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EFFECT OF ALCOHOL AND FOLIC ACID ON PREGNANCY OUTCOME OF SWISS ALBINO MICE

[1]Uttam Shrestha, [2*]Mandavi Singh

[1]Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Utter Pradesh-221005
[2]Department of Anatomy and Dean, Faculty of Medicine, Institute of Medical sciences, Banaras Hindu University, Varanasi, Utter Pradesh-221005

ABSTRACT

Exposure to ethanol in utero is known to produce many congenital malformations in developing fetus. Ethanol depletes folic acid from the body which is essential for synthesis of DNA, RNA and protein during cell division and its requirement is increased during pregnancy. The objective of the present study was to observe whether folic acid reduces the adverse pregnancy outcome induced by ethanol exposure during pregnancy. Pregnant mice were divided into different experimental groups. Group I termed as control received distilled water, group II received ethanol, group III received ethanol and folic acid and group IV received folic acid only from gestational days 6 to 15. On GD 18, the dams were sacrificed. The fetuses were extruded and observed. In alcohol exposed dams the number of live fetuses was reduced while the numbers of fetuses with birth defects as well as resorbed/dead fetuses were significantly increased. Similarly the weight, CRL and tail length of fetuses of alcoholic dams were also severely affected. Folic acid reduces the adverse effect of ethanol. So the study showed that the adverse pregnancy outcome in alcoholic pregnancy might be due to folic acid deficiency which can be neutralized by folic acid supplementation.

Kew words: Ethanol, birth defects, fetal alcohol syndrome, gestation

Alcohol consumption during pregnancy is harmful to the healthy development of the fetus as well as continued growth and development after birth. Consumption of large amounts of alcohol during pregnancy often results in a specific pattern of malformations as well as mental retardation in the progeny known as fetal alcohol syndrome (FAS)[1]. The major clinical features of FAS include pre- and post-natal growth retardation, central nervous system dysfunction and craniofacial malformations are among many other abnormalities[2]. The toxicity of ethanol during development depends upon the dose and the time of exposure[3].

Folic acid (Folate) is water soluble B-vitamin whose biologically active form is tetrahydrofolate, is essential for cell division and for the synthesis of DNA, RNA and protein. Adequate folate intake during the periconception period helps protect against a number of congenital malformations, including neural tube defects[4]. The risk of neural tube defects is significantly reduced when supplemental folic acid is consumed in addition to healthy diet prior to and during the conception[5]. Supplementation with folic acid has also been shown to reduce the risk of congenital heart defects, cleft lips, limb defects and urinary tract anomalies[6].

Folic acid deficiency is a common feature in pregnancy, being more severe in alcoholics[7]. Ethanol ingestion induces a noted increase in urinary excretion of folic acid depleting serum and hepatic folic acid [8], leading to folic acid deficiency[9]. It is also suggested that ethanol reduces folic acid uptake by intestinal bacteria and its metabolism in liver. Animal

***Corresponding Author :**
mandavi5@rediffmail.com

and in vitro studies have found that ethanol may impair transport of folic acid across placenta by decreasing expression of folate transport proteins[10]. Folate deficiency during pregnancy may increase the risk of preterm delivery, infant low birth weight and fetal growth retardation, as well as increasing homocysteine level in blood, which may lead to spontaneous abortion and pregnancy complications, such as placental abruption and pre-eclampsia[11]. So we proposed that when folic acid is supplemented along with alcohol especially during organogenesis may reduce the alcohol related birth defects.

MATERIAL AND METHODS:

Animals:

In the present study female Swiss albino mice weighing approximately 27gm (±2gm) 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 1:2. The next day, 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 6gm/kg/day orally through oral gavage needle at 9 a.m. Mice in the group III group were given ethanol 6 gm/kg/day and Folic acid 60 mg/kg/day on the same gestational days two hours later. Group IV mice received only folic acid 60mg/kg/day. Group I mice received equal volume of distilled water only. The weight of mice was measured daily from GD 0 to GD 18.

