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Effect of folic acid supplementation in early pregnancy on spinal cord in mice embryos

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Abstract: *The present study was conducted to evaluate the effect of folic acid (FA) administration in early pregnancy on spinal cord in mice fetuses. Virgin female albino mice, 8-10 weeks old were were obtained from the animal breeding house of veterinary medicine collage, Omar Al mukhtar University, EL Beida ,Libya. The animals mated 1 male: 2 female and next morning examined to confirmed successful mating by vaginal smear . Appearance of vaginal plug was considered a day zero of pregnancy. The pregnant females were divided into equal three groups. GI : Control group received orally distilled water, G II (Normal dose group) : given orally folic acid at dose level 80ug /kg bw for a week and G III(High dose group): given orally folic acid at dose level 160ug/kg bw for a week. Oral administration of folic acid was achieved by oral gavage from the 7th day until the 14th day of gestation then the pregnant females were observed till 18 th day of gestation (per natal) . On 18th day of gestation the contents of the uterus from both control and treated mice were examined with microscope for gross abnormalities and external morphological defects in the brain or spinal cord. Transverse sections of spinal cord at cervical, thoracic and lumbar levels were prepared and stained with haematoxylin and eosin (H&E) for histological study. Neither clinical signs nor abnormalities in behavior and external features were observed in pregnant mice treated with normal or high dose of folic acid. Also, no mortality was recorded in control and folic acid treated groups during experimental period. Microscopic examination of the embryos of mice treated with normal dose of folic acid revealed no obvious defects in the brain or spinal cord compared to control group. No outstanding differences in the mean number and body weight of fetuses between control and normal dose of folic acid treated group. While, high dose of folic acid induced marked decrease in the number and size of fetuses. In histological study administration of normal dose of folic acid revealed normal architecture of spinal cord regions with distinct grey and white matter, normal pattern of cellular density, nuclear shape and arrangement with slight increase in the extracellular space ,intact lining ependymal cells of central canal and normal widened anterior median fissure compared to the corresponding spinal cord regions of the control . While, administration high dose of folic acid in early pregnancy had adverse defects on mice fetuses. This study suggested that administration of normal dose of folic acid for a week in early pregnancy had no noticeable effect on spinal cord of mice fetuses .*

Key words: *folic acid , spinal cord ,brain , mouse embryo, histological*

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I. Introduction

Neural tube closure is an early developmental process that gives rise to the central nervous system, including the spinal cord and brain[1]. Neural tube defects are serious and common birth defects resulting from both genetic and environmental factors[2] [3]. Failure of the neural tube to close which leads to different clinical types of neural tube defects depending on the site and timing of closure failure[3] [1]. Folic acid (FA) is B-9 vitamin prescribed commonly for pregnant women to prevent neural tube defects in the fetus, patients under chemotherapy, pernicious anemia and to reduce the risk of stroke and cardiovascular disease. Acute or chronic ingestion of a large dose of folic acid generally manifests as neurological complications[4]. The United States implemented mandatory fortification of FA in 1998 to prevent neural tube defects during pregnancy. The health benefits of folic acid are well documented however, there are potential risks of exceeding the upper tolerable limit [5]. Murphy and Westmark [5] also, concluded that national fortification with folic acid is not associated with a significant decrease in the prevalence of neural tube defects at the population level. Other study reported that the possible negative effect of high levels of maternal FA supplementation during pregnancy and lactation. Such alterations potentially lead to neurobehavioural changes in the adult offspring of Wistar rats [6]. Folic acid supplementation decreased the incidence of the defects neural tube caused by certain drugs as progesterone, but did not obviate them [7]. The toxicity with LD50 values of folic acid by the i.p. route in different strains of mice showed convulsions, ataxia and weakness. Histopathological study in some strains (BDF1, DBA/2 and

