



ORIGINAL ARTICLE

Prenatal Effect of Methotrexate Exposure on the Fetal Brain of Albino Rats and the Possible Protective Role of Folic Acid

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ABSTRACT

Background: Methotrexate is a folate antagonist, used to treat several diseases by interfering with cell growth. The objective of this study was to evaluate the effect of methotrexate on rat fetal telencephalon and the protective role of folic acid. **Methods:** 20 Pregnant rats weighting 200 – 250 gm were divided into four equal groups; control negative group, control positive group: received 60 mg/kg folic acid daily via gastric gavage from the first gestational day. Methotrexate treated group which treated with a single dose of 30 mg/kg methotrexate at gestation day 16. Methotrexate and folic acid treated group: received combined methotrexate and folic acid by the same manner mentioned before. The pregnant females were sacrificed over 2 gestational days (17th and 19th). The cerebrum was excised from each embryo, gross measurements were taken then processed for hematoxylin and eosin and caspase-3 staining for light microscopic examination. **Results:** Weight, volume, anteroposterior and transverse diameters of total cerebrum in methotrexate group were decreased in comparison to the control group. Histopathological examination revealed distributed vacuolated cells, hemorrhagic spots and congested blood vessels with in meninges in methotrexate treated group. Caspase-3 was widely expressed in fetal telencephalon in day 19 of methotrexate treated group in when compared with control group. All these findings were considerably improved in the combined methotrexate and folic acid treated group. **Conclusions:** Methotrexate induces microcephaly and apoptosis in the fetal telencephalon which is partially improved by folic acid supplementation
Keywords: Methotrexate; Telencephalon; Microcephaly; Folic acid; Apoptosis

INTRODUCTION

The developing cerebral cortex contains six temporary embryonic zones, the most inner is the ventricular zone (VZ) and the sub-ventricular zone (SVZ). Then there are the intermediate zone (IZ), the sub-plate (SZ), the cortical plate (CP) and lastly the most superficial is the marginal zone (MZ) [1].

The balance between the expansion of neuronal progenitor cells and the cell death is established and that is the main determinant of cortical size. Generalized apoptosis occurs in the ventricular and subventricular zones of cortices (the proliferative zones) during embryonic development. Excessive increase in apoptosis

of progenitor cells leading to brain with small size [2].

Methotrexate (MTX) is an antifolate and used to cure several types of diseases through its main mechanism of action which is DNA synthesis inhibition [3]. It is applied in several autoimmune diseases such as rheumatoid arthritis and systemic lupus [4]. Also it has been widely needed in treatment of variety of malignancies such as breast cancer and acute type of lymphoblastic leukemia [5].

It is known that MTX interferes with cell division, especially with rapidly proliferating cells. The dangerous period for its teratogenic effect is 6–8 weeks after gestation [6].

Many pregnant females unintentionally exposed to methotrexate in low doses in early gestation have normal offsprings. However, some fetal syndrome has been established and described as a result to methotrexate administration [7].

Folic acid (FA) is known as water-soluble of vitamin B complex. It is considered a synthetic form of the naturally presented folate which is found in vegetables like spinach, broccoli, green beans and also in fruits such as bananas and melons [8, 9].

Generation and maintenance of new cells are enhanced by folate and folic acid, especially throughout periods of growth and rapid cell division, as in pregnancy and infancy. Defects in development have been associated with an insufficient folate intake [9].

Based on these previous findings, this study was carried out to uncover the effect of prenatal exposure of methotrexate on fetal cerebrum and the ameliorating effect of folic acid co-treatment.

METHODS

The study was performed according to The Institutional Animal Care and Use Committee Zagazig University (ZU-IACUC) Instructions.

Drugs:

- **Methotrexate:** Methotrexate ampoule was bought from Mylan pharmaceutical company
- **Folic acid:** Folic acid was bought in the form of powder from El Gomhouria company for trading chemicals and medical appliances (Egypt), Zagazig branch

Experimental animals and dosing:

The study was carried out on 20 adult pregnant female Wistar albino rats weighting 200 -250 gm. The animals were obtained from the Animal House of Zagazig Scientific and Medical Research Center (ZSMRC).

For mating, Females were housed with adult males at ratio of 2:1 respectively in each cage. The day after the day in which sperms were noticed in the vaginal smear was considered as the day one of gestation.

