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Folic Acid Ameliorates Prenatal Alcohol Induced Bone Malformations in Mice Fetuses.

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ABSTRACT

Consumption of ethanol during pregnancy can result in different types of skeletal malformations by acting directly on the cells of the developing bone or by affecting the systemic factors required for bone development. Folic acid is one of the important water soluble B-vitamin which is required during pregnancy for proper development of fetus. Alcohol consumption has negative impact on maternal folic acid level. The objective of present study is to observe whether administration of folic acid along with alcohol reduces alcohol related bone malformations. The plug positive mice were randomly divided into four groups. Group I mice were termed as control, group II mice were given alcohol 6gm/kg body weight of mice, group III mice were administered alcohol 6gm/kg body weight and folic acid 60 mg/kg body weight of the mice while group IV mice were given only folic acid 60 mg/kg body weight. On GD 18 the pregnant mice were sacrificed and fetuses were collected. The fetuses were processed for alizarin red staining. Prenatal alcohol exposed fetuses showed different types of axial and appendicular bone malformations. When folic acid was given along with alcohol such malformations were greatly reduced showing protective effects of folic acid.

Keywords: alizarin red, mineralization, pregnancy, organogenesis, birth defects

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INTRODUCTION

Ethanol, present in alcoholic beverages, is the most widely used psychoactive drug in our society causing an anesthetic type response in the brain [1,2]. Maternal ethanol consumption during pregnancy can produce several birth defects in the developing fetus that manifest as congenital malformations in postnatal life. This teratogenicity of ethanol was first described decades ago which is characterized by the ability of ethanol to induce malformations in the developing embryo or fetus [3].

It has been clear that prenatal alcohol exposure results in different types of fetal bone malformations. Alcohol crosses the placenta and reaches in concentration that is similar to the maternal blood [4]. So it may exert its effects on fetal bone development by acting directly on the cells of the developing bone or by affecting the systemic factors required for bone development [5]. In vitro and in vivo studies had shown that alcohol inhibits bone formation by inhibiting osteoblast proliferation and activity [5]. Fetal alcohol syndrome which is one of the predominant feature of prenatal alcohol exposure is characterized by short stature or reduced crown-rump length indicating defects in bone development [6]. It has been reported that exposure to increased levels of alcohol can result in permanent short stature and delayed mean bone age in children up to 14 years of age [7,8]. Studies in rodents have identified reduced body length and length of individual bones, delayed ossification and decreased skeletal maturity in response to prenatal alcohol exposure [5,9].

Folic acid is one of the water soluble B-vitamin required to synthesize, repair and methylate DNA and acts as a cofactor in many biological reactions. The requirement for folic acid during pregnancy is increased as it is the period of rapid cell division. However the alcohol consumption has negative impact on maternal folic acid level as it decreases its intestinal absorption and increases urinary excretion [10,11]. So the developing fetus is compromised for the folic acid which might result into different malformations related to the bone. So the current study was done to observe whether maternal folic acid supplementation along with alcohol reduces alcohol related bone malformations in the fetuses.

MATERIALS AND METHODS

Animals

In the present study Swiss albino mice approximately 12 weeks of age were used. The mice were allocate in propylene cages 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 feeding with standard animal chaw and tap water *ad limitum*. All experimental protocol was made accordance with Principles of animal research ethics and was approved by Ethical committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi.

Study design and drug exposure

Female and male mice of the same stock were matted overnight in the ratio of 2:1. In the next morning appearance of vaginal plug was checked. Presence of vaginal plug was considered as gestational day (GD) 0 of pregnancy.

The plug positive mice were randomly divided into four groups (n=6). Group I mice were termed as control and given normal saline, group II mice were introduced alcohol 6gm/kg body weight of mice, group III were administered alcohol 6gm/kg body weight and folic acid 60 mg/kg body weight of the mice while group IV mice were given only folic acid 60 mg/kg body weight from GD 6 to GD 15 orally through oral gavage needle.