DBA/2fNCri) showed acute renal tubular necrosis [8]. The available literature suggests that either a deficiency or excess of FA, especially during the critical periods of development, affects embryos, which are unable to successfully repair damage or undergo catch-up growth after a toxic insult [9]. lterations in maternal dietary FA levels during pregnancy and lactation are associated with an increased risk for cardiovascular, renal, and metabolic diseases laterin the life of the infant[10]. Folic acid and "folate" mean the same thing helps the body make healthy new cells. It is found naturally in some foods . It has been shown to have a role in preventing congenital malformations, especially those related to defects in the process of neurulation and neural tube closure [11]. Folic acid plays a role in both the folate cycle for the production of thymidylate and purines mediating cell division, and in the methylation cycle of homocysteine metabolism resulting in epigenetic regulation of gene expression[12] [13] [14]. Van Amsterdam et al. [15] reported that folic acid is important for protein synthesis, and DNA- and RNA-synthesis. Consequently, folic acid is required in rapid growing tissues, like the development and outgrow of the foetus, blood forming organs and the epithelium. In addition, folic acid is required for the synthesis of S-adenosylhomocysteine, which is extremely important for the further biosynthesis of brain neurotransmitters (serotonin and dopamine). Nishigori et al. [16] found that there was no association between preconceptional folic acid supplements and the incidence of neural tube defects. Therefore, this investigation was planned to study the effect of supplement different doses of folic acid during pregnancy on the spinal cord in mice.

II. Material and Methods

2.1 Animals and Treatment

Virgin female Swiss albino mice (*Mus-musculus*), 8-10 weeks of age, weighing (25-27g) were obtained from the Animal Breeding House of faculty of veterinary medicine, Omar Al mukhtar University, EL Beida ,Libya. They were housed in the laboratory animal in clean plastic cages (2 mice/ cage) under controlled conditions of temperature (20 ± 2)°C and photoperiod (12h light: 12h dark) cycle . The animals were maintained on standard commercial pellet diet and clear drinking water. For one month, vaginal smear cytology was performed for determination of mice estrous cycle phases and to ensure regular cycles. Mating was done between proesterous virgin females and potent male (1 male: 2 female) overnight and checked for vaginal plugs the next morning. Success of mating process was confirmed by vaginal smear on the next morning [17]. Presence of spermatozoa means successful mating and appearance of vaginal plug was considered a day zero of gestation. A 18 adult female mice after formation of vaginal mating plaque (zero day of gestation) were selected caged separately, weighted and divided into three groups (6 mice each). Group I: Control mice received orally distilled water at dose level 4ml/kg for one week. Group II (Normal dose group): Experimental mice were given orally folic acid at dose level 80ug /kg bw (equivalent to normal human therapeutic dose) for a week . Group III(High dose group): Experimental mice were given orally high dose of folic acid at dose level 160ug/kg bw (represent duplicate to human therapeutic dose) for a week. Oral administration of folic acid was achieved by oral gavage from the 7^{th} day until the 14^{th} day of gestation then the pregnant females were observed till 18^{th} day of gestation (per natal). In mice gestation lengths vary between strains from 19 to 21 days [18].

2.2 Material used:

2.2.1.Folic acid and dose Preparation

Folic acid was purchased from (Sigma Co,Germany). Mice were received orally folic acid at dose levels 80ug/kg and 160ug/kg (0.1 and 0.2 ml for each mouse) for a week. One tablet of folic acid (5mg) was dissolved in 250 ml of DW. A dose was determined according to Paget and Barnes [19].

2.2.2. Clinical signs study

Animals were observed daily during pregnancy to record any changes in the behavior, depression, food intake and signs of difficulty breathing, salivation, diarrhea, muscular weakness and any signs of toxicity and mortality.

2.2.3.Gross morphological examination

At the end of the experimental period (on 18^{th} day of gestation) the pregnant mice from control and experimental groups were sacrificed and necropsies after anesthetized with light chloroform and fetuses delivered by hysterectomy. The contents of the uterus were examined with naked eye for gross abnormalities and the weight of each fetus was recorded. All the embryos were fixed in freshly prepared 10% formalin and immediately examined for any external morphological defects in the brain or spinal cord under microscope low power magnification. For further histological analyses, mid line incision was performed in each fetus and fixed in Bouin's fluid.

