

Effect of folic acid in prenatal alcohol induced behavioral impairment in Swiss albino mice

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KEY WORDS

Folic Acid
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ABSTRACT

Background: Alcohol is a potent teratogen inducing oxidative stress as well as a massive wave of apoptosis in the developing brain as well as oxidative stress. It affects brain including cerebellum, hippocampus and cerebral cortex resulting into motor and cognitive deficits. Alcohol depletes folic acid from the body which is essential for synthesis of DNA, RNA and protein during cell division and proved to prevent many brain related malformations. **Purpose:** The objective of the present study was to study whether folic acid reduces behavioral impairments that were induced by prenatal exposure to ethanol in mice. **Methods:** Pregnant mice were divided into different experimental groups. Group I termed as control receiving distilled water, group II received ethanol, group III ethanol and folic acid and group IV folic acid only from gestational days 6 to 15. The dams were allowed to deliver their offspring naturally and until weaning the pups remained with their natural mothers. At the age of 8-9 weeks, they were subjected to battery of various behavioral tests. **Results:** The alcohol exposed dams showed decreased motor activity in open field test and decreased exploration and increased anxiety in elevated maze test as compared to controls. Folic acid administration reduced the intensity of these effects of alcohol in mice. **Conclusion:** The exposure to alcohol in utero produces long lasting effect on the developing pharmacological character of brain affecting postnatal behavioral expression which may be reduced by prenatal folic acid administration.

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Introduction

Chronic maternal ethanol abuse during pregnancy is associated with important teratogenic effects on the offspring. It is the leading cause of mental retardation and congenital malformations.¹ However, the extent and severity of malformations depends upon the dose and time of consumption during pregnancy.² The most common abnormality is Fetal Alcohol Syndrome which is characterized by growth retardation, microcephaly, poor coordination, underdevelopment of mid- facial region and minor joint anomalies.³

Ethanol readily crosses the placenta and reaches concentrations in the fetus that are similar to those in the maternal blood.⁴ Due to low activity of hepatic dehydrogenase, the fetus is limited in its ability to metabolize alcohol. In addition, the rate of diffusion of alcohol from amniotic fluid is slow which can act as reservoir for alcohol and the fetus can be actually exposed to it for a longer period.⁵ It induces a massive wave of apoptosis in the developing brain and increases neuronal losses within different regions of brain.⁶⁻⁹ According to some authors, these neuronal deficits are permanent, extending into adulthood^{10,11} It also increases oxidative stress and activates caspase-3 effector proteins.¹²⁻¹⁴ Due to low fraction of antioxidant enzyme activity the developing brain is thought to be more susceptible to the neurotoxic effects of oxidative stress than the adult brain.¹⁵ Cerebellum and hippocampus have been shown to be particularly vulnerable to oxidative stress and suffer most from alcohol insult.¹⁶⁻¹⁸ Therefore the motor and cognitive deficits are the most common consequences of the alcohol intoxication.¹⁹

Folic acid (Folate), a water soluble B-vitamin whose biologically active form is tetrahydrofolate, is essential for cell division and for the synthesis of DNA, RNA and protein. Ethanol ingestion induces a marked increase in urinary excretion of folic acid and decreases hepatic and serum folic acid, resulting into folic acid

deficiency.²⁰⁻²³ Gestational folic acid deficiency leads to impressive net reduction of cells in fetal brain and spinal cord.²⁴ It can cause a wide array of disorders in the fetal brain ranging from subtle changes in intelligence to profound mental retardation manifesting as severe damage in learning capabilities or impaired adaptation abilities in their environments.^{25,26} We therefore wanted to test whether folic acid when given along with alcohol may reduce the neurobehavioral impairments induced by alcohol in mice.

Methods

Animals

In the present study female Swiss albino mice weighing approximately 27 gm (± 2 gm) were used. The animals were housed in Animal house of Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University under a temperature-controlled environment with a 12-h light/12-h dark cycle. They were permitted access to standard animal feed and tap water *ad libitum*.

Determination of pregnancy

The male and female were mated in the ratio of 2:1. The occurrence of a vaginal plug was considered as gestation day 0 (GD 0). Plug positive dams were housed individually in polypropylene cages in the same laboratory conditions.

