twice, dried, and their radioactivity measured. Na^+ containing medium was used (Krebs-Henseleit medium) for low-affinity uptake, whereas for high-affinity uptake Na^+ was replaced by sucrose by using an iso-osmotic concentration of NaCl, and the medium was designated as Na^+ free medium.

Receptor binding assay. Dopamine D_2 receptor binding assay was carried out using the method of Briley and Langer (9). ^3H-Spiperone (specific activity—85 mCi/mmol) is used as a ligand to study the receptor binding in the presence and absence of 10 μM haloperidol, which is an antagonist for dopaminergic receptor. The "nonspecific binding" is thus carried out in the presence of high concentration of haloperidol. The binding in the presence of haloperidol subtracted from total binding gives the "specific binding." Binding was measured in the medium containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. Tissue was homogenized and washed twice with 40 vol of buffer by centrifuging at 49,000 × *g* for 30 min at 4°C. Finally, the pellet was resuspended in the buffer. Binding was determined by incubating membrane suspension with varying concentrations of ^3H-spiperone (0.05–0.5 nM) at 37°C for 15 min. The reaction was terminated by the addition of 2.0 ml of ice cold buffer, and the mixture filtered through GF/B filters presoaked in polyethylenimine (0.5% w/v). Filters were washed with ice cold buffer, dried, and placed in toluene-based scintillation fluid to count the radioactivity absorbed onto the filters. The specific binding was obtained by subtracting nonspecific binding from the total spiperone bound. The values of K_d and B_{max} were obtained from the Scatchard plot analysis (52), as is explained by Bylund and Yamamura (10).

Proteins. Proteins were quantitated by the method of Lowry et al. (36).

Neurobehavioral Studies

Motor function tests. Spontaneous locomotor activity of animals was measured according to the method of Dews (18) in an animal activity meter (Columbus Instruments, Columbus, OH). The cage space was transacted by two pairs of infrared beams. The interruptions were monitored by a computer (Apple lle)-based system. The system was programmed to count the interruptions at intervals of 15 min for a period of 60 min after allowing 5 min of acclimatization from the time the animals were placed in the activity meter cage. The experiments were performed between 0800–1200 h in a dimly lit room with the temperature in the room being kept constant at 25°C.

The rotarod test was carried out by the method of Dunham and Miya (21) to test the motor control and coordination of animals. Rats were placed on a metallic turning at the rate of 8 rpm. The circular section divides the line of space of the rod into four lengths so that four rats could be tested together. The rats were initially screened for their ability to maintain themselves on the rotating rod for more than 3 min. If the experimental animals could not remain on the rod for three successive trials of 2 min each, the test was considered positive, meaning thereby that the treatment has affected the motor coordination of animals.

Aggressive behavior. The aggressive behavior of animals was tested as per the method described by Tedeschi et al. (61) by placing a pair of rats from each group in a Persepex chamber (8 × 8 × 7") with a grid stainless steel rods to employ foot shock. A 100-V, 2-mA shock at 5 Hertz was administered. The pair of rats that exhibited at least one fighting episode in 1 min were selected for the study. Preliminary screening was done to exclude nonresponders in all pairs of rats. The fighting episode was considered positive when the rats converged abruptly to close quarters and stood face to face on their hind limbs and stuck a bit savagely at each other. The return of the rats to quadrupedal posture was considered as the demarcation between fighting episodes.

Statistical Analysis

ANOVA was used for statistical analysis of the data followed by Newman–Keuls test for multiple comparisons. *p*-values of less than 0.05 were considered statistically significant.

TABLE 1

EFFECT OF LEAD, ETHANOL, AND THEIR COEXPOSURE ON THE DIETARY INTAKE, BODY WEIGHT, AND BRAIN WEIGHT OF RATS

	Gain in Body Weight (g) (Final–Initial Body Weight)	Brain Weight (g)	Mean Dietary Intake (g)/Day/Rat
Control group	82.0 ± 3.59	1.81 ± 0.06	18.7 ± 1.6
Lead-treated group	53.3 ± 5.1†	1.68 ± 0.08†	17.9 ± 1.7
Ethanol-treated group	62.8 ± 5.2†	1.68 ± 0.12†	15.0 ± 3.1
Lead + ethanol-treated group	47.0 ± 4.2†‡	1.59 ± 0.09†	17.7 ± 2.4

Values are mean ± SD of six animals/group. Multiple comparisons are done when F-value is $p < 0.05$.