2.2.4.Histopathological studies

After fixation in aqueous Bouin's fluid for 24 hours. The specimens were dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin wax, the paraffin serial sections of 5 μm thickness were cut at cervical, thoracic and lumber levels and stained with Harri's Hematoxylin and Eosin (H&E) according to **Bancroft & Gamble**[20]. The sections were made into permanent slides and examined under high-resolution microscope with photographic facility (Nikon Eclipse E400, Japan) and histopathological changes were recognized and photographed.

2.2.5.Statistical analysis

Data are presented as mean ± SD .The significance of differences among the groups was assessed using one-way analysis of variance (ANOVA) .The significance level was set at $P \leq 0.05$.

III. Results

3.1.Result of Clinical signs study

The pregnant mice supplemented orally with normal or high dose of FA for a week did not show any notable alteration in the behavioral and external features including body furs, feces, activity, food and water consumption compared to control group. In addition, there was no weakness and any signs of toxicity. Additionally, no mortality encountered in control and FA treated groups during experimental period.

3.2. Result gross morphological examination

Insignificant increase in the mean body weight (0.922±0.33) of fetuses of pregnant mice administered FA at dose level 80ug /kg bw during pregnancy was recorded compared to control group (0.867±0.03). However, few fetuses with reduction size were notes in normal dose of FA treated group. Examination of uterus contents of most mice treated with FA at dose level 160 ug/kg bw showed empty uterus with necrotic dead embryos appeared as dark spots in the uterus. The average number of fetuses was found to be 10 and11 in mice treated with normal dose of FA and control group respectively. Microscopic examination of the embryos of mice treated with normal dose of folic acid revealed no obvious defects in the brain or spinal cord compared to control group.

3.3. Result of histological study

Histological examination of spinal cord at deferent levels of fetuses from control group showed normal architectural of central canal with intact lining ependymal cells, inner gray matter of H- shape with normal nerve fibers, neurons population, and neuroglial cells, peripheral white matter contain thick nerve fibers and neuroglia cells were seen. Likewise, intact meninges membranes, posteromedian sulcus and anteromedian fissure with normal widened feature were noticed (Fig.1a-d). No obvious histological alteration in spinal cord at different levels of fetuses from normal dose of FA treated group compared to the corresponding control group. Since all spinal cord regions showed normal architecture with distinct grey matter with normal cellular density , normal pattern of white matter ,central canal with intact lining ependymal cells and normal widened anteromedian fissure. However, few scattered dense nuclei in white and grey matter, less cellular density and increase in the extracellular space were more pronounced in spinal cord regions of normal dose of FA treated group (Fig.2a-d). Increase in the extracellular space, shrinkage, degeneration, less arrangement, reduction cellularity and dense irregular nuclei were observed in spinal cord sections of high dose treated group.

IV. Discussion

In the current study nor remarkable differences in the behavioral and external features neither mortality were observed in the mice administrated normal or high dose of FA in early pregnancy indicating non toxic doses were used in this work. Since, Beliles [21] demonstrated that the LD50 values of FA by the oral route in mice is 10g/kg. Also, following intra-venous treatment with dose up to 150 mg, no adverse effects have been reported[22] [23]. In contrast one early study reported in 'The Lancet' in 1970 reported neurotoxic symptoms like malaise, sleep disturbances, and mental changes in 14 healthy volunteers who took daily 15 mg of folic acid for one month[24]. In the current study administration of normal dose of FA in early pregnancy had no noticeable defects on brain and spinal cord of mice fetuses. It was previous reported in the literature that daily doses of 5 to 15 for up to 3 years were applied did not show any evidence of folate associated neurotoxicity[15]. Our results are in harmony with the previous report by Chung et al. [25] who reported that the female rats were supplemented FA (50 mg/kg b.w. per day) from days 7-17 of gestation, by gavage showed no significant maternal or embryotoxicity, as compared with vehiculum. Our findings indicate that supplementation of normal dose of FA in early pregnancy during gestational period reduced number fetuses and induce insignificant increase in the mean body weight of fetuses, also reduced number of fetuses. These results were in agreement with previous studies which had demonstrated that average number of pups born to pregnant dams rats fed with