On GD 18 pregnant mice of each group were sacrificed by cervical dislocation, their uterine horns were cut open and live fetuses were collected. The fetuses of each group (n=24) were rinsed in tap water, skinned out and eviscerated. It was transferred to 70 % alcohol for 24 hours. Next day it was transferred to 90% alcohol and kept in it for at least 5 days for proper fixation. It was transferred to 1% KOH. Solution was changed daily till all the soft tissues got dissolved and bones became clearly visible. The specimen was then transferred in 1% KOH solution containing a few drops of 0.1 % alizarin red. The fluid was changed daily till the bones were properly stained. The solution was replaced by 40% glycerol solution for 24 hours which was

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further replaced by 80% glycerol. Finally, 80% glycerol was replaced by absolute glycerol after 24 hours. The specimen was kept in the same solution till analysis.

RESULTS

Alizarin red staining of whole fetus was done to reveal the gross malformations of different bones in all the groups. In group II, the developing maxillary bones were less developed and separated as compared to group I. The ossifications of parietal as well as occipital bones were also reduced in comparison to group I. The ossifying occipital bones were widely separated in some fetuses. In one fetus, the bony orbit was bigger. The phalyngeal ossification was deficient in both fore limbs as well as hind limbs. Similarly, the ossifying humerus, radius and ulna in the fore limbs were comparable, while the ossifying femur, tibia and fibula were shorter than that of group I indicating delayed ossification of these structures. Only four sternebrae were visible ossifying while in group I there were five. The two halves of xiphoid process hadn't fused yet resulting into divided xiphoid process. In some fetuses the vertebral column was curved laterally (scoliosis) as compared to group I. The number of ossifying coccygeal vertebrae was reduced in some specimens (Fig. 1-3). In many fetuses, the bones were faintly stained with alizarin red indicating deficient calcium deposition in developing bones (Fig. 4). In group III, no such skeletal malformation and faulty ossification was observed indicating improvement in skeletal development (Fig. 1-3). In group IV also, the developing bones were comparable with group I (Fig. 1-3).

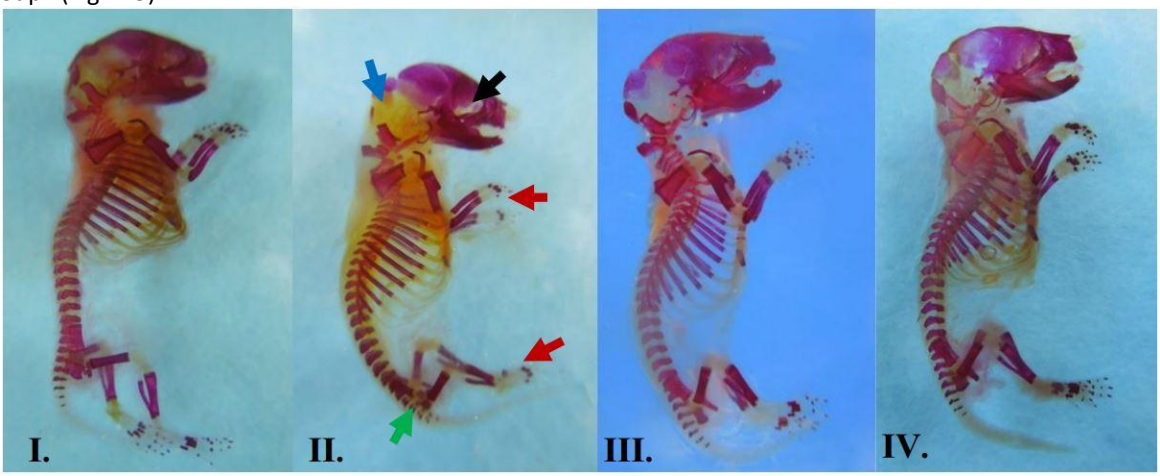

Figure 1: Lateral view of alizarin red stained fetuses: Group II fetus showing reduced parietal & occipital bone ossification (blue arrow), enlarged orbit (black arrow), deficient phalangeal ossification of fore limb and hind limb (red arrow), and reduced ossification of coccygeal vertebrae (green arrow).

Figure 2: Dorsal view of alizarin red stained fetuses: Group II fetus showing scoliosis (lateral curvature) of vertebral column (black arrow).

