*Annals of African Medicine*
*Vol. 2, No. 1; 2003: 17 - 21*

# PRE-NATAL EFFECTS OF ETHANOL AND FOLIC ACID SUPPLEMENTS ON THE MINERALISATION OF BONES IN WISTAR RAT

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**Abstract**
*Background:* Alcohol consumption has long been implicated as capable of inducing folic acid deficiency, in particular at pregnancy; thus inflicting severe skeletal dysgenesis on the conceptuses particularly the mineralisation of the bones.
*Methods:* In the present study, 120 adult female Wistar rats were grouped into three: A, B and C. Group A received 0.79g/kg of 30% ethanol from day 1 to day 10 of gestation, group rats received same dosage of ethanol plus 0.14g/kg folic acid supplements for the same period, and group C served as the control. Bone calcium and phosphorus contents were assessed daily from day 12 to 21 in the conceptuses of the three groups; and also the detailed sequence of calcification in the foetal bones were simultaneously monitored with alizarin red S stains.
*Results:* Low mineral levels and a lag or delay in calcification of about 2 days were recorded in the ethanol rats compare with the folate supplement group; with respect to the control, reparative or 'catch-up' growth was displayed in the ethanol plus folate treated rats.
*Conclusion:* These observations attest to the toxic consequences of gestational ingestion of ethanol on bone, and the possible alleviating effects of folic acid supplementation.

**Key words:** Ethanol, folic acid, bone mineralisation, rat

## Introduction

Consumption of ethanol, particularly at pregnancy had long been associated with abnormal development of skeletal tissues of the resulting offsprings.[1, 2] Commencing the ingestion of ethanol at the onset of conception inflicts more consequences on the fetuses. One of the ways ethanol is thought to effects its toxicity is by the inhibition of folic acid uptake by the intestinal bacteria, and its metabolism in the liver. Folic acid is a well-known essential co-factor in the synthesis of purine and pyrimidine components of DNA and RNA, which are important in the formation of protein for normal development, growth and reparation of tissues. Folic acid deficiency is a common feature in pregnancy, being more severe in the alcoholics.[3-7]

Moreover, bones are the reservoir of calcium and phosphorus needed for the normal functioning of mineral deposition on the skeleton when needed. In young actively growing animals, bones are sensitive to changes in intake of calcium and phosphorus for instance in extreme deficiencies of either of them. This usually necessitates profound alteration in the state of mineralisation[8] Factors instigating a deficient absorption or utilization of the minerals particularly in the presence of adequate in take had been assessed with low retention of calcium in rickets[8, 9] Calcium is the main constituent of bone and more than 99% of the mineral in the body is concentrated in the skeleton. Adequate calcium and phosphorus is important for normal skeletal growth, health and integrity. In alcoholics, calcium along with other minerals deficiencies is part of the general malnutrition, resulting from impairment of its absorption. The effects of ethanol ingestion with the concurrent folic acid supplements at pregnancy on the mineralisation of bones of the conceptuses are investigated in this study, using the Wistar rat animal model.

## Materials and methods

One hundred and twenty adult nulli - parous Wistar rats weighing between 200g and 250g were procured from the animal holdings of the Faculty of Pharmaceutical Sciences, A.B.U., Zaria, for the experiment. They were kept in the animal holdings of Department of Human Anatomy and fed on rat pellets (Agro Feeds Ltd., Ibadan), with clean drinking water

provided ad - libitum. The animals were grouped into three: A, B and C with 40 rats in each group. The animals were subsequently caged in twos with one adult male rat for mating. Confirmation of pregnancy and commencement of gestation dating was done according to Asling methods.[10] The group A rats were intubated at a dose of 0.79g/kg of 30% v/v ethanol per day from day 1 to 10 of gestation (dosage calculated from the equivalent g/ml of ethanol). Group B rats were also intubated with the same dosage of ethanol plus 0.14g/kg of folic acid per day for the same period. This dosage of folic acid is equivalent of the recommended 5g/day at pregnancy in Nigeria. The group C rats serve as control.

*Assessment of bone calcification*

Four rats were sacrificed daily from each group by chloroform inhalation from day 12 to day 21; the abdominal wall opened up and the fetuses retrieved from each uterine horn; immediately fixed in 10% formalin and carefully inspected to detect any morphological distortion. The skin, soft tissues and viscera were dissected off to expose the skeleton and subsequently processed in alizarin red S stains according to the methods of Dix. [11] Calcification in the individual bone of the fetuses was monitored daily by the aid of the stereoscopic microscope.

*Bone mineral quantification*

From days 12 to 21 four rats were sacrificed daily from each group by chloroform inhalation; the abdominal wall opened up and the fetuses retrieved from each uterine horn and weighed. The skin, soft tissues and viscera were dissected off, and the weight of the exposed skeleton recorded. These were subsequently processed according to the methods of Sampson and co-workers [12] to assess the bone minerals and materials levels.