Experimental design and drug treatment

On GD 6 the pregnant mice were randomly assigned to Control group (Group I, n = 8), Alcohol group (Group II, n = 8), Alcohol and Folic acid group (Group III, n = 8) and Folic acid group (Group IV, n = 8). From GD 6 to GD 15, the mice in group II were given ethanol at a dose of 6 gm/kg/day orally through oral gavage needle. Mice in the group III group were given ethanol 6 gm/kg/day and Folic acid 60 mg/kg/day on the

same gestational day. Group IV mice received only folic acid 60 mg/kg/day. The Folic acid was given 2 hrs after ethanol administrations. Group I mice received equal volume of distilled water. The weight of mice was measured daily from GD 0 to GD 18.

In 50% of dams, the fetuses were collected for histological and biochemical study (not included in this report) and remaining 50% dams were allowed to deliver their offspring normally. The day of birth was considered as postnatal day 0 (PND 0). At birth, pups from each group were culled to four pups (2♀, 2♂) per litter on the basis of body weight. The culled pups were weaned with their natural mothers till 6th week. Four male pups (n = 4) and four female pups (n = 4) were selected randomly from each group (n = 8/group) for the behavioral study. The selected mice were kept in separate cages. The remaining mice were sacrificed for neurohistological study (not included in this report). The weight of pups was taken every week from the time of birth to PND 70 (11th week). The use of animals in this research was approved by, and conducted in compliance with the guidelines of Animal Ethics Committee of Banaras Hindu University.

Behavior testing

From 8 weeks (PND 52) onwards male and female pups from each group were tested for locomotor activity, anxiety, exploration, learning, memory and depression in a series of behavioral tasks which included open field (first 3 days) and elevated plus maze (1 day).

Open field test

The mouse was placed on one of the corners of open field (60 cm × 60 cm × 60 cm) the floor of which was divided into 16 squares (15 cm × 15 cm) by white painted lines. The field was lit by a 100 watt bulb which was kept 2 m above. The test lasted for 5 minutes. The observed parameters were ambulation, rearing, freezing time, grooming and number of faecal boli. Before each trial, the floor and the walls were cleaned with cotton soaked in 70% ethanol. The test was done for 3 consecutive days in the morning. The scores of three days were averaged and average value of each parameter was used for further calculations.

Elevated plus maze test

The apparatus consist of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) which are connected through central platform (10 cm × 10 cm). the arms are arranged in a cross shape with the two open arms facing each other and two closed arms facing each other. The maze was kept 45 cm above the floor. The mouse was placed at the centre of the plus maze with its face directed towards one of the closed arms and observed for 5 minutes. The number of entries into open arm, closed arm and central square as well as period of permanence in those areas were observed. The floor and the walls of the open and closed arms were cleaned with 70% alcohol before each trial.

Statistical analysis

The experimental results were expressed as mean ± SD. Data was analyzed by ANOVA and Kruskal-Wallis test using SPSS (Version 16) system to determine their significance was if the comparison between the groups was significant, Mann-

Whitney U test and SNK test were used for *post hoc* analysis, $p \leq 0.05$ was considered as significant.

Results

Body weight

During pregnancy, the gain in maternal body weight from GD0 to GD18 differed significantly between the different groups ($F = 7.864$, $P = 0.001$). The maximum gain in maternal body weight was found in group IV followed by group III and group I and least in group II (Fig. 1). Post hoc analysis showed that the gain in maternal weight was significantly lower in group II as compared to group I ($p = 0.037$), group III ($p = 0.032$) and group IV ($p = 0.001$) but among group I, group III and group IV the difference was insignificant ($p > 0.05$).

The weight of pups was recorded every week till postnatal day (PND) 70. There was significant difference in mean weight of pups between the groups in each week till 10th week. On post hoc analysis the mean weight of pups of group II was found to be significantly lower but on PND 70, the difference disappeared statistically (Fig. 2).