Maternal and fetal assessments:

On GD 18, pregnant dams of each group were weighed and sacrificed by diethyl ether inhalation. The abdomen of the mouse was dissected. The uterine horns were cut open and carefully inspected for all implantations. The number of live, dead and resorbed fetuses were counted. Live fetuses were extracted from the uterus, examined for the presence of external malformations and their weight, crown-rump length and tail length was recorded. Two fetuses from each litter were kept in 70% alcohol for bone staining (data not shown) and remaining fetuses was preserved in 10% neutral formalin for soft tissue examination. The use of animals in this study was approved by, and conducted in compliance with the guidelines of Animal Ethics Committee of Banaras Hindu University.

Statistical analysis:

The experimental results were expressed as mean±SD. Data were analyzed by one way ANOVA using SPSS (Version 16) system to determine their significance. If the comparison between the groups were significant, SNK test was used for *post hoc* analysis, $p \leq 0.05$ were considered as significant.

RESULTS

Each experimental group comprised of 8 pregnant dams. Their weight gain from GD0 to GD 18 significantly differed between the groups ($p<0.01$) when analyzed by one way ANOVA. On post hoc analysis using SNK test the dams weight gain in group II was significantly lower than that in groups I, III and IV ($p<0.01$). There was no significant difference in dams weight gain between groups I , III and IV ($p>0.05$), although mean maternal weight gain was highest in group IV. The data showed that alcohol consumption during pregnancy has severe effect in maternal weight gain while these effects were reduced by folic acid administration.

There was no significant difference among the groups in terms of number of implantation per dam ($p>0.05$) although highest mean implant per dam was observed for group II and lowest for group I. The number of live fetuses per dam significantly differed between the different groups ($p<0.01$). Post hoc analysis showed significantly lower number of live fetuses in group II as compared to group I($p<0.05$), III($p<0.05$) and IV ($p<0.01$) but such difference was not observed between groups I, III and IV($p>0.05$) although the number of live fetuses per dam was highest in group IV.

Significant difference among the groups was observed for number of resorptions and dead fetuses per dam ($p<0.01$). Highest numbers were observed in group II and lowest in group I. On post hoc analysis resorptions and dead fetuses per dam

was significantly ($p<0.01$) higher in group II as compared to groups I, III and IV. While no such difference was observed between groups I, III and IV ($p>0.05$), The data showed that ethanol exposure to pregnant dams (6g/kg/day) from GD6 to GD 15 significantly decreases the number of live fetuses and increases the number of resorptions and death of fetuses as compared. While such findings were reversed after folic acid administration.

III and IV while such difference was not observed among groups I, III and IV ($p>0.05$)(Table 2). Similarly, mean crown rump length and mean tail length of the fetus significantly differed among the groups ($p<0.01$)(Fig 1 & table 1). The highest mean crown rump length and tail length was observed in group I and lowest in group II. Post hoc analysis showed that the crown rump length was significantly decreased in prenatally alcohol exposed fetuses as

Table 1: Distribution of maternal and fetal findings in different groups of mice

Findings	Group I	Group II	Group III	Group IV	P-value
Maternal weight gain (gm)	13.75±3.01	7.12±4.02	14.87±3.99	15.87±3.64	0.001
Implants per dam	7.50±2.13	8.63±1.51	7.87±0.83	8.37±0.92	0.421
Live fetuses per dam	7.25±2.25	4.75±1.16	7.00±1.30	7.62±1.30	0.004
Resorptions/dead fetuses per dam	0.25±0.46	3.87±0.83	0.87±0.83	0.75±0.88	0.001
Mean fetal weight (gm)	1.18±0.15	0.85±0.12	1.09±0.11	1.12±0.15	0.001
Mean crown-rump length (mm)	25.03±2.36	20.66±2.25	24.31±1.58	24.31±1.45	0.001
Mean tail length (mm)	11.97±0.90	10.26±1.59	11.33±0.86	11.54±0.86	0.001

Table 2: Group wise comparison of findings (p-value)

Groups	Maternal Wt Gain	Implants per dam	Live fetuses per dam	Resorptions / dead fetuses per dam	Mean fetal weight	Mean crown-rump length	Mean tail length
Groups I Vs II	<0.01	>0.05	<0.05	<0.01	<0.01	<0.01	<0.01
Groups I Vs III	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05
Groups I Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Groups II Vs III	<0.01	>0.05	<0.05	<0.01	<0.01	<0.01	<0.01
Groups II Vs IV	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01
Groups III Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

The fetal weight was compared between different groups. There was significant difference in mean fetal weight among the groups ($p<0.01$)(Fig. 1 & table 1). The highest fetal weight was observed for group I and least for group II. Post hoc analysis revealed that the mean fetal weight in group II was significantly ($p<0.01$) lower as compared to groups I,

compared to remaining groups (Table 2). The crown rump length and tail length of fetus was increased after folic acid supplementation.