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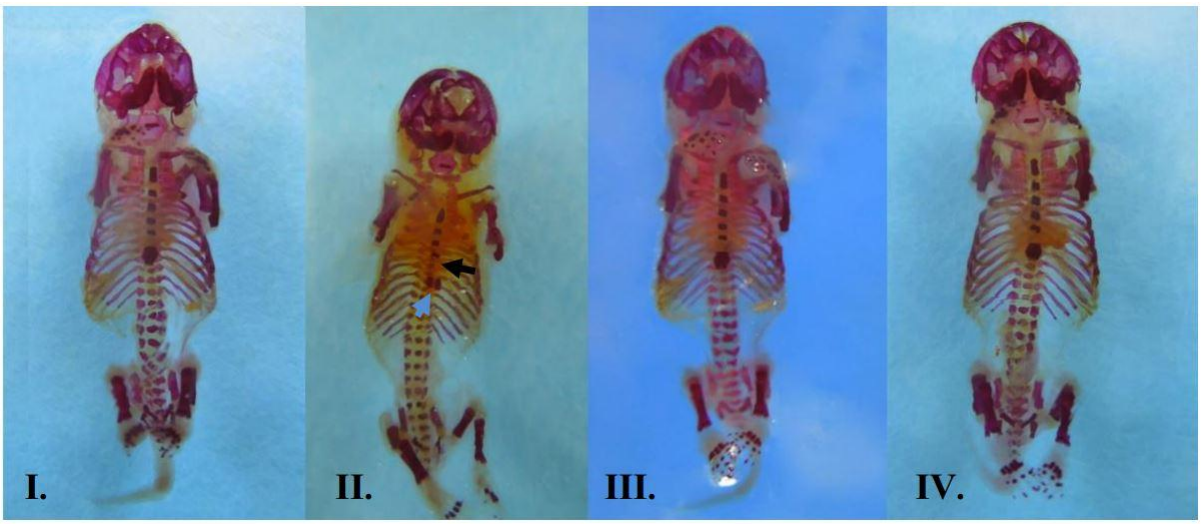

Figure 3: Frontal view: Group II fetus showing missing fifth sternebrae (black arrow) and divided xiphoid process (blue arrow). No such malformations were seen in remaining groups.

Figure 4: Alizarin red stained fetus of group II showing faint staining of developing bones

DISCUSSION

In the present study, several skeletal malformations were observed in prenatally alcohol exposed fetuses of group II. Many fetuses were lightly stained with alizarin red indicating decreased calcium deposition in the developing bone. The enhanced skeletal malformations in the prenatally alcohol exposed fetus might be due to oxidative stress induced by alcohol and defect in methionine metabolism during their development. Decrease in calcium deposition might be due to facilitatory effect of ethanol on calcium inhibitors which are family of inorganic phosphates, phosphonates and diphosphonates. The calcium inhibitors normally act to prevent calcium deposition in soft tissues [12]. Pyrophosphate enzymes, which are normally secreted in vesicles, destroy the inorganic calcium inhibitors. Ethanol suppresses these pyrophosphates enzyme activities due to which calcium inhibitors are activated resulting in delayed bone ossification [13]. Furthermore, alcohol consumption also reduced osteoblast activity which inhibited the synthesis of matrix during ossification [14]. Chronic alcohol consumption also decreased folic acid level in the body as described earlier due to which the homocysteine level increases. Elevated homocysteine levels also leads to defective bone matrix by interfering with the proper formation of collagen, the main protein in the bone. It is believed to interfere with cross links of newly formed collagen and, consequently, with bone strength and bone mineralization [15]. These could explain the poor calcification of the developing bones in the present study. Decreased bone mineralization and bone density due to chronic alcohol exposure was also reported by other studies [16]. Folic acid supplementation along with alcohol reduced the skeletal malformations as well as increased the calcium deposition in the developing bones in group III as compared to those in alcohol exposed fetuses of group II. This might be due to decrease in homocysteine level after folic acid administration. Folic acid has antioxidant property as it can scavenge free radicals [17]. So when folic acid was administered along with alcohol, it would also reduce the oxidative stress induced by alcohol. This decreased oxidative stress due to prenatal folic acid

increases osteoblast activity [18] which will induce bone mineralization as seen in our study. So the current study showed that prenatal alcohol exposure during the period of organogenesis resulted in different axial and appendicular bone malformations in developing fetus by interfering with different factors which could be reduced by folic acid administration.

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