Open field test

In the open field test, used to measure the locomotor activity and exploratory behavior, the values for ambulation, rearing, freezing time and number of faecal boli were observed to be significantly different in different groups of mice when

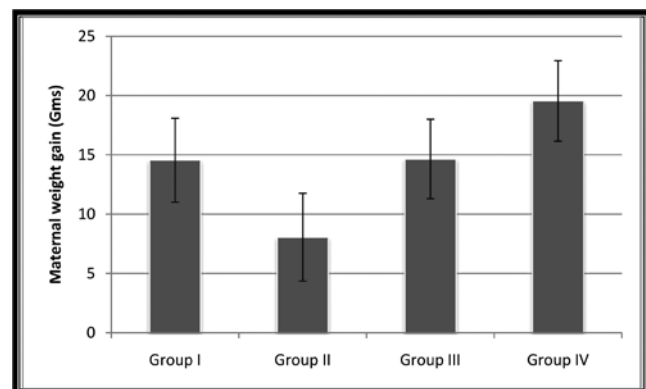


Fig. 1: Maternal weight from GD0 to GD18 in different groups of mice.

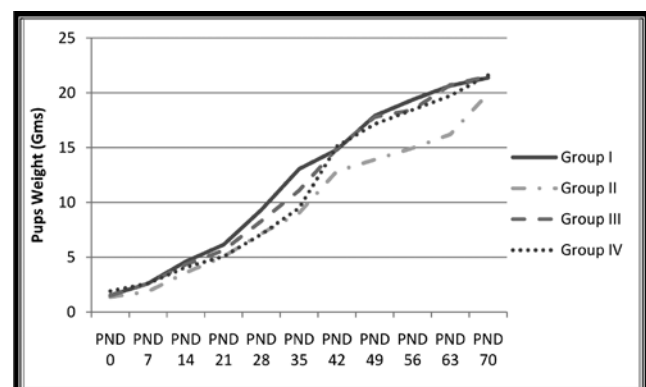


Fig. 2: Weights of pups in different groups of mice from PND 0 to PND 70.

analyzed by one way ANOVA and Kruskal-Wallis test. Highest ambulation and rearing scores were observed in group IV and lowest in group I (Fig. 3). Similarly freezing time and defecation was maximum in group I and minimum in group IV while the grooming score was highest in group I and lowest in group III (Fig. 4)

Post hoc analysis indicated significant decrease in ambulation and rearing and increase in freezing time in group II when compared with Group I ($p = 0.015$) and group IV ($p = 0.001$). But when Group II and group III were compared, group III showed significant increase in rearing scores ($p = 0.017$) and significant decrease in freezing time ($p = 0.001$) but the increase in ambulation scores were not significant ($p = 0.231$). The data shows that alcohol intoxication during pregnancy significantly decreased locomotor activity in offspring and folic acid administration reduced this deleterious effect of alcohol. The defecation scores were significantly higher in group II when compared with group I ($p = 0.033$), group III (0.003) and group IV (0.001). Grooming scores didn't show significant difference between different groups ($p > 0.05$) though highest grooming score was observed in group I and III and lowest in group IV (Fig. 4).

Elevated plus maze test

In the elevated plus maze test there was significant difference between the groups ($p = 0.01$) for open arm entries, open arm duration, central square entries, central square duration, closed arm entries and closed arm duration when analyzed by

one way ANOVA and Kruskal-Wallis test. Highest open arm entries and open arm duration was observed in group I, highest central square entries and central square duration in group IV, highest closed arm duration in group II and highest closed arm entries in group IV (Fig. 5 & 6).

Post hoc analysis showed that the mice in group II spent significantly less time in open arms as compared to group I ($p = 0.001$), group III (0.018) and group IV (0.001). Although mean open arm duration was highest in group I, than in group IV and in group III, but the values were not statistically significant when compared between the three groups (Fig. 6). The open arm entries were significantly lower in group III as compared to group I ($p = 0.018$) and group IV ($p = 0.047$). Central square duration was significantly higher in group IV when compared with group II ($p=0.001$) and group III ($p = 0.035$). Central square entries were also significantly higher in group IV when compared with group I ($p = 0.015$), group II (0.001) and group III (0.001). Closed arm duration was significantly higher in group II as compared to group I ($p=0.001$) and group IV ($p = 0.001$). Closed arm entries was significantly higher in group IV as compared to group I ($p = 0.001$), group II ($p = 0.001$) and group III ($p = 0.001$). The data show that ethanol administration during pregnancy significantly increased the anxiety and decreased the exploration in mice in terms of increased closed arm duration and decreased open arm duration and folic acid exposure along with ethanol lower this effect.

