EFFECTS OF LOW DOSE OF METHRATAXATE (MTX) ON WEIGHT, STILLBIRTH (INTRAUTERINE DEATH) OF MICE EMBRYOS AND HISTOLOGICAL STUDY OF EMBRYONIC BRAIN

*Ban Thabit Al-Ani, Mohammad Oda Salman¹: MBChB, FIBMS (CAA) and Rana R. Al-Saadi

¹High Institute of Infertility Diagnosis and ART, Al-Nahrain University.

ABSTRACT

Background: MTX a medicine stop growth of cells and interfere with immune system. Its efficacy is wide but it may cause serious or life-threatening toxicities on liver, lungs, kidney and immune system. In humans, a severe malformation called the “aminopterin syndrome” has been described in outcomes of pregnancies exposed to MTX from four to 12 weeks. This syndrome consists of CNS abnormalities (spina bifida, mental retardation, hydrocephaly, anencephaly). Closed neural tubes but poor brain development were seen in rat embryos cultured on folate-deficient serum, and neural tube defects have been demonstrated in embryos of folic-acid-deficient rats. The Objective is to Observed the effect of low dose MTX on weight, mortality rate and the toxic effect of MTX on the brain of mice embryos. A group of pregnant female mice (10 pregnant mice) injected by low dose MTX (0.02 mg/kg), at day 4, 5, 6 of gestation (G1), another (10 pregnant mice) injected by the same dose of MTX day 14, 15, 16 (G2). Four pregnant female mice served as a control group. Pregnant females were sacrificed on gestational day 7, 17 then examine the embryos weight and the incidence of intrauterine death. For histological study 20 mice embryos were fixed in Bouin fixative, paraffin infiltration, then embedded sections stained with haematoxylin and eosin, the specimens independently read. The Results shows a highly significant decrease in weight of embryos at days 7, 17 (P< 0.01) as comparison with control groups. None of embryos died in control group, there is a highly significant increase in mortality rate were observed in G1, G2(P<0.05). And 60% of embryos died at day 7 and 30% of embryos died at day 17. The histological investigation showing
embryonic brain damage in all brain regions. In Conclusion low dose MTX discontinued as early as possible in pregnant women because of its toxic effect on embryos.

**KEYWORD:** Methotrexate, embryo, weight, intrauterine death, brain toxicity.

**INTRODUCTION**

Methotrexate (MTX) is the drug more frequently used by rheumatologists to control rheumatoid arthritis (RA) and other rheumatic diseases. MTX is a folic acid antagonist that impairs dihydrofolate reductase and interferes with the production of purines.[1] The importance of folic acid, a B group vitamin, in haematopoiesis was realized in the early 1940s and by 1946 it had been synthesized and given its chemical term, pteroylglutamic acid.[2] By the following year, the synthesis, anti-bacterial and anti-metabolic actions of its 4-amino derivative, aminopterin, had been described.[3] The megaloblastic appearance of marrow in some cases of leukaemia, similar to that seen in folic acid deficiency, led early researchers to speculate that folic acid analogues could be useful in treating this form of malignancy. Paradoxically, it was found that a folate-deficient diet could cause depletion of leukaemic cell lines, which led to the use of the competitive analogues in haematological malignancies.[4]

Folic acid is required for normal development and its deficiency may be harmful in pregnancy. MTX used as effective anticancer agents[2]. In animal studies (rats, mice and rabbits) MTX has been shown to cause embryotoxicity and teratogenicity.[3] In humans, a severe malformation called the “aminopterin syndrome” has been described in outcomes of pregnancies exposed to MTX from four to 12 weeks. This syndrome consists of CNS abnormalities (spina bifida, mental retardation, hydrocephaly, anencephaly).[4] the drug was tested to ascertain its specific detrimental effects on the neural development of the mouse embryo.[5] The 7th to 12th day of gestation is a particularly sensitive period. At this time the neural plate has formed and neural morphogenesis is at its height and should be especially susceptible to the action of teratogens.[6,7]