## Results

*Calcification*

Detailed daily recordings of the calcification are presented in Tables 1a, b and c. The control rats displayed normal, proximo - distal sequence of bone growth.

The ethanol plus folic acid supplements rats displayed an attempted 'catch-up' with the control rats in the period of calcification The ethanol treated rats exhibited delay in calcification, with lag time of about 2 days.

*Minerals*

The dry weight steadily increased with the foetal age from day 12 through the gestational period in all the groups but at relatively slower rates in the ethanol, and the folate supplement groups with respect to the control group, except on days 15 and 19 respectively when the weights closely match up with the control rats.

Table 1. First day of appearance of calcification in the bones of Wistar rat foetuses monitored from days 12 to 21 of gestation (foetuses n= 15/group/day): (a) *Skull bones*

| Bones | A (n=40) | B (n=40) | C (n=40) |
|---|---|---|---|
| Mandible | 14 | 13 | 13 |
| Maxilla | 17 | 16 | 15 |
| Frontal | 18 | 17 | 15 |
| Nasal | 17 | 16 | 16 |
| Temporal | 19 | 17 | 16 |
| Parietal | 18 | 17 | 16 |
| Occipital | 19 | 17 | 16 |

Table 1. First day of appearance of calcification in the bones of Wistar rat foetuses monitored from days 12 to 21 of gestation (foetuses n= 15/group/day): (b) *Axial bones*

| Bones | A (n=40) | B (n=40) | C (n=40) |
|---|---|---|---|
| Clavicle | 14 | 13 | 13 |
| Sternabrae | 18 | 17 | 16 |
| Ribs | 18 | 17 | 16 |
| Scapula | 20 | 18 | 18 |
| Vertebrae | | | |
| Cervical | 18 | 17 | 17 |
| Thoracic | 19 | 17 | 17 |
| Lumbar | 21 | 19 | 18 |
| Sacral | 21 | 20 | 19 |
| Caudal | 21 | 20 | 19 |

Table 1. First day of appearance of calcification in the bones of Wistar rat foetuses monitored from days 12 to 21 of gestation (foetuses n= 15/group/day): (c) *Appendicular bones*

| Bones | A (n=40 | B (n=40) | C (n=40) |
|---|---|---|---|
| Humerus | 18 | 17 | 16 |
| Ulna | 18 | 17 | 16 |
| Radius | 18 | 17 | 17 |
| Carpals | 19 | 17 | 17 |
| Meta-Carpals | 19 | 18 | 17 |
| Fore- Phalanges | 21 | 19 | 18 |
| Femur | 17 | 16 | 16 |
| Tibia | 18 | 16 | 16 |
| Fibula | 18 | 17 | 16 |
| Tarsal | 20 | 18 | 17 |
| Meta-Tarsal | 19 | 17 | 17 |
| Hind-Phalanges | 20 | 19 | 18 |
| Pelvis | 20 | 19 | 18 |
| Astragalus | 20 | 19 | 18 |

Fat-free weights were less in the ethanol group. Higher weights were recorded for the folate supplement and the control groups; the mean percentage fat-free weights were 25%, 35% and 38% respectively. Likewise, the mean percentage ash-weights calculated from the dry weights were 5%, 23% and 27% respectively (Table 2).

The bone calcium and phosphorous contents are expressed in mg/g, and this was low throughout the foetal age; likewise the phosphorous, the level apparently plateaued through the gestational days and was considerably less in the ethanol group than in the ethanol + folate and the control foetuses (Table 3).

Table 2. Analysis of bone size (mg/g, from day 12 – 21 of gestation) in the Wistar rats' foetuses in the ethanol, folate supplements and control groups

| Group | | Foetal age (days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Control | Dry-weight | 200 | 215 | 220 | 220 | 228 | 235 | 240 | 245 | 254 | 260 |
| | Fat-free | 185 | 201 | 206 | 195 | 200 | 201 | 210 | 212 | 216 | 220 |
| | Ash-weight | 85 | 89 | 90 | 93 | 96 | 110 | 109 | 100 | 115 | 120 |
| Ethanol + folate | Dry-weight | 210 | 210 | 215 | 218 | 220 | 230 | 240 | 240 | 248 | 250 |
| | Fat-free | 194 | 202 | 190 | 197 | 206 | 202 | 210 | 210 | 211 | 212 |
| | Ash-weight | 60 | 85 | 101 | 96 | 100 | 108 | 110 | 118 | 112 | 115 |
| Ethanol | Dry-weight | 205 | 212 | 208 | 220 | 216 | 218 | 228 | 226 | 194 | 236 |
| | Fat-free | 160 | 165 | 170 | 180 | 170 | 165 | 182 | 190 | 194 | 205 |
| | Ash-weight | 50 | 60 | 65 | 60 | 78 | 80 | 75 | 87 | 91 | 95 |