FA supplementation (40mg/kg body weight/day) or normal diet for 4 weeks before mating and through their pregnancy were 8 and 9 respectively [26]. Birth weight of pups born to pregnant dams fed with FA supplementation was found to be significantly increased compared to normal diet [26]. Our data may be support and explain from finding of Iyengar and Rajalakshmi [27] who reported that the infants born to mothers receiving FA supplements in addition to iron during pregnancy were heavier than those born to mothers receiving iron alone. Higher intake of FA is associated with higher bone mineral density[28]. Regarding reduction size of some fetuses of pregnant mice administered normal dose of FA in this study is in line with the previous publications of Huot et al. [29] who reported that that a 10-fold increase in folate content of the diet (20 mg folic acid/kg diet) during pregnancy reduced body weight gain of the offspring. While, the present study showed that high dose of folic acid during early gestational period has deleterious effects on development mice embryo as shown above. Some drugs are safe during pregnancy while others may have side effects on the fetus[30]. Our results regarding high dose of FA in line with previous published reports who reported that some drugs act on the fetus as teratogens causing preventable congenital anomalies[31]. However, Harden et al. [32] stated that there is a relationship between dose of teratogen, its effect and time of administration during pregnancy period. The effect of teratogen ranges from no effect to malformations or intrauterine fetal death depending on the dose[32]. Nakouzi and Nadeau [33] who examined the effect of FA supplementation, at 5-fold level in the control diet, on the neural tube defects (NTDs) in Apob^{tm1Unc} and Vang*l2*^{Lp} mice models stated that parental FA supplementation did not reduce the incidence or severity of NTDs in these two mouse models. In the present study histological examination showed no obvious alterations in spinal cord only few scattered dense nuclei in white and grey matter, less cellular density and increase in the extracellular space were more pronounced in spinal cord regions of normal dose of FA treated group. Our results are in harmony with the previous report by Lee *et al.* [34] who investigated the effects of dietary FA on the expression of myelin basic protein in the maternal brain and spinal cord during pregnancy and lactation. They reported that no significant change was observed in the hippocampus or spinal cord in rats fed with folic-acid-supplemented diet (8 mg/kg diet). In the present study increase in the extracellular space, shrinkage, degeneration, less arrangement, reduction cellularity and dense irregular nuclei were observed in spinal cord sections of high dose treated group. Our findings are in a good agreement with several authors who have reported deleterious effect of FA in nervous tissue and other tissue. Vinaykumar et al. [6] demonstrated that excess FA supplementation in pregnancy contributed to neuronal loss in offspring, especially in the CA1 and CA3 regions of the hippocampus, and resulted in deficient short-term learning and memory. Other studies have also shown that the parenteral administration of high doses of folic acid into rats (100 - 400 mg/kg b.w.) or mice (75 mg/kg b.w.) produces precipitation of the compound in the renal tubules and renal hyperplasia, hypertrophy and necrosis[35] [36] [37]. Mikael et al. [38] reported that maternal FA supplementation (pregnant mice with FA-supplemented diet (10-fold higher than the recommended intake, 20 mg/kg diet) was associated with embryonic loss, embryonic delays, a higher incidence of ventricular septal defects, and thinner left and right ventricular walls compared to mothers fed control diet. It has been reported that high FA intake may mask vitamin B-12 deficiency [39], accelerate cancer progression[40]**,** associated with increased risks of cardiovascular diseases in the offspring [41]. Additionally, high supplementation of FA (20 mg/kg diet) during gestation and pregnancy dysregulated gene expression of several genes including those associated with neural development in the cerebellum of neonatal pups[42]. Chu et al. [43] suggested that FA supplementation during early life stage affected gene expression in weaning mice, and exhibited long-term impairments in adult behaviors in a dose-sensitive manner. Implicated folic acid in reproduction and fertility in human have been reported in several studies[44] [45] [46] [47]. Excess folic acid intake may promote changes to one carbon metabolic pathways and gene expression patterns, leading to liver injury[48] and impaired immune function[49]. Kelly et al. [50] reported that the body weight of rats in the high fat diet - excess folic acid group after 12 weeks was 14% higher than in rats in the high fat diet - adequate folic acid group, with increased fat mass accounting for this difference in weight. Histological examination of adipose tissue revealed that high fat diet - excess folic acid fed rats had larger adipocytes. They suggested that in adult males the dual insult of a high fat diet combined with excess folic acid may promote fat mass gain, adipose tissue inflammation and systemic glucose intolerance. In obesity, excess adipose tissue accumulation precedes immune cell infiltration and production of pro-inflammatory cytokines [51]. Oxidative stress that is sensitive to folate levels and stimulates apoptosis may be a more important factor in neurodegeneration [52]. Irregular supplementation of folic acid increased indicators of lipid peroxidation in first trimester of pregnancy where significant increase in the malondialdehyde (MDA) and significant decrease Glutathione (GSH) were recorded in pregnant women[53]. On the other hand Huang et al. [54] demonstrate that in vitro folate supplementation exerts differentially protective effects against 7-ketocholesterol (KC)-induced apoptosis. High-dose supplementation (>1000μmol/l) alleviates oxidative stress, mitochondria-associated death signaling and apoptosis induced by 7-KC. However, the in vivo relevance is not clear and requires further study.