If the actions of MTX are mediated via inhibition of folic acid, then folic acid deficiency occurring in pregnancy may give clues to the effect of MTX on the fetus.[8] MTX, is an immunosuppressive drug, act as folic acid antagonist that binds to the enzyme dihydrofolatereductase(DHFR). This enzymes inhibits the synthesis of thymidylate, serine, and methionine, which disrupts synthesis of DNA, RNA and protein leading to cell death[9],
MTX is further metabolized to MTX polyglutamates, which are long-lived metabolites, inhibiting other folate-dependent enzymes.\textsuperscript{[10,11]}

Further rat studies showed that folic acid deficiency could also cause embryonic deformities, the most common being cleft palate and limb abnormalities. As would be expected, folate deficiency after the thirteenth day of a 21-day gestation (i.e. after the critical period of organogenesis) was associated with a marked reduction in the number of abnormalities.\textsuperscript{[12]} Closed neural tubes but poor brain development were seen in rat embryos cultured on folate-deficient serum,\textsuperscript{[13]} and neural tube defects have been demonstrated in embryos of folic-acid-deficient rats.\textsuperscript{[14,15]}

**MATERIALS AND METHODS**

The experiments were performed on 24 mature female Swiss-Webster mice; their ages ranged between 6-8 weeks with a body weight (B.wt) ranging between 28-30g. These mice were divided into three groups: two experimental (G1, G2)(10 animal/group) and four pregnant female mice served as a control group (G3). Vaginal smear were performed to all the adult female mice to diagnose the stages of estrus cycle, to detect heat stage for mating. Females in the metestruus phase were left with mature healthy males for mating (1male/2female). The occurrence of vaginal plug considered as the first day of pregnancy (17), the subsequent days were sequentially numbered. The pregnant female was removed into separate cages. A group of pregnant female mice (10 pregnant mice) injected by low dose of MTX (0.02 mg/kg), at day 4,5,6 of gestation concerned as group one (G1) and another group of pregnant female mice (10 pregnant mice) injected by the same dose of MTX days14, 15, 16 concerned as group two (G2). Pregnant females were sacrificed on gestational day 7, 17 and examine the embryos weight and the incidence of intrauterine death. The total number of implantations was 111 embryos (76 embryos in treated groups and 35 embryos in control group).

When the female in G1 and two pregnant mice of control group (G3) reach day 7 of gestation, animals were sacrificed, while G2 animals and two pregnant mice of control group were sacrificed at day 17. After abdominal incision a number of died and live fetuses in each horn were recorded, then the muscle layer of the uterus were dissected to shelled out the live embryos with the tips of closed forceps. Each fetus were washed and weighted for all groups. For histological study 20 treated embryos were fixed in Bouins fixative for 24 hr, then dehydration, infiltration with paraaffin and embedded sections were stained with haematoxylin
and eosin, the specimens were independently read and reviewed by two pathologists who were unaware of the drug and the dose.

RESULTS
In this experimental study, the results were categorized into two main parts including the outcomes of parameters of weight, mortality rate and the histological findings of certain embryos brain features as follows:

The weight and mortality results shows a highly significant reduction ($P < 0.01$) in weight of embryo at day 7 of G1 in comparison with control group as show in (Figure.1), whereas there is a highly significant reduction ($P < 0.01$) in weight at day 17 was noted in the same group in comparison with control groups as show in (Figure.2). None of the embryos died in the control group while 60% of embryos died in MTX treated group at day 7 and 30% of embryo were died at day 17. There is a highly significant increase ($P < 0.01$) in mortality rates comparison with control group as show in (Figure. 3).

Figure (1): A diagram showing the effect of low dose injection of (MTX) on the embryonic weight during day7 at gestational period.
Figure (2): A diagram showing the effect of low dose injection of (MTX) on the embryonic weight during day 17 at gestational period.

Figure (3): A diagram showing the effect of injection of 0.02 µm of (MTX) on the rate of embryonic mortality in day 7 and day 17 from pregnancy period.