Pregnant females were equally divided into four groups with 5 females in each group. All rats

were bred in standard plastic cages at constant room temperature (21 –22 °C) and in a 12 h light/12 h dark cycle (light on at 07:00 a.m.) and were given free access to food and water.

The four groups were:

Control negative group: the pregnant females were given distilled water via gavage once daily and a single saline intraperitoneal injection on the gestational day16.

Control positive group (Folic acid treated group) (FA group): The pregnant females received 60 mg/kg body weight of folic acid daily via gavage (10) from the first gestational day.

Methotrexate treated group (MTX group): The pregnant females were treated with a single intraperitoneal dose of 30 mg/kg body weight of methotrexate (11) on the gestational day 16.

Methotrexate and folic acid treated group (MTX+FA group): The pregnant females received 60 mg/kg body weight of folic acid daily via gavage from the first gestational day and a single intraperitoneal dose of 30 mg/kg body weight of methotrexate on the gestational day 16.

Experimental methods:

The pregnant females were sacrificed over 2 gestational days (17th and 19th). Laparotomy and dissection of the gravid uterus was done for all animals. Whole embryos were excised and fixed in 10% neutral buffered formalin. After 48 hours, the cerebrum was excised carefully from each embryo.

Gross measurements were performed after excision of the cerebrum. Anteroposterior diameter and transverse diameter of the telencephalon were measured. In addition, the weight and volume of the whole cerebrum were estimated. The volume was assessed by estimating the formalin volume increase in falcon tube after adding the cerebrum.

Cerebrum specimens were processed for light microscope examination according to **Bancroft and Gamble [12]**. Sections of 5 µm thickness

were obtained and then stained with Hematoxylin and eosin staining (H&E).

Some sections from the paraffin blocks were obtained for immuno-histochemical staining according to Kanemitsu et al (13). Caspase 3 rabbit polyclonal primary antibody was used for localizing apoptosis in paraffin sections. Kits were delivered from Lab Vision Laboratories.

Statistical analysis

The collected data were computerized and statistically analyzed using Graph Pad Prism 5.01. Quantitative data were expressed as mean \pm SD (Standard deviation). Differences between mean values were tested with analysis of variance (ANOVA). Tukey's multiple comparison test was carried out as post hoc test of ANOVA. The results were considered statistically significant when the P value <0.05 .

RESULTS

Gross features:

Age 17 day post coitum (dpc)

Estimation of the fetal cerebral weight showed statistically significant decrease in the MTX treated group compared to the control group as P value <0.05 . However, there was a non-significant difference between MTX+FA group and MTX treated group.

The volume estimation revealed a significant decrease in the MTX treated group as P value <0.05 while a significant improvement in MTX+FA treated group compared to MTX group.

On the other hand, telencephalic transverse and anteroposterior diameters decreased significantly in the MTX treated group as P value <0.05 with a significant improvement in MTX+FA group (Table 1).

Age 19 day post coitum (dpc)

There was non-significant decrease in the weight of the cerebrum in MTX group. However, the decrease in cerebral volume was highly significant in MTX treated group as P value < 0.001 with a mild improvement in MTX+FA group.

Slight decrease was detected in the transverse and anteroposterior diameters of telencephalon in MTX treated group. No improvement was found in MTX+FA treated group (Table 1).

Hematoxylin and eosin features

Age 17 day post coitum (dpc)

The telencephalic wall in the control group showed six zones arranged as follow: The first two zones from inside to outside were of ventricular and subventricular zones that were difficultly demarcated from each other so considered as one layer. The third was the intermediate zone with slightly separated and dispersed cells, the fourth was small subplate, the fifth was the closely compacted cortical plate and lastly small superficial marginal zone.

There were immature neurons that were prominent in the cortical plate. They were rounded and small with darkly stained intensely basophilic nuclei and minimally present cytoplasm. In the intermediate zone, the cells were variably shaped, and irregularly distributed in the whole zone. Beside it there was closely disorganized cells in the ventricular and subventricular zone as shown in (Fig. 1A).

In MTX treated group there were congestion in the blood vessels mostly in meninges and hemorrhagic spots clearly demonstrated in the cortical plate as shown in (Fig. 1B). In addition, vacuolation in the cells was prominent in the intermediate zone as shown in (Fig. 1C).