Maximum numbers of fetuses with congenital malformations were observed in group II which received ethanol only followed by group III while such malformations were not observed in other

groups. In group III there were two fetuses with hemorrhagic patches. In group II congenital malformations was seen in 31.2% of the fetuses. There were 4 fetuses with hemorrhagic patches in body, limbs and tail, 3 fetuses with amelia, 2 fetuses with bilateral anopthalia, 2 fetuses with kyphosis and 1 fetus with defect in face development with elongated snout (Fig. 2)

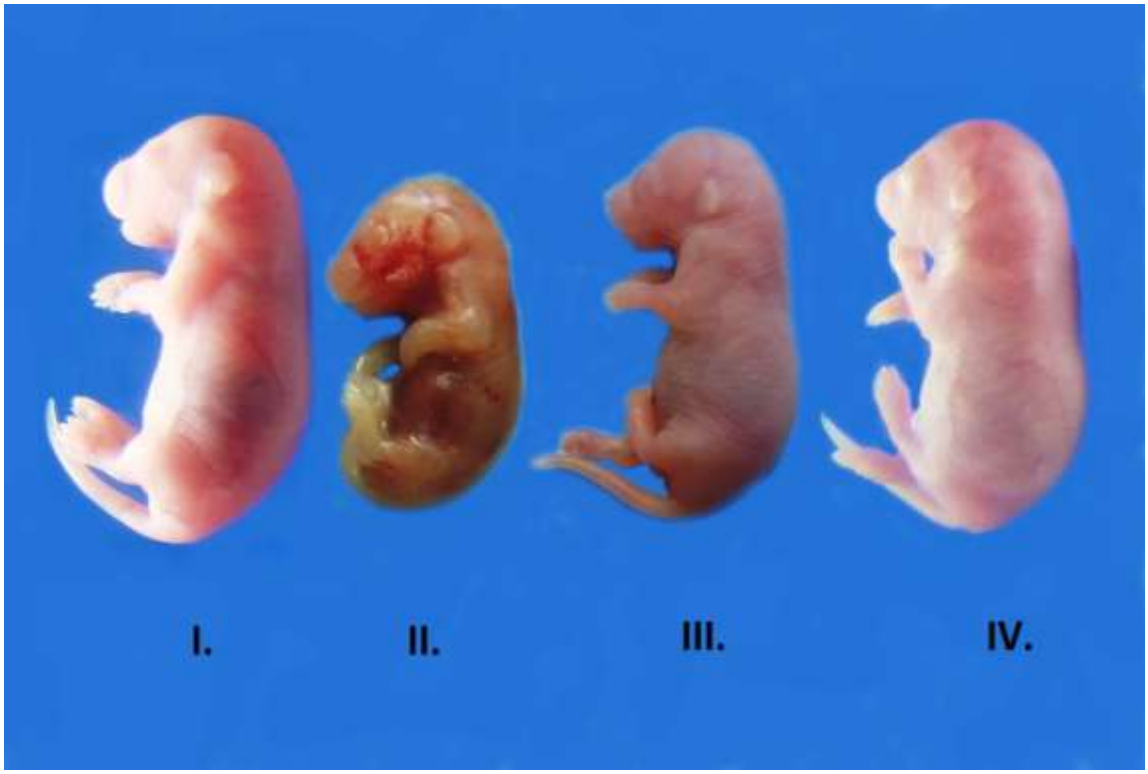

Figure 1: Fetuses prenatally exposed to D/W (I.), Ethanol 6gm/kg/day(II.), Ethanol 6gm/kg/day+Folic acid 60 mg/kg/day(III.) and Folic acid 60 mg/kg/day (IV.) from GD6 to GD15.