(Chi2 value = 4.978), (P-value = 0.026) column with star was significant different $(p<0.05)$

* Means within each columns is significantly different $(P<0.05)$.

**Microscopic examination of the fetuses**

Representative fetuses from experimental group was sectioned as previously described. They were compared with normal fetuses obtained from untreated control pregnant females. Figure[4] shows representative sections of a portion of the developing brain of a normal fetus. In the treated group the basic neural organization was maintained but the tissues were clearly
undergoing degenerative changes. The damage was more extensive in the brain region than posteriorly. The surrounding tissues and especially the mesenchyme were sparse. The cells of the neural epithelium were few in number and loosely and abnormally arranged. The neural epithelium was generally thin, often discontinuous and thrown into numerous irregular folds figure 2.

Figure (4): Longitudinal Section in the brain of mice embryo at day 17. This slide show embryonic brain damage was more extensive in all brain regions H & E, 40x.
Figure (5): This slide show the histological appearance of brain embryo day 7. Group of brain cell show The neural epithelium was generally thin, often discontinuous, and thrown into numerous irregular folds shrinking (arrows). H & E, 400x.

DISCUSSION

Despite the limited scope of the experiments it is clear that the MTX drug is detrimental to the embryos when administered to pregnant females during the sensitive period in the morphogenesis of the nervous system. The results showed a grossly visible defects only in the treated fetuses, microscopic examinations of the superficially untreated fetuses of the control group, however, revealed degenerative changes and abnormal histogenesis of neural tissues. The methotrexate-induced effects were not as easily evaluated because of high embryo lethality which resulted from the toxic dosage administered. Although no visible alterations were observed among the surviving fetuses, the possibility of detrimental effects on the nervous system cannot be excluded.\textsuperscript{16} The increased incidence of hematomas among the fetuses might be incidental, as these occurred in methotrexate-treated mice and control...
groups. The preliminary nature of the data precludes speculations on the mechanisms of the teratogenic action of the MTX. A drug may produce its noxious effect a) directly on the fetus, or more indirectly b) at the fetoplacental site. Initial work by Nelson et al., (1949)\cite{17} using rat, had suggested that foliate deficiency had a lethal effect on embryos; the number of dying being directly related to the duration of deficiency prior to conception. Embryonic growth delay induced by folic acid antagonists was first described in chickens by Karnofsky, (1949).\cite{18} In other experimental studies performed by Skalko and Gold, (1974)\cite{19} observed the intrauterine death after single intraperitoneal injection of MTX of 0.3-50 mg/kg, to ICR mice (Imprinting Control Region mouse). Females were killed on gestational day 17, litters were observed for intrauterine death and malformations whereas the doses of 10 mg/kg caused an increase in the intrauterine death rate. This observation is agreement with our finding in this experimental study.

Studies by Wilson (1973)\cite{20} reveals that MTX which was taken during pregnancy resulted in fetal death, Wilson revealed that 20% of infant mortality is due to major birth defects. Of those defects about 25% are of genetic origin (genetically inherited diseases, new mutations and chromosomal abnormalities) and 65% are of unknown etiology (multifactorial, polygenic, spontaneous errors of development and synergistic interactions of teratogens) and only 2-3% defects are thought to arise in association with drug treatment.

The MTX drugs could perpetrate teratogenic effects by causing nutritional deficiencies, inhibiting enzymes and altering cell membrane functions, or furthermore by interfering with the mitotic apparatus, nucleic acid metabolism, energy metabolism and osmolar balance.\cite{21} Moreover, the drugs may be modified by the mother to a different array of metabolites, which further complicates the analysis of the mechanism of drug action at the cellular level. Schleuning and Clemm, (1987)\cite{22} suggested that, it is not necessary for drugs to cross the placenta in order to exert a teratogenic effect; MTX is known to pass to the fetus, even after maternal administration. Also Lewden, (2004)\cite{23} and his collageous reveal that low dose MTX in the first trimester of pregnancy assess in the risk of major malformations in pregnant women with chronic inflammatory disorders. All observations are agreement with our finding in this study.

**REFERENCE**