On the other hand, the group received MTX+FA showed a degree of partial improvement in accordance to vacuolation and complete disappearance of vascular congestion or hemorrhage as shown in (Fig.1D).

Age 19 day post coitum (dpc)

Regarding the control group, the telencephalic wall of fetal rat brain at day 19 dpc showed nearly the same embryonic zone with the same characters as the previous age. The control group showed irregularly distributed cells in ventricular and subventricular zone then migrating cells in the deep part of intermediate zone in slight

organized radially arranged manner and dispersed cells in the superficial part of intermediate zone and subplate interwoven with loose plexiform network of fibers beside radially arranged columns in cortical plate as shown in (Figs. 2A and 2B).

In the MTX treated group there was a marked congestion in the blood vessels mostly in meninges as shown in (Fig.2C). There were also multiple cell clusters with pyknotic nuclei clearly apparent in the ventricular and subventricular zone. Vacuolation in cells and hemorrhagic spots were prominent also in MTX treated group as shown in (Fig. 2D).

There was a partial improvement in the group received MTX+FA. The clusters of cells and hemorrhagic spots were completely disappeared however, the vacuolated cells only decreased in density but did not disappear as shown in (Figs. 2E , 2F).

Caspase-3 immunohistochemistry features:

Age 17 day post coitum (dpc)

At embryonic day 17 dpc there was a positive immune reaction for caspase-3 in the control group in all embryonic zones especially in ventricular and subventricular zone and intermediate zone while in MTX treated group there was decrease in expression of immune reaction with a mild increase in MTX+FA treated group as shown in (Fig. 3).

Age 19 day post coitum (dpc)

At embryonic day 19 dpc there was a positive immune reaction in the control group in all embryonic zones while, there was a marked increase in expression of immune reaction in MTX treated group with a marked improvement in MTX+FA treated group as shown in (Fig. 4).

Table 1. (Gross measurements): Statistical comparison between mean values of gross parameters in the different studied groups at embryonic ages 17 and 19 dpc. Ts: Transverse diameter, AP: anteroposterior diameter, *** (highly significant): P value < 0.001, ** (moderately significant): P value < 0.01, * (mildly significant): P value < 0.05 and ns: Not significant.

Age	Parameter	Control Means \pm SD	FA Means \pm SD	Mtx Means \pm SD	Mtx + FA Means \pm SD	ANOVA	Post hoc (Tukey's test)	
							Control vs Mtx	Mtx vs Mtx + FA
17dpc	Weight (gm)	0.168 \pm 0.020	0.165 \pm 0.018	0.124 \pm 0.012	0.14 \pm 0.01	<0.0001***	***	ns
	Volume (ml)	152.5 \pm 11.34	151.75 \pm 12.31	121.25 \pm 21.17	151.25 \pm 13.56	0.0004***	**	**
	Ts (mm)	5.88 \pm 0.24	5.74 \pm 0.34	4.14 \pm 0.29	5.75 \pm 0.32	<0.0001***	***	***
	AP (mm)	5.24 \pm 0.41	5.54 \pm 0.51	3.38 \pm 0.70	5.59 \pm 0.38	<0.0001***	***	***
19dpc	Weight (gm)	0.24 \pm 0.022	0.23 \pm 0.044	0.19 \pm 0.038	0.21 \pm 0.029	0.0603ns	ns	ns
	Volume (ml)	238.75 \pm 33.57	239.38 \pm 36.88	162.5 \pm 34.64	212.5 \pm 26.05	0.0002***	***	*
	Ts (mm)	7.38 \pm 0.52	7.13 \pm 0.83	6.7 \pm 0.39	6.82 \pm 0.54	0.1235ns	ns	ns
	AP (mm)	6.31 \pm 0.56	6.21 \pm 0.38	5.69 \pm 0.46	6.15 \pm 0.32	0.0375*	*	ns