DISCUSSION:

It is well known that different exogenous and endogenous factors affect the developing fetus and alters the pregnancy outcome. This is thought to occur through fetal programming, which refers to the ability of changes in environmental factors (e.g., nutrition, stress, exposure to toxicants) at critical periods during development to permanently alter the structure, physiology or metabolism of the body, resulting in a lifelong effect on the developing fetus[12]. One such environmental factor is ethanol. Exposure to ethanol in utero is known to have serious long-term implications for the offspring and can lead to a diverse array of health problems, described collectively as Fetal Alcohol Spectrum Disorder (FASD)[13]. Fetal Alcohol Syndrome (FAS), the most severe manifestation of FASD, is characterized by a distinctive craniofacial dysmorphology, central nervous system abnormalities and growth retardation [2].

In the present study the dosage of ethanol use (6g/kg/day) seemed appropriate to induce developmental toxicity in Swiss albino mice. Oral route was used in the present study since it is more relevant to human ethanol exposure, and may be less stressful to the pregnant mice for daily exposure for 10 days period. Other investigators have used intraperitoneal (i.p.) injection, inhalation, intravenous infusion and voluntary drinking methods[14]. Since, intraperitoneal injection, inhalation and intravenous infusion methods may be fairly stressful and prenatal stress is known to adversely affect fetal development[15] these routes were avoided. In voluntary drinking it is difficult to monitor the dose of the ethanol used. Most rodents find the taste of ethanol aversive and so their fluid and food intake in response to ethanol in the drinking water is reduced[14]. Since, Fetal alcohol syndrome is more common among babies born of alcoholic mothers (who consume alcohol throughout pregnancy) the mice in our experiment were exposed to alcohol from GD6-15 as it reflects the