†$p < 0.01$, significantly different from control group; ‡$p < 0.05$, significantly different from the lead-treated group.

RESULTS

Lead exposure (50 mg/kg b.wt.) and ethanol exposure (3 g/kg b.wt.) for a period of 8 weeks did not result in any apparent sickness of the animals, but it had a detrimental effect on the growth of the animals. Exposure to lead as well as ethanol resulted in a significant decrease in the net gain in body weight of animals. As is evident from Table 1, there was a significant decrease in brain weight following respective treatments in all the groups. Mean food as well as water intake of animals was not significantly affected in any of the treatment groups; however, the animals showed sickly demeanor. Behavioral abnormalities such as hyperactivity, excessive self grooming, and aggressiveness were observed in only lead-treated and lead and ethanol-coexposed animals.

Levels of lead in the blood of rats (Table 2) exposed to only lead were found to be approximately eightfold compared to control animals. However, when animals were coexposed to lead and ethanol, blood lead levels increased to 13-fold, thus significantly higher than only the lead-treated group. Lead exposure resulted in a significant accumulation (12-fold) of this heavy metal in the cerebrum of rat brain compared to controls (Table 2). Exposure to ethanol along with lead further enhanced the levels by two-fold compared to only lead-treated animals. To study the effect of lead, ethanol, and their coexposure on the dopaminergic neurotransmitter system, levels of catecholamines were quantified in the cerebrum of rat brain. As can be seen in Table 3, approximately 60% decrease in the levels of dopamine was observed in only lead-treated animals compared to controls. Coexposure of lead and ethanol also resulted in a significant decrease in the dopamine levels compared to control animals. Norepinephrine, an associated neurotransmitter in the dopaminergic system, formed by the hydroxylation of dopamine at the beta carbon atom, was also measured in the cerebrum of rats following lead and ethanol exposure independently and in combination. As shown in Table 3, there was a 1.7-fold increase in the norepinephrine level following lead exposure compared to control animals. Ethanol exposure, however, did not affect the norepinephrine content in the cerebrum. When the animals were given both lead and ethanol, norepinephrine levels increased by 1.84-fold compared to the control group.

Figure 1 shows a significant increase (35%) in the high-affinity tyrosine uptake, following lead exposure, and 30% following ethanol exposure. Exposure to both lead and ethanol resulted in a 56.2% increase in the high-affinity tyrosine uptake. However, no significant effect on the low-affinity tyrosine uptake was observed. The decrease in the levels of dopamine and an increase in norepinephrine levels could be due either to the alterations in synthesis or degradation of these neurotransmitters. Therefore, the effect was observed on the activity of key enzymes of dopaminergic system. The activity of tyrosine hydroxylase was significantly increased in lead-treated animals (Table 4) compared with the control animals. Ethanol exposure also elevated the enzyme activity in the cerebrum of rats compared to control animals, the increase being twofold. When animals were coexposed to lead and ethanol, it was found that the activity of this enzyme increased to 2.2-fold when compared to control animals, thus being significantly more compared to the lead-treated group ($p < 0.01$).

There was a significant increase (37%) in the activity of DBH in the cerebrum of animals compared to control animals. Ethanol exposure alone did not affect the enzyme activity significantly, whereas coexposure to lead and ethanol resulted in approximately 40% increase in the activity of DBH compared to control animals. Lead exposure to animals as responsible for significantly increasing the activity of monoamine oxidase (15.7%) in the cerebrum (Table 4). Exposure to ethanol also resulted in a significant increase (29.2%) in the activity of MAO. However, when the animals were coexposed to lead and ethanol, activity of MAO was synergistically increased by 55.6% compared to control animals. Thus, it was observed that exposure to ethanol along with lead has resulted in the enhancement of both synthesis and degradation of dopamine in the cerebrum of the rat brain.