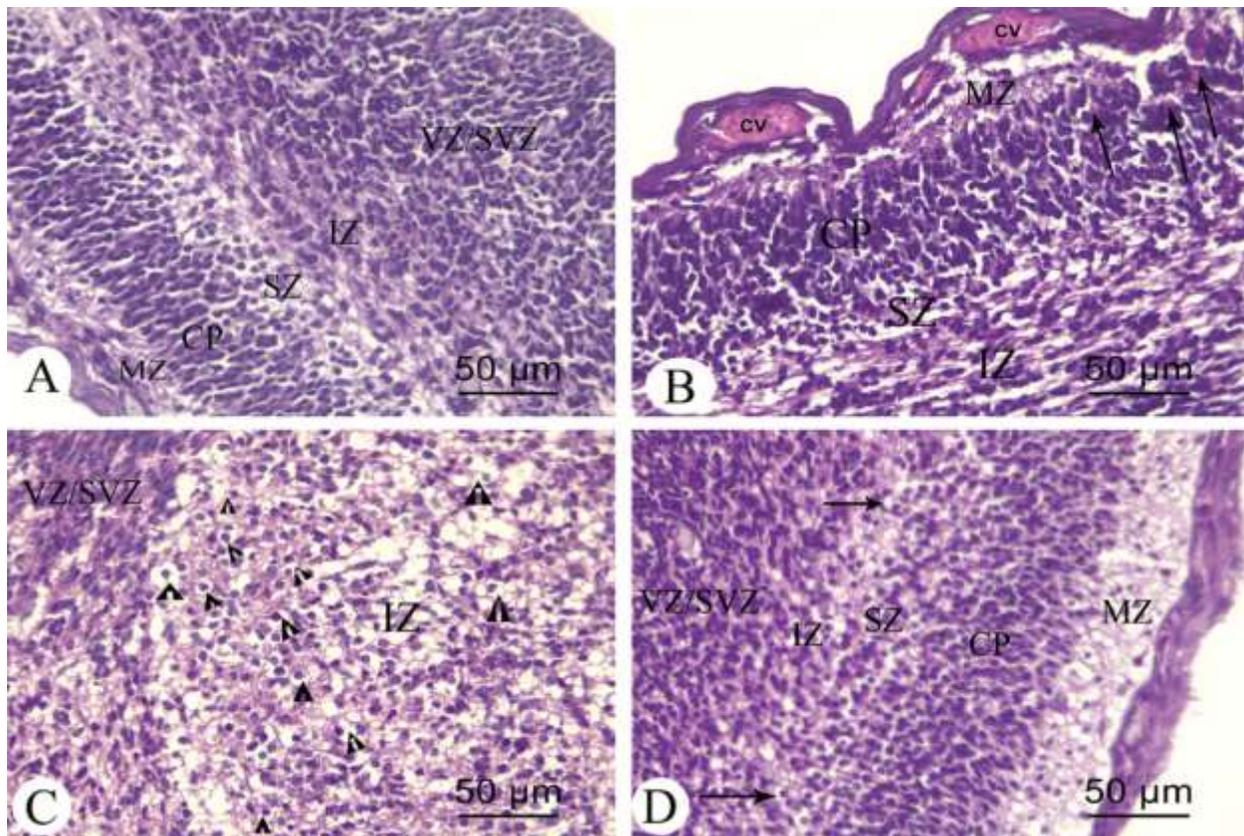


Fig. 1. (Histopathological sections at 17 dpc):

Photomicrographs of histological sections in the zones of the rat telencephalic wall; ventricular and subventricular zones (VZ/SVZ), intermediate zone (IZ), subplate (SZ), cortical plate (CP) and marginal zone (MZ) at embryonic age 17dpc; A: Control group showing all the normal embryonic zones features; B: MTX treated group showing congestion of cerebral vessels mostly in meninges (CV) and hemorrhagic spots in the cortical plate (Arrows); C: MTX treated group showing distributed vacuolated cells in the intermediate zone (Arrow heads); D: MTX+FA treated group showing a decrease in number of vacuolating cells (arrows). (H&E X 400)