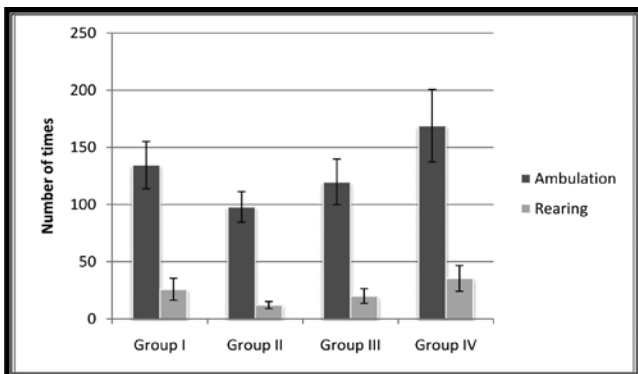


Fig. 3: Ambulation and rearing scores in different groups of mice.

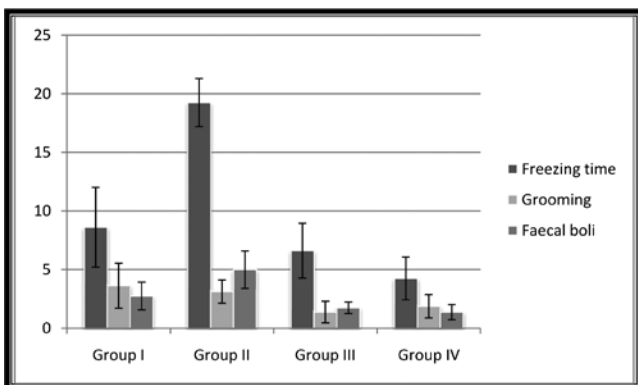


Fig. 4: Freezing time, grooming and number of faecal boli in different groups of mice.

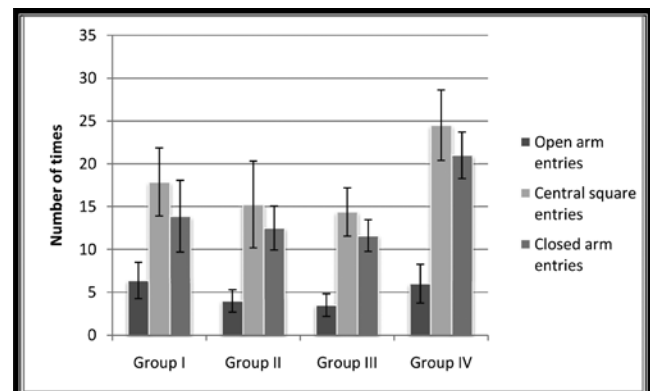


Fig. 5: Number of entries in different areas of elevated plus maze by different groups of mice.

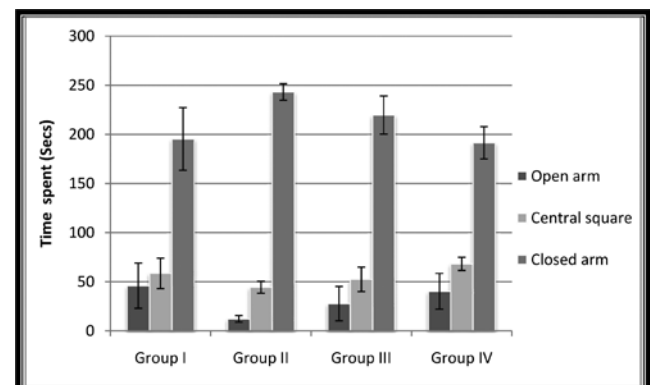


Fig. 6: Time spent in different areas of elevated plus maze by different groups of mice.