Dopamine receptors play an important role in coupling the neurotransmitter release to its propagation and postsynaptic effects. An attempt was made to study the effect of lead,

TABLE 2

EFFECT OF LEAD, ETHANOL, AND THEIR COEXPOSURE ON THE LEVELS OF LEAD IN BLOOD AND BRAIN (CEREBRUM) OF RATS

	Lead Levels	
	Blood (μg/dl)	Cerebrum (μg/g Tissue wt.)
Control group	10.3 ± 3.4	0.21 ± 0.02
Lead-treated group	83.8 ± 8.7†	2.51 ± 0.24†
Ethanol-treated group	10.2 ± 2.9	0.20 ± 0.02
Lead + ethanol-treated group	142.6 ± 7.8†‡	4.97 ± 0.38†‡

Values are mean ± SD of six animals/group. Multiple comparisons are done when F-value is $p < 0.05$.

†$p < 0.001$, significantly different from control group; ‡$p < 0.001$, significantly different from lead-treated group.

TABLE 3
EFFECT OF LEAD, ETHANOL, AND THEIR COEXPOSURE ON THE LEVELS OF DOPAMINE AND NOREPINEPHRINE IN THE CEREBRUM OF RAT BRAIN

	Dopamine	Norepinephrine (ng/g Tissue Wt.)
Control group	1173.24 ± 374.37	421.21 ± 91.91
Lead-treated group	473.5 ± 103.97†	718.64 ± 182.33†
Ethanol-treated group	855.7 ± 133.05*	446.68 ± 90.03^{NS}
Lead + ethanol-treated group	520.33 ± 181.60†	775.74 ± 152.64‡

Values are mean ± SD of six animals/group. Multiple comparisons are done when *F*-value is $*p < 0.05$.

$*p < 0.05$, $†p < 0.01$, $‡p < 0.001$, significantly different from control group; NS—not significant.

ethanol, and their combination on the dopamine D_2 receptor binding using [^{3}H]spiperone as an antagonist. Scatchard plot analysis (Table 5) revealed no change in the binding affinity (K_d) following any of the treatments; however, the value of B_{max}, which gives the number of binding sites, was found to increase by 38% following coexposure to lead and ethanol when compared to control animals. Contrary to several studies in the literature, we are getting very high K_d values for D_2 receptors. This is possibly due to a high concentration of haloperidol used to displace nonspecific binding.

Spontaneous locomotor activity of the animals was monitored in an automatic manner using a microprocessor-based animal activity meter. Lead caused a significant increase in the spontaneous locomotor activity (3.3-fold) of animals compared to control animals (Fig. 2). Exposure to ethanol also resulted in a twofold increase in the spontaneous locomotor activity compared to control animals. When the animals were coexposed to lead and ethanol, the increase in spontaneous locomotor activity was found to be 4.5-fold compared to control animals. As evident, the animals in the lead- and ethanol-coexposed group exhibited a significantly high locomotor activity that was 1.3-fold more compared to only lead-treated animals.

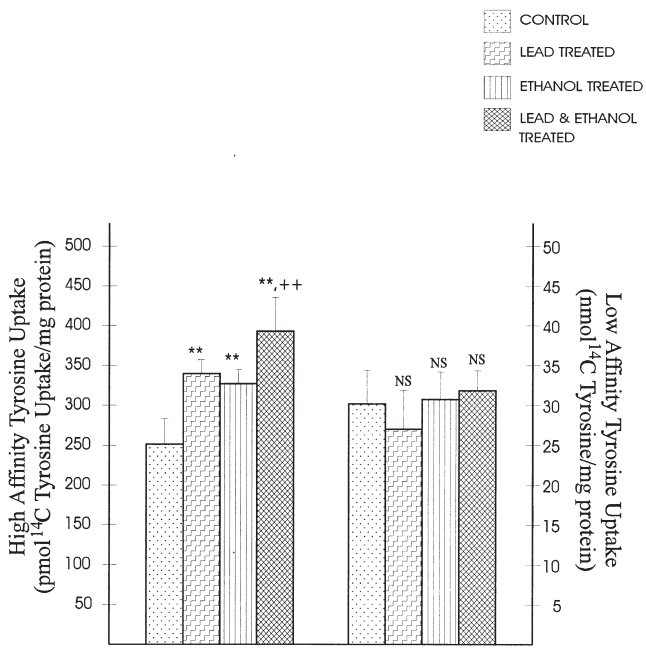

FIG. 1. Effect of lead, ethanol, and their coexposure on the uptake of tyrosine in synaptosomes prepared from cerebrum of rat brain. Values are mean ± SD of six animals/group. $**p < 0.01$, significantly different from control group, $^{++}p < 0.01$ significantly different from the lead-treated group; NS—not significant.