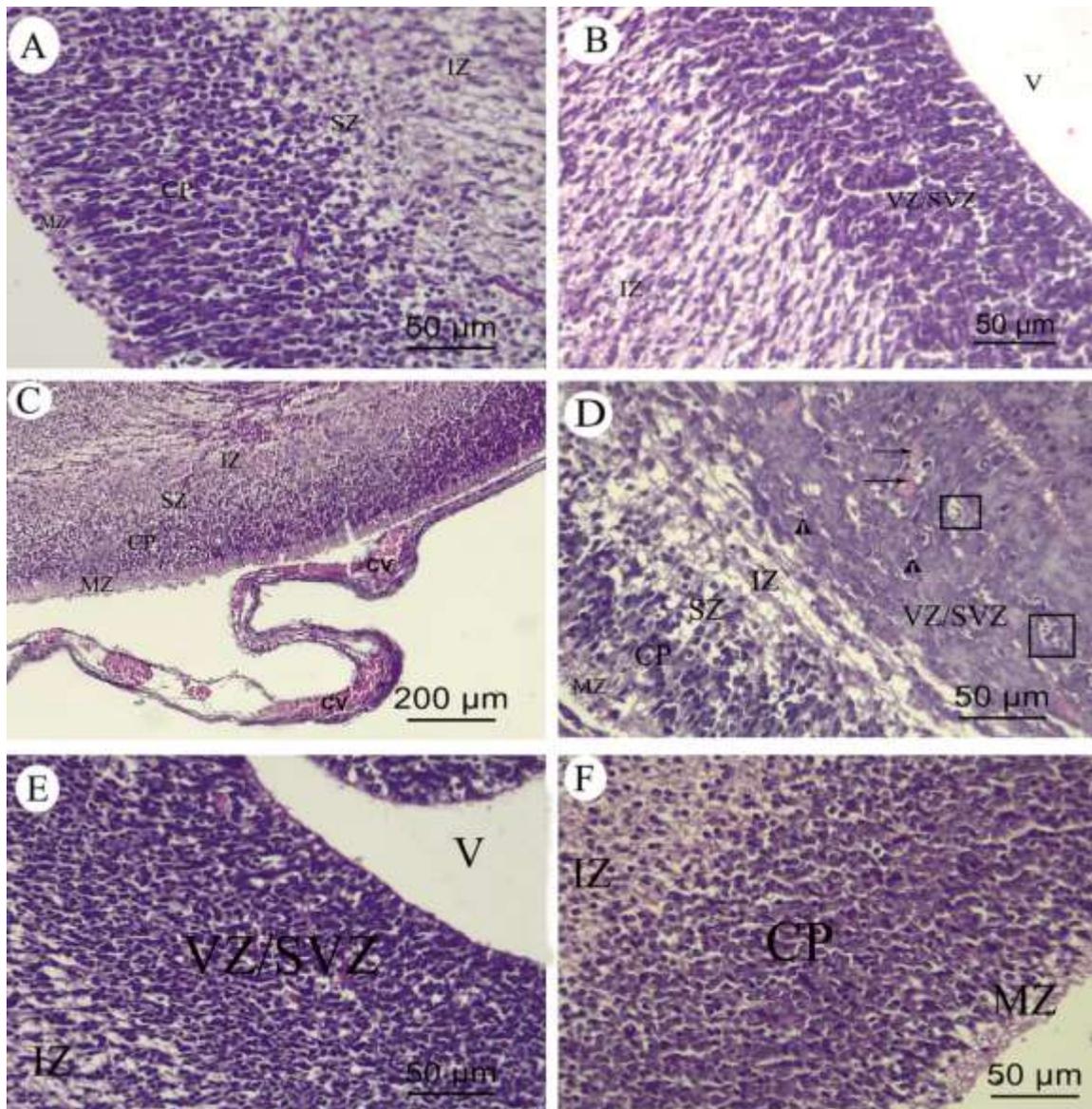


Fig. 2. (Histopathological sections at 19 dpc):

Photomicrographs of histological sections in the rat telencephalic wall; ventricular and subventricular zone (VZ/SVZ), intermediate zone (IZ), subplate (SZ), cortical plate (CP) and marginal zone (MZ) at embryonic age 19 dpc; A: Control group showing; intermediate zone (IZ), subplate (SZ), cortical plate (CP) and marginal zone (MZ); B: Control group showing intermediate zone (IZ) and ventricular and subventricular zones (VZ/SVZ); C: MTX treated group showing highly congested cerebral vessels mostly in meninges (CV) (H&E x100); D: MTX treated group showing vacuolated cells (arrow heads) and clusters of cells with pyknotic nuclei (square) and hemorrhagic spots (arrows); E & F: MTX+FA treated group showing an improvement in vacuolation, hemorrhage, clusters and congestion. (C: H&E X 100) (Others: H&E X 400).