V. Conclusion

This study suggested that administration of normal dose of folic acid in early pregnancy had no remarkable defects on spinal cord of mice fetuses. While, administration high dose of folic acid in early pregnancy (during gestational period) may be induce deleterious effects on mice fetuses as manifested by marked decrease in the number and size of fetuses and degeneration, less arrangement and reduction cellularity. However further studies are needed to elucidate adverse defects of high dose of folic acid on mice fetuses and other species and must be undertaking to avoid exposure to high dose of folic acid in early pregnancy.

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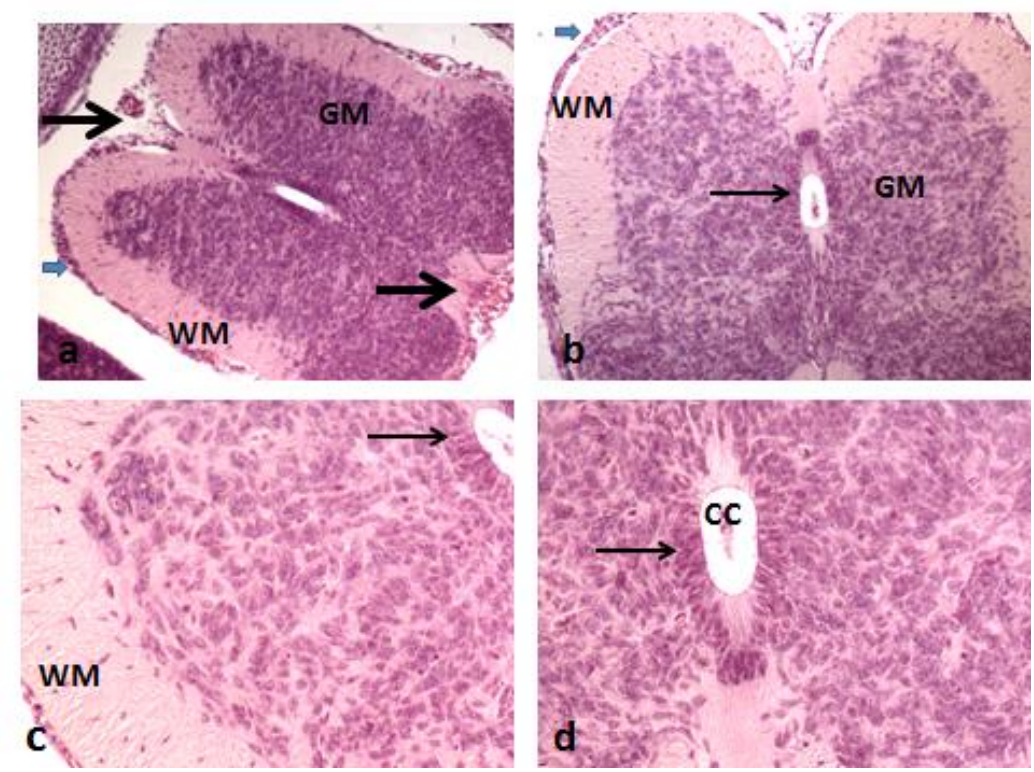