Table 3. Analysis of bone mineral values (mg/g, from day 12 – 21 of gestation) in the Wistar rats' foetuses in the ethanol, folate supplement and the control group

| Bone mineral | Foetal age (days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Control | | | | | | | | | | |
| Phosphorus | 8.92 | 9.00 | 8.46 | 9.24 | 9.42 | 9.24 | 9.29 | 9.22 | 9.55 | 4.10 |
| Calcium | 3.00 | 3.20 | 3.40 | 3.30 | 3.40 | 3.64 | 3.40 | 3.30 | 4.10 | 4.40 |
| Ethanol + folate | | | | | | | | | | |
| Phosphorus | 8.25 | 8.87 | 7.85 | 8.87 | 8.25 | 8.22 | 8.25 | 6.42 | 8.86 | 8.94 |
| Calcium | 2.10 | 2.30 | 2.50 | 2.20 | 2.00 | 3.00 | 2.70 | 2.80 | 2.50 | 3.00 |
| Ethanol | | | | | | | | | | |
| Phosphorus | 4.60 | 4.46 | 4.65 | 4.42 | 4.46 | 4.23 | 3.45 | 4.86 | 4.58 | 4.99 |
| Calcium | 3.40 | 4.50 | 4.42 | 4.50 | 5.67 | 4.82 | 4.92 | 4.86 | 5.00 | 5.30 |

## Discussion

The pattern of calcification in the control group is in agreement with those observed in earlier studies in rodents; [13, 14] the delay in calcification in the ethanol treated group could be attributable to the fascilitatory effect of ethanol on the calcification inhibitors. These are family of inorganic phosphates, phosphonates and diphosphonates, usually present in matrix of osteoid tissues undergoing calcification. They normally act to prevent calcium deposits from forming in soft tissues[15]. On the other hand, pyrophosphates enzymes are normally secreted in the vesicles; these usually destroy the inorganic inhibitors. Ethanol is thought of capable of suppressing these enzyme activities, thereby slowing down bone mineralisation [16 – 17]

Moreover, earlier reports indicated that alcohol consumption results in reduced osteoblast activity with consequent inhibition of the process of matrix synthesis. This leads to poor formation of bone protein (collagen); in addition, the possibility of ethanol - potentiation of folic acid deficiency could therefore explain the defects in the calcification seen in the present investigation.

Furthermore, the fact that the high demand for calcium and phosphorus to the manifold functions

in the organism recalls its important role and the effects of its deficiency in the feature of calcification of bones and in osteoporosis.[8, 18,19] An increase in the production of $Ca^{2+}$ x $PO4^{2-}$ to a point beyond the solubility product constant of calcium hydroxy - apatite (CaHPO4) is critical for deposition of the bone mineral and also determines the formation of a colloidal calcium phosphate in the plasma.

Deficiency in bone minerals usually account for a relatively short supply of stored calcium, high osteoblast and osteoclast activity, reflecting in maximum demand for stored calcium and a subsequent porous shaft[8] Unfortunately, there is no special storage mechanism for calcium and phosphorus to meet the needs of pregnancy and lactation in mammals because these organisms are able to adapt to low intake of calcium to some extent without demonstrable pathological changes; but in the face of high demand such as in calcification, inadequacy of minerals is manifested in poor skeletal health and integrity[9]

Malnutrition which usually accompany alcoholics contributes to hypocalcaemia and phosphate depletion, having its maximal impact on the skeleton particularly in early childhood of the resulting offspring; the low mineral levels and poor calcification in the experimental rats concur with the earlier studies.[12, 20] Earlier workers examined the chronic effects of ethanol consumption on 10-month old rats and similar decrements in bone density and mineralisation were observed. [21, 22]

The apparent compensatory growth in the folate supplement group rats could have probably improved perhaps with higher dosage of folate. The hypothesis that growth retardation of bone due to ethanol consumption is modified by impairment of folate uptake by the foetal tissues tested in this study is exhibited in the differential bone growth and mineralisation in the foetuses. The fact that there is a relative improvement in the foetal group receiving the same dosage of ethanol with folate supplementation substantiate this point of a possible rehabilitatory growth. Folate supplement was administered to alleviate the hypothetical ethanol - induced deficiencies.

The anomalies in bone mineralisation seen in the ethanol foetuses were not fully redressed before birth as indicated in the folate supplement group, and such pathology persists even to adulthood and usually manifests in cases like foetal alcohol syndrome; however, a higher dosage of folate supplementation in a future study may be necessary in order for a conclusive picture.

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