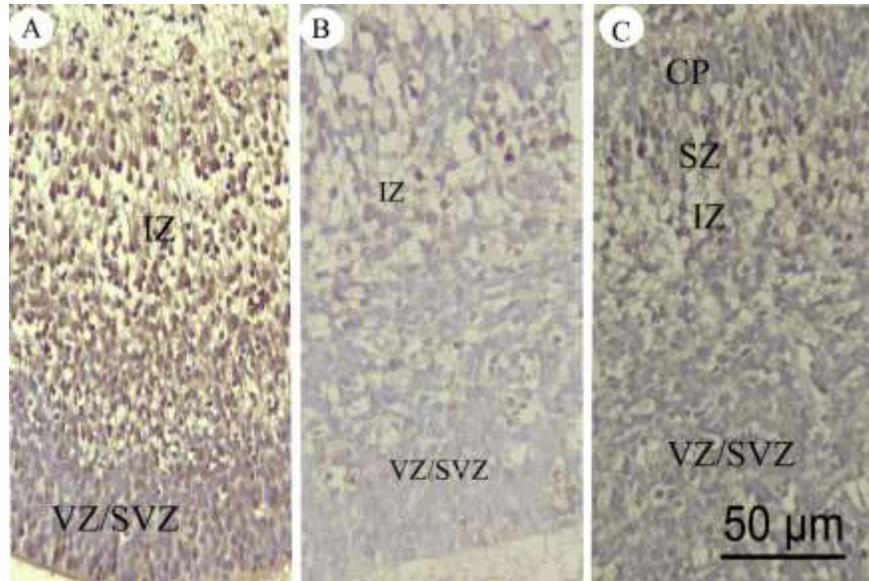


Figure 3. (immunohistochemical sections for Caspase-3 at 17 dpc):

A photomicrograph of sections in the telencephalic wall; ventricular and subventricular zones (VZ/SVZ), intermediate zone (IZ), subplate (SZ), cortical plate (CP) and marginal zone (MZ) at embryonic day 17dpc in three different groups; the control group (A), the MTX treated group (B) and the MTX+FA treated group (C), showing Caspase-3 +ve cells decrease in MTX treated group with a very mild improvement in MTX+FA treated group. (Immunohistochemistry for Caspase-3 X 400).

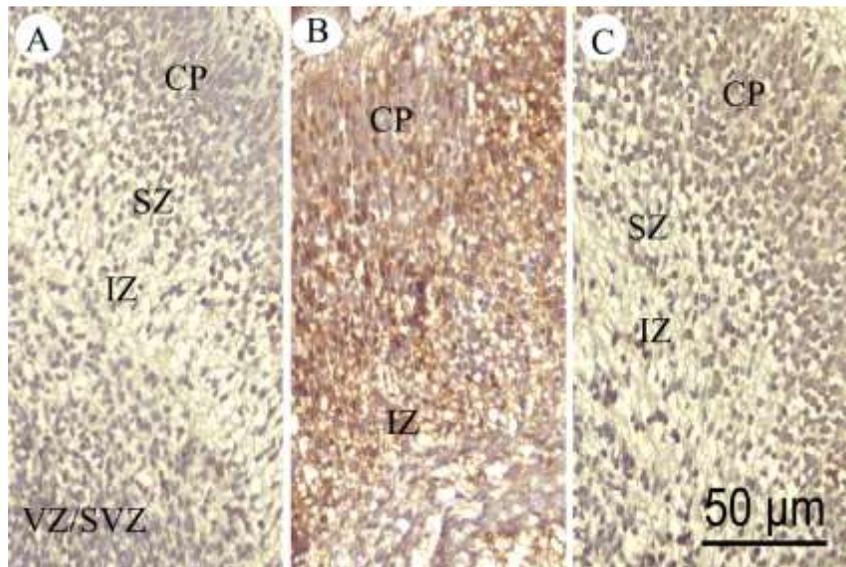


Figure 4. (immunohistochemical sections for Caspase-3 at 19 dpc):

A photomicrograph of sections in the telencephalic wall; ventricular and subventricular zones (VZ/SVZ), intermediate zone (IZ), subplate (SZ), cortical plate (CP) and marginal zone (MZ) at embryonic day 19dpc in three different groups; the control group (A), the MTX treated group (B) and the MTX+FA treated group (C), showing Caspase-3 +ve cells increase in MTX treated group with a marked improvement in MTX+FA treated group. (Immunohistochemistry for Caspase-3 X 400).