Fig.(1a-d): A cross section of spinal cord at deferent levels from mice fetuses of control group showing normal architectural : Central canal (CC) with intact lining ependymal cells (Thin Arrows),Grey matter (GM), White matter (WM) Posteromedian sulcus and anteromedian fissure (Thick Arrows), Meninges (Bold pointers) (H&E stain, a&bX100 and c&d X200).

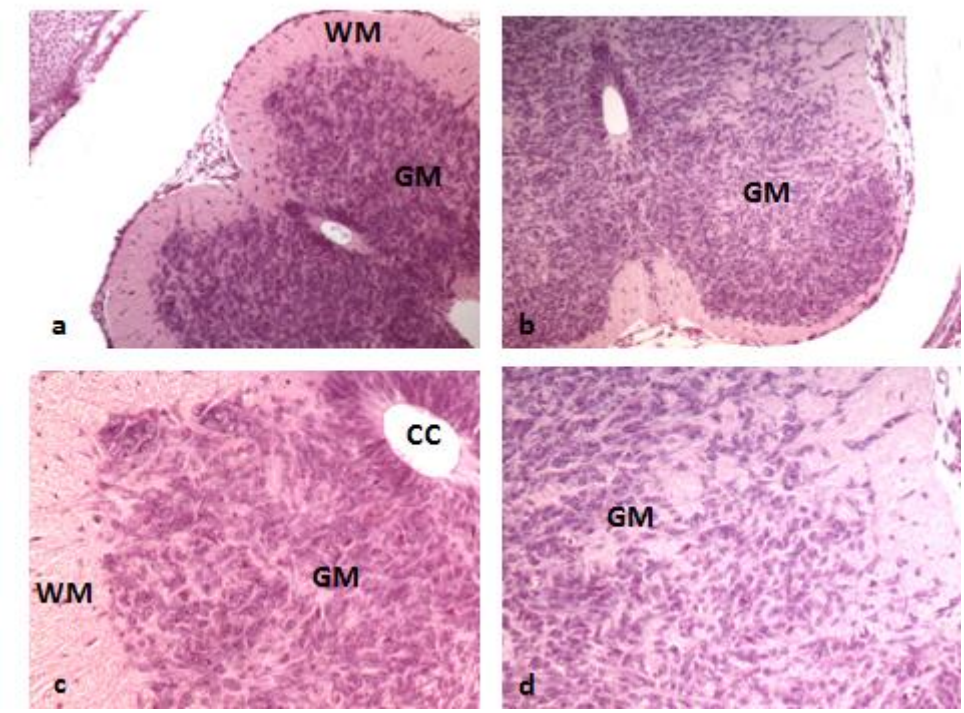

Fig. (2a-d): A cross sections of spinal cord at deferent levels from mice fetuses of normal dose of folic acid treated group showing no abnormalities : Central canal (CC), Grey matter with slight increase extracellular space (GM), White matter (WM) (H&E stain, a&bX100 and c&d X200).

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