Performance on the rotarod apparatus was assessed in terms of the mean retention time for animals for which they could maintain themselves on the rotating rod. As shown in Fig. 2, performance on the rotarod apparatus was significantly diminished when animals were exposed to only lead. Significant reduction in the mean retention time was also observed in case of ethanol-treated animals compared to control animals. But when animals were coexposed to lead and ethanol, performance on the rotarod apparatus was further deteriorated or impaired, mean retention time being significantly less compared to lead-treated animals. Unlike control animals, none of the tested animals could maintain themselves on the rotating rod for the full quota of the cut-off time (120 s).

Regarding the aggressive behavior of animals, Fig. 3 clearly shows that exposure to lead significantly increased the number of fighting episodes in lead-treated animals compared to control ones. Ethanol exposure, however, did not affect the aggressive behavior of animals. When animals were coexposed to lead and ethanol, the mean fighting score of animals were higher than only lead-exposed animals, thus indicating the aggressive behavior of animals.

DISCUSSION

A detailed investigation into the effect of lead, ethanol, and their coexposure on the dopaminergic transmission has been presented. Exposure of rats to lead or ethanol had a detrimental effect on the growth of animals; the decrease in body weight could be due to toxic effects of lead and ethanol on cellular metabolism (63) and not due to underfed or undernourished conditions. It has been suggested that loss in body weight in general on heavy metal exposure can be attributed to the enhanced synthesis of glucose from noncarbohydrate sources, that is, metabolization of fat deposits (59). Alcoholism is associated with increase in metabolic rate and increased brown fat thermogenesis in rats and humans, which in turn, may be responsible for the decrease in growth of the animals (1). Further, lead and ethanol are possibly acting together, resulting in poor growth of the animals.

When animals were given ethanol along with lead, a substantial increase in blood lead levels were observed, which

TABLE 4

EFFECT OF LEAD, ETHANOL, AND THEIR COEXPOSURE ON THE ACTIVITY OF TYROSINE HYDROXYLASE, DOPAMINE-β-HYDROXYLASE, AND MONOAMINE OXIDASE IN THE CEREBRUM OF RAT BRAIN

	Control Group	Lead-Treated Group	Ethanol-Treated Group	Lead + Ethanol-Treated Group
Tyrosine hydroxylase (nmol (^{14}C) Dopa formed/h/mg protein)	0.14 ± 0.03	0.21 ± 0.04*	0.30 ± 0.06‡	0.32 ± 0.05‡§
Dopamine-β-hydroxylase (pmol octopamine formed/min/mg protein)	23.79 ± 2.14	32.71 ± 2.85†	25.07 ± 2.02¶	33.33 ± 3.31†^{NS}
Monoamine oxidase (nmol benzaldehyde formed/min/mg protein)	4.44 ± 0.44	5.14 ± 0.26*	5.74 ± 0.52§	6.91 ± 0.51‡#

Values are mean ± SD of six animals/group. Multiple comparisons were done when F-value is $*p < 0.05$.

$*p < 0.05$, $\dagger p < 0.01$, $\ddagger p < 0.001$, significantly different from control group; $\S p < 0.01$, $\# p < 0.001$, significantly different from lead-treated group, ¶not significant.

could be due to increased absorption of lead from gastrointestinal tract as has also been observed by Bortoli et al. (7). It has also been suggested that alcohol might induce a modification in the mechanism of absorption, mobilization, and distribution of lead, with resultant increased blood lead concentration (37). This is also supported by higher blood lead levels in alcoholic industrial workers, as reported by Dally et al. (16) and Cezard et al. (12). The high blood lead levels in the animals receiving alcohol might result in impaired heme availability, which may lead to impaired cellular energetics, thus affecting neuronal functions. Lead levels as high as 150 μg/dl have been reported in the industrial workers and in those who are exposed to lead from Asian traditional remedies (6,31). Appreciable amount of lead reached the cerebrum of animals exposed to lead treatment. This, however, increased substantially when animals were coexposed to lead and ethanol. This is possibly due to an effective blood–brain barrier (BBB), known to restrict the entry of heavy metals in brain, and many such examples in literature confirm our findings (23,45). This is also supported by the observation that immature animals accumulate more lead in the brain because of the underdeveloped blood–brain barrier (64). Further, our results are in agreement with those of Flora and Tandon (23), who reported significantly higher accumulation of lead in the whole brain of rats coexposed to lead and ethanol. It is well established that the interaction of ethanol with cellular membranes has a profound effect on various cellular functions. Studies by other workers have demonstrated that the transport of various substances through the BBB is influenced by the presence of even a small concentration of ethanol. It can, thus, be suggested that although lead is not able to cross the BBB effectively, increased accumulation of lead in the brain following lead and ethanol coexposure could be due to alterations in the permeability of the BBB by ethanol.

Activity of tyrosine hydroxylase was seen to increase in lead-treated animals as well as in ethanol-treated animals compared to the control group. Tyrosine hydroxylase activity was further increased in the lead and ethanol coexposed groups compared to only lead-treated animals. The hydroxylation of tyrosine to form DOPA by the enzyme tyrosine hydroxylase is the rate-limiting step in the biosynthesis of dopamine and norepinephrine. A preliminary study of the synaptosomal conversion of tyrosine to dopamine (4,5,66), suggested enhancement of catecholamine synthesis in the presence of lead at concentrations of lead ranging from 0.1 μM–10 mM. Regulation of tyrosine hydroxylase is known to be mediated by a phosphorylation-dependent process, wherein both cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases have been implicated (8). Because in vivo exposure to lead has been reported to increase the activity of calmodulin in the brain (51), activation of tyrosine hydroxylase by lead may involve enhanced Ca^{2+}/calmodulin-dependent protein phosphorylation (27), an observation in line with reports of Wince and Azarro (65), who also observed enhanced tyrosine hydroxylase activity following in vivo lead exposure. Increase in the activity of tyrosine hydroxylase following ethanol exposure may constitute a compensatory mechanism for dopamine synthesis to accommodate for accelerated dopamine catabolism (41). When animals were coexposed to lead and ethanol simultaneously, activity of tyrosine hydroxylase increased further. This increase in tyrosine hydroxylase activity could be because of enhanced calcium/calmodulin-dependent phosphorylation, which corresponds to increased lead levels in this group (50).

As presented in Table 3, lead exposure decreased the levels of DA in the rat brain. Coexposure to lead and ethanol also resulted in a significant decrease in DA levels. This was contrary to our previous findings of an increase in the activity of tyrosine hydroxylase, implying that the levels of DA might increase following lead exposure. Another possibility for decreased DA levels could be its conversion to NA. Our results show that NA, formed as a result of hydroxylation of DA was found to increase following lead exposure. Coexposure to

TABLE 5

EFFECT OF LEAD, ETHANOL, AND THEIR COEXPOSURE ON THE BINDING CONSTANTS FOR DOPAMINE RECEPTORS IN RATS EXPOSED TO LEAD, ETHANOL, AND COMBINATION OF THE TWO

Group	K_d (nM)	B_{max} (fmol Spiperone/mg Protein)
Control group	0.55	48.43 ± 7.9
Lead-treated group	0.48	60.74 ± 4.22
Ethanol-treated group	0.52	52.67 ± 2.05
Lead + ethanol-treated group	0.45	75.40 ± 2.54†‡

K_d—dissociation binding constant. B_{max}—maximum number of binding sites. Value of B_{max} was found to be 425 fmol/mg protein in corpus striatum using 0–0.5 nM ^{3}Hspiperone and 1 μM haloperiodol.

Values are mean ± SD of six animals/group. K_d values are antilog of mean of six log values. Multiple comparisons were done when F-value is $p < 0.05$.

$\dagger p < 0.01$, significantly different from control group; $\ddagger p < 0.01$, significantly different from lead-treated group. NS—not significant.

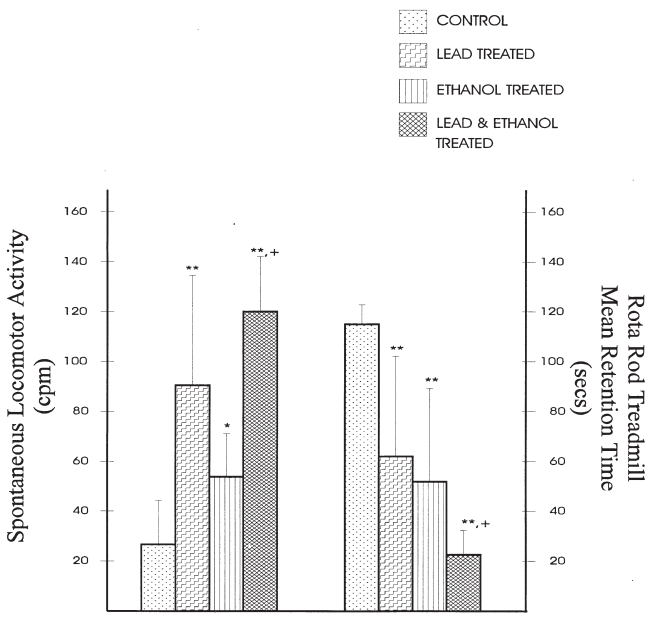

FIG. 2. Effect of lead, ethanol, and their coexposure on motor functions. Values are mean ± SD of six animals/group. $*p < 0.05$, $**p < 0.01$, significantly different from control group, $^{+}p < 0.05$ significantly different from the lead-treated group; NS—not significant.

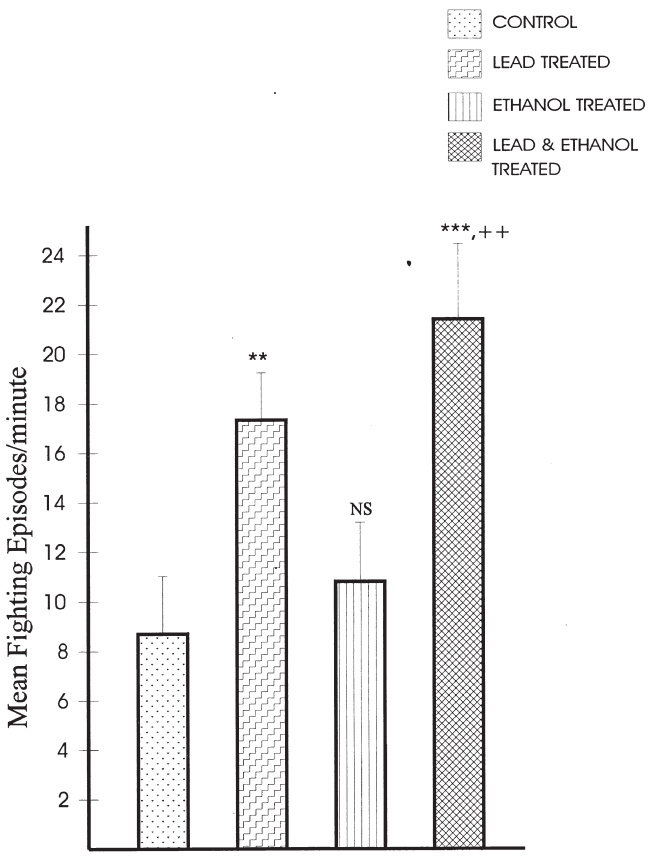

FIG. 3. Effect of lead, ethanol, and their coexposure on the aggressive behavior of rats. Values are mean ± SD of six animals/group. $**p < 0.01$, $***p < 0.001$, significantly different from control group, $^{+++}p < 0.001$, significantly different from the lead-treated group; NS—not significant.

lead and ethanol also resulted in an increase in the NA levels in the cerebrum compared to control animals. Our results are supported by a number of studies in which decreased steady-state levels of DA have been observed in the whole brain as well as various brain regions (20,23) following lead exposure.

The decreased levels of DA and increased NA levels observed following lead exposure could be the results of altered activity of biosynthetic and degradative enzymes, or alternatively, could be due to altered availability of their precursor amino acid tyrosine. Our results presented in Fig. 1 indicate an increased high-affinity tyrosine uptake following lead as well as ethanol exposure. When animals were coexposed to lead and ethanol, the tyrosine uptake was further increased. In vitro, higher lead concentrations have been shown to increase the uptake of tyrosine by rat brain synaptosomes (55); herein it has been suggested that tyrosine uptake is a calcium-sensitive process, and lead appears to interfere with this process by interacting with Ca^{2+} (51). An increase in tyrosine uptake following ethanol exposure could be the result of its effect on the membrane structure. Perturbations in membrane lipid and protein conformations upon ethanol intoxication have also been suggested as a possible mechanism to modify the influx and efflux of dopamine from synaptosomes (35). Ethanol, like other anesthetics, is known to disorder and fluidize the membranes, but chronic ethanol treatment has been shown to result in the development of a number of adaptive modifications (34). However, evidences are available in the literature indicating that chronic ethanol administration changes the normally occurring interaction between various proteins and lipids and thus affecting the functioning of various membrane bound enzymes and ion channels that are dependent on their immediate surrounding lipids for optimal activity (58). Thus, our results imply that both lead and ethanol exposure resulted in a significant activation of dopamine synthesis by increasing tyrosine uptake and dopamine synthesizing enzyme tyrosine hydroxylase.

Lead exposure has been shown to cause a significant increase in the activity of DBH in the cerebral region of rat brain compared to control animals. Ethanol exposure, however, did not affect the enzyme activity compared to control animals. Coexposure to lead and ethanol on the other hand elicited a significant increase in activity. Prolonged stress accompanied by chronic sympathetic nerve activity is known to result in the induction of DBH that could be a possible means of adapting to physiological stress (26). Appreciable accumulation of lead in the brain of animals exposed to lead and lead- and ethanol-coexposed animals might be the possible cause of increased DBH activity observed. Jacob and Monod (29) indicated that proteins undergo a regulation in response to changes in the microenvironment. Increased DBH, in turn, could be responsible for the enhanced formation of norepinephrine.

Our results presented in Table 4 revealed a significant increase in the activity of monoamine oxidase following lead and ethanol exposure individually. When animals were coexposed to lead and ethanol, the activity of MAO increased synergistically. Monoamine oxidase is a membrane-bound enzyme, and several investigations have indicated that agents that disrupt the lipid environment can alter the substrate kinetic characteristics of MAO (58). High activities of MAO have been observed in alcoholics by Carlsson et al. (11). An increase in MAO activity observed following ethanol exposure might increase the levels of DA metabolites as observed by Honkanen

et al. (28). Coexposure to lead and ethanol increased the activity of MAO further compared to lead-treated animals, possibly activating it in a synergistic manner. Our previous results corroborate the studies of Shih and Hanin (53) and Silbergeld and Chisolm (54), where a consistent increase in the levels of two catecholamine metabolites, homovanillic acid (HVA) and vanillylmandelic acid (VMA), has been reported in the brain and urine of lead-intoxicated animals and humans. Because MAO is the principle enzyme involved in the metabolism of dopamine and norepinephrine, an increase in its activity, as observed following lead exposure in our study, could be responsible for the increase in HVA and VMA in brain and urine of lead-intoxicated animals. However, to further understand the reduced DA levels with its increased biosynthesis, release and reuptake of dopamine and specific turnover studies measuring DA metabolites against total DA shall be planned in the future.

The nerve impulse is propagated from one neuron to the other via the binding of a specific neurotransmitter released from the presynaptic terminus, to the receptors on the postsynaptic terminus. It is evident from Table 5 that exposure to either lead or ethanol did not affect the binding affinity as well as maximum number of binding sites for dopamine D_2 receptors in the cerebrum of the rat brain. However, coexposure to lead and ethanol resulted in a marked increase in the dopamine receptor sites. These results indicate alterations in dopaminergic transmission at the postsynaptic site following lead and ethanol coexposure, which could be due either to direct effects of elevated brain lead levels or because of altered membrane structure by ethanol (57). Further, alterations in the B_{max} of D_2 receptors may result in the G-protein activation and thus changes in the second messenger system. Following lead exposure, changes in the G-protein–mediated signal transduction are well documented in the literature (47,51).

Studies were also carried out to assess the neurobehavioral deficits to investigate whether the changes at the biochemical level are of sufficient magnitude and biological relevance to have an impact at the level of the whole animals. Therefore, it was important to conduct the neurobehavioral studies, because behavior is regarded as the net output of sensory, motor, and cognitive functions in the nervous system, making it a potentially sensitive end point of chemically induced neurotoxicity (2,13).

Both chronic lead exposure and ethanol exposure independently resulted in a significant increase in the spontaneous locomotor activity of animals compared to control animals, which was further increased when animals were coexposed to lead and ethanol. It has been suggested that hyperactivity, a serious clinical aspect associated with lead intoxication, may occur as a consequence of specific alterations in neurotransmitter systems (40). Impairment of the dopaminergic system as reported in this study, involving enhanced dopamine synthesis, increased tyrosine uptake, aberrations in DA receptor regulation and function, along with altered dopamine metabolites could be responsible for hyperactivity of animals exposed to lead (13,60).

Our results of increased spontaneous locomotor activity following ethanol exposure are in line with those of Rommelspacher et al. (49), who reported a marked increase in the locomotor activity of ethanol-intoxicated animals. In addition, other psychoactive drugs such as cocaine and amphetamine (32,48) share an ability to produce locomotor stimulation in rodents. This drug-related increase in locomotor activity is apparently correlated with the activation of the dopaminergic system produced by these drugs of abuse (28). Following exposure to ethanol, an increase in the activity of both synthesizing and degrading enzymes suggests an activation of the dopaminergic system, which could be responsible for the hyperactivity of animals observed in ethanol-exposed animals.

Chronic lead administration also resulted in a marked impairment of the motor coordination of animals as revealed by the significant reduction in the retention time on the rotarod apparatus. Ethanol exposure also resulted in significant alterations in the performance on the rotarod apparatus. In animals coexposed to lead and ethanol, retention time on the rotarod apparatus was further diminished, indicating a possible synergistic action of lead and ethanol in producing motor incoordination. Neurotoxic manifestations of this heavy metal as reported by WHO (67), typically include weakness of the extensor muscles, particularly those used most heavily for performing the various motor functions.

Aberrations in motor functions following ethanol exposure have also been observed in alcoholics. Alcoholic peripheral neuropathy is a mixed motor sensory impairment affecting the distal regions, primarily the legs, and occurs in more than 80% of patients with severe neurological disorders such as Wernicke-Korsakoff syndrome, a neuronal disease associated with chronic alcoholism (1). Thus, the neurochemical aberrations, indicating an activation of the dopaminergic system, were reflected in the observed deficits in motor functions following lead and ethanol coexposure. Hence, alterations in the dopaminergic transmission that could be because of the marked increase in lead levels in the brain of animals coexposed to lead and ethanol compared to only lead-treated animals, might be responsible for motor deficits of greater magnitude in this group of animals.

Impairment of dopaminergic transmission is further reflected by the aggressive behavior of animals exposed to lead. Coexposure to lead and ethanol resulted in further accentuation of aggressive behavior of animals. A study of the mechanisms of stimulus-evoked aggression has revealed the participation of brain dopaminergic system in aggression (44). Our results are supported by Petit and Alfano (46), who have also reported aggression in lead-intoxicated animals as well as in children. Thus, on the basis of the results obtained in the present study, it can be speculated that although relatively low levels of lead are able to reach the brain because of an effective blood–brain barrier, exposure to lead along with ethanol enhances the accumulation of lead by twofold. Thus, elevated brain lead levels, along with well-known membrane-disrupting effects of ethanol, might be responsible for marked disruptions in the dopaminergic transmission with resultant aggressive behavior, motor incoordination, and hyperactivity in animals.

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