DISCUSSION

Methotrexate is known as an antimetabolite and antifolate utilized to cure many autoimmune diseases like rheumatoid arthritis and psoriasis. In addition, used as a chemotherapeutic drug for a lot of cancers like lymphoma and breast cancer [14]. It is known as a DNA damaging drug to which the developing brain is very sensitive [15].

In the present study, the weight and volume of fetal cerebrum at embryonic age 17 dpc decreased after maternal MTX treatment. In addition, the volume at embryonic age 19 dpc decreased after the same treatment. As well as, Famurewa et al [16] reported decrease in the adult cerebrum weight after exposure to methotrexate compared to the control group.

In addition, Zajac-Spychała et al [17] reported decrease in the volume of different parts of the brain in magnetic resonance imaging after chemotherapy by methotrexate when compared with control.

In the current study, the transverse diameter and anteroposterior diameter of the fetal telencephalon in the MTX treated group at embryonic age 17 dpc were markedly decreased. This supported what previously mentioned by Sugiyama et al [18] who observed significant decrease in cerebellum dimensions after methotrexate injection of 1 mg/kg body weight on postnatal day 6.

The decrease in gross measurements was explained by testing the expression of Caspase-3, a marker of apoptosis. Shao et al [19] demonstrated increase caspase-3 positive cells in mouse spinal cord and brain with increase in astrocyte apoptosis.

It was highly expressed in the fetal telencephalon at embryonic age 19 while at embryonic age 17 it showed slight decrease in MTX treated group when compared to control group. These findings confirmed the previously stated that there was increase in number of cells showed positive caspase-3 staining in rat brain after methotrexate injection [20].

Vacuolation in cerebral sections are a common histopathological finding that can be associated with neurotoxicity. As a result of neuronal injury, cell death and disintegration occur and the classical excitotoxic materials as domoic acid may induce variant brain tissue spaces and also neuronal spaces. [21].

In this study, methotrexate exposure induced some pathological changes like widely distributed vacuolated cells, hemorrhagic spots at embryonic age 17 and congestion of blood vessels mostly in meninges at embryonic ages 17 and 19 in fetal telencephalon.

These results coincide with Bhushan et al [22] that reported intracerebral hematoma and brain hemorrhage as a complication of intrathecal (direct CSF) methotrexate administration. In addition, intraperitoneal injection of methotrexate in adult rat showed severe vacuolar changes, inflammatory cells infiltration and necrosis at cerebral sections [16].

Excessive death of neurons is one of the most important mechanisms that disturb the neuronal migration in the developing telencephalon. Disorders of neuronal migration are considered major causes inducing microencephaly in many experimental animals [23].

Interruption of neurons migration leads to clumping of cells in the proliferative zones; ventricular and subventricular zones to form aggregation of cells [24]. This theory explained the cell clusters with pyknotic nuclei that were clearly demonstrated in the ventricular and subventricular zones at embryonic age 19 dpc in the MTX treated group.

Folic acid is essential for metabolism and cell growth and is considered a common co-drug prescribed by physicians. It has a low cost and an ability to decrease or eliminate the toxicity, while preserving the effect of MTX [25].

The protective role of folic acid during neuronal development was previously described by Craciunescu et al [26] who reported that, folate deficient diet leads to a decrease in

numbers of proliferating neuronal progenitor cells.

Adding folic acid with methotrexate administration in the current study showed considerable improvement in the form of decreased number of vacuolated cells, disappearance of both hemorrhagic spots and congested blood vessels.

This confirmed the previously reported increased the possibility that sufficient amount of folic acid was required for cerebral cortex development in late pregnancy [11].

In addition, folic acid supplementation decreased the expression of caspase-3 in the current study. This protective effect was previously stated by Soliman [27] and Mohamed & Nor-Eldin [28] in other different organs. They reported that administration of folic acid with methotrexate treatment revealed marked protection of the liver cells and cardiac muscles from the degenerative changes caused by methotrexate treatment and decreased the number of positive cells stained by caspase-3.

CONCLUSION

Intraperitoneal injection of 30 mg/kg MTX on gestational day16 produced a visible decrease in gross parameters of cerebrum and severe apoptotic changes shown by increased caspase-3. Excessive apoptosis of neurons were linked to neuronal migration disorders that lead to microcephaly. Adding folic acid provided a visible improvement. So far, to our knowledge, this is the first study that discusses the effect of folic acid on fetal telencephalon after methotrexate exposure in the late gestation.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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