

Developmental Toxicity of Oral Administered Low- and High-Dose of Folate Antagonist, Methotrexate in Female CD-1 Mice

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Abstract: ***Objective:** The present study was carried out to evaluate potential adverse effects of the low and high doses of MTX through implantation and organogenesis. **Materials and Methods:** Pregnant female mice were given doses of 0, 0.13 and 0.42 mg/kg bw/week methotrexate orally on gestation day zero through day 15 along with vehicle-treated control. Status of gravid/non-gravid uterus, the number of corpora lutea in the ovary, implantation status, fetal resorption, fetal body weight, and external, visceral and skeletal malformations were recorded. **Results:** Maternal toxicity, as evidenced by reduction in body weight gain and signs of toxicity was observed at the low- and high-dose groups. Developmental toxicity that included significantly reduction in the number of live fetuses, mean fetal weight and increased resorption sites was observed only in the treated group of 0.42 mg/ kg bw/week. Also, there was significant increase in the incidence of fetuses with external, skeletal or visceral malformations in 0.42 mg/ kg bw/week dose group. It seems likely that marked maternal toxicity contributed to the observed fetotoxicity. Also, these results reveal that the malformations by MTX treatment are correlated to the disrupted circulating levels of reproductive hormones and histoarchitecture of ovary. **Conclusion:** The results suggest that the MTX had developmental toxicity and teratogenicity at high dose level, which could affect pregnancy, implantation and gestation. The no-observed-effect level (NOAEL) in the present study for developmental toxicity was 0.13 mg/ kg bw/week.*

Keywords: Methotrexate; Implantation; Fertility; Fetus, Anomalies; Resorption.

1. Introduction

Methotrexate (MTX) (N-10-methyl-aminopterin) is a methyl-derivative of folate antagonist aminopterin and is a potent antimetabolite of folic acid (FA) (4-amino-10-methylfolic acid) was first described in 1947 [1]. Like aminopterin, MTX is a folate analogue and a folate antagonist [2] that antagonizes the synthesis of folate in various cells by inducing a pseudo-irreversible inhibition of dihydrofolate reductase (DHFR), the key enzyme in folic acid metabolism and nucleic acid synthesis [3]. It is used increasingly in most childhood leukemias [4], breast cancer [5], osteosarcoma [6] and brain tumours [7]. Low-dose methotrexate is widely used in the treatment of rheumatic conditions [8], gynaecological [9] and neurological diseases [3] while high-dose methotrexate is used in anti-cancer therapy. High-dose methotrexate is one of the most prescribed agents in many malignant diseases affecting girls and young women of reproductive ages [10]. In addition, high-dose methotrexate induced senescence in human adenocarcinoma cells [11