Discussion

In the present study, the dam's weight gain in group II (Alcohol) was significantly lower than group I (Control). In group III (Alcohol + Folic acid), the body weight of dams were equivalent to group I. In group IV (Folic acid) the body weight was significantly higher in comparison to group I, II and III. The weight of pups also increased or decreased respectively. In ethanol treated group the weight of pups was significantly low till 10th week of postnatal development. From 11th week onwards the increase was noticed. Similar observations were reported by Dursun et al. (2006)¹⁹ and Wang et al. (2009)²⁷ also. This result may be attributed solely to the effect of alcohol after considering that it alters the food intake and nutrient absorption including micronutrients.^{28,29} Therefore, the prenatally undernourished mice undergo prolonged catch-up growth.

Central nervous system dysfunctions are the most severe and permanent consequences of maternal alcohol intake.³⁰ The impaired cognitive and behavioral functions resulting from damage to the central nervous system have been described as distinguishing features of prenatal alcohol exposure.³¹⁻³³ Both human and animal research provide strong evidence of the damaging effects of ethanol on central nervous system during development.³⁴

The open field test and elevated plus maze test was used to access the effects of folic acid on behavioral changes induced by prenatal alcohol exposure in Swiss albino mice. The data showed that decrease in locomotor activity and increase in anxiety levels induced by prenatal alcohol exposure was reduced by subsequent folic acid supplementation. These results suggest that folic acid is depleted during prenatal alcohol exposure which is necessary for development of brain.

In the present study prenatal ethanol administration at the dose of 6 g/kg/day from GD6 to GD15 to pregnant dams significantly decreased their locomotor activity as assessed by open field test in terms of decreased ambulation, rearing and increased freezing time in alcohol exposed mice. Several authors have also reported decreased locomotor activity in offspring after alcohol intoxication during pregnancy as compared to control.^{19,34} The folic acid administration along with alcohol significantly increased the locomotor activity. Decreased open field activity in folate deficient animals has also been reported by Ferguson et al. (2005).³⁵

In elevated plus maze test, the ethanol exposed mice spent significantly less time in open arm and more time in closed arm as compared to controls. This observation shows lower ambulation score in this group as compared to controls in open field test suggesting increased level of anxiety in alcohol pretreated mice as also reported earlier.¹⁹ Elevated anxiety in alcohol treated mice offspring as assessed by their plus maze behavior is consistent with reports by several authors that animals exposed to alcohol in utero are typically hyper responsive to stressors in adulthood as indicated by increased activation of the hypothalamic-pituitary-adrenal axis.^{19,36-38} It is also consistent with the report that prenatal exposure to alcohol decrease sensitivity to gamma amino butyric acid (GABA) receptor's allosteric modulators such as endogenous neurosteroid allopregnanolone which is thought to act as an endogenous anti-anxiety agent in novel or stressful situations.³⁹ In the present experiments when folic acid was administered along with ethanol, the open arm duration was significantly increased and closed arm duration significantly decreased as compared to only alcohol group.

There was no significant difference in open arm and closed arm duration between control, alcohol plus folic acid and folic acid groups. The results show that prenatal alcohol exposure significantly increased the anxiety level in mice which is subsequently decreased by folic acid administration. In folic acid deficient mice decreased open arm duration and increased closed arm duration has also been reported by Ferguson et al. (2005).³⁵

The behavioral impairments which were seen in prenatal alcohol exposed mice in the present study might be due to deficiency of folic acid during development of the fetus because it has been shown that alcohol ingestion induces noted increase in urinary excretion of folic acid depleting the hepatic and serum folic acid level which leads to folic acid deficiency.²⁰⁻²³ *In vivo* and *in vitro* studies have suggested that alcohol may impair transport of folic acid across placenta by decreasing expression of folate transport proteins.⁴⁰⁻⁴² It has been postulated that behavioral impairments associated with lower folate status are the neurodevelopmental consequences of alcohol exposure during pregnancy.⁴³⁻⁴⁵ Under the state of folic acid insufficiency, nucleic acid biosynthesis is inhibited, and cells are unable to manufacture enough DNA for mitosis. Additionally, inhibition of the methylation cycle results in an inability for methylation of proteins, lipids and myelin. Thus, many of the mechanisms proposed to explain congenital birth defects resulting from folic acid deficiency or genetically rooted errors in folic acid metabolism are based on disturbances of biosynthesis and methylation cycle.⁴⁵⁻⁴⁸

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