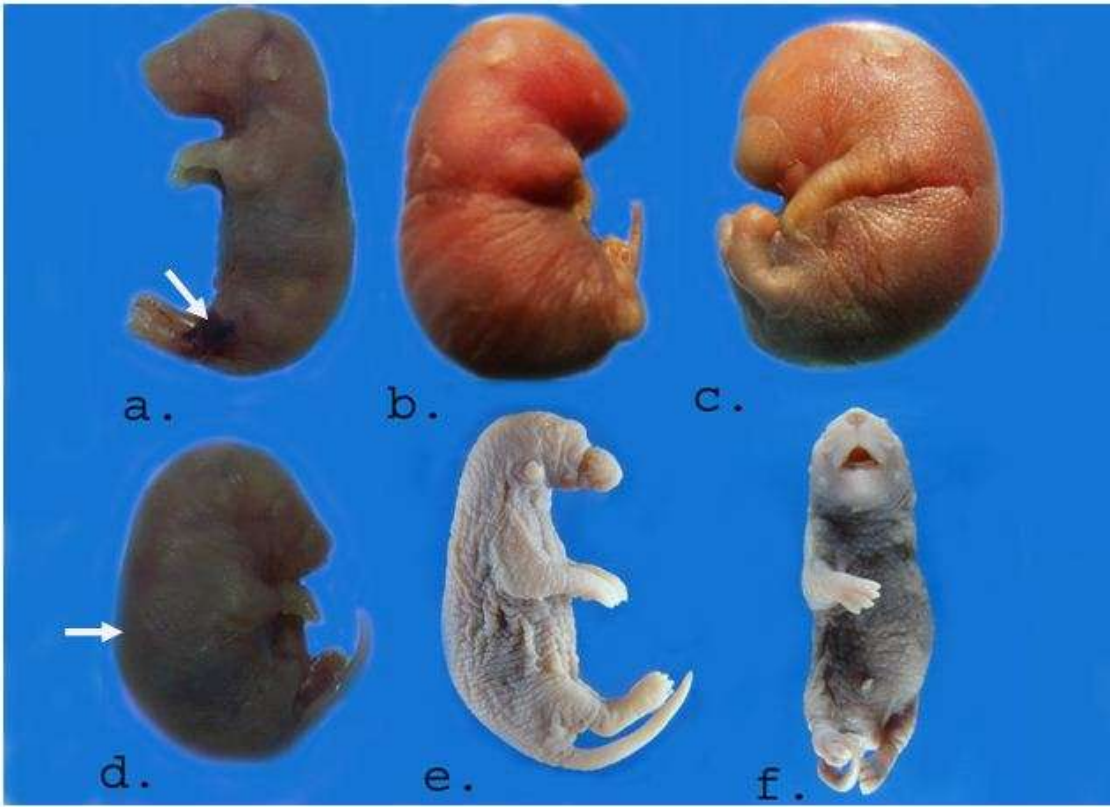

Figure 2: A fetus with hemorrhagic patches in lower left limb(⇲) (a.), fetuses with bilateral anopthalmia (b & c), a fetus with kyphosis(→) (d), a fetus with defect in development of face with elongated snout (e), a fetus with amelia of left upper limb with opening of mouth (f).

gestational exposure for human Fetal alcohol syndrome.

The weight gain during pregnancy in alcohol exposed dams was severely affected as compared to remaining groups. In our experiment the dams were provided food and water ad libitum during the whole test period to mimic the human situation. The food consumed in alcohol exposed mice was equivalent to those in other groups. Alcohol can interfere with digestion of ingested food and their absorption including those of micronutrients and vitamins like folic acid[16]. The dose related decrease in maternal weight gain during pregnancy in ethanol treated mice was also observed by Wang et al, 2009. Excess folic acid that was given in group III mice along with alcohol reestablished the weight loss due to alcohol in our study. Chronic alcohol exposure is known to decrease the serum folic acid level by decreasing it's intestinal absorption and increasing the urinary excretion[8]. Folic acid deficiency may lead to occurrence of anemia, by impairing erythropoesis, hence inducing hypoxia which is again a factor inducing congenital malformations[17]. This might result in decreased weight gain in alcohol exposed dams.

In the present study, the number of implantations didn't significantly differ between the groups because the treatment was started from GD 6 onwards at which implantation already completed. The number of live fetuses was significantly lowered in alcohol exposed group and the number of resorptions and the dead fetuses was greatly increased. Similarly, ethanol exposure during organogenesis resulted in developmental retardation of term fetuses. The weight, crown rump length and tail length were also severely affected. Similar findings were observed by other investigators. It is known that folic acid deficiency is a common feature in pregnancy, so it becomes more severe in alcoholics during pregnancy[7]. Ethanol ingestion induces a noted increase in urinary excretion of folic acid depleting serum and hepatic folic acid[8], leading to folic acid deficiency [9]. It is also suggested that ethanol reduces folic acid uptake by

intestinal bacteria and its metabolism in liver. Animal and in vitro studies have found that ethanol may impair transport of folic acid across placenta by decreasing expression of folate transport proteins[10]. So the adverse pregnancy outcome in the present study might be due to deficiency of folic acid induced by alcohol which was reversed by the administration of folic acid which neutralizes the effect of ethanol on the pregnancy outcome. Xu et al, 2005 also observed that birth defect induced by ethanol is reduced by maternal folic acid and Vit B12 supplementation. But the ethanol dose that was used in that study was lower than the present study[18]. Wang et al, 2009 also observed that teratogenic effects of ethanol can be suppressed by folic acid supplementation[19].

Folic acid deficiency causes elevated serum homocysteine (HCY) in animals and humans[20]. Elevated HCY during pregnancy may result in different developmental defects[18]. So the increased number of fetuses with congenital malformation in alcohol exposed mice that was observed in the present study might be due to elevated HCY level. Folic acid supplementation decreases the homocysteine level. This may be the reason of lowering the number of malformations that was seen in folic acid supplemented group in our study. Folic acid is an important cofactor for enzymes that are essential in DNA and RNA synthesis that is involved in the transfer of methyl groups in the amino acid methylation cycle. It is an essential step in the recycling of homocysteine back to methionine. The demand for folic acid must be increased during pregnancy as the synthesis of the nucleic acids and proteins increases during rapid embryonic and fetal growth during development. The function of specific proteins, lipids, or even myelin might be impaired by Inhibition of the methylation cycle. These mechanisms might explain the adverse pregnancy outcomes resulting from folic acid deficiency [17,21,22] due to alcohol exposure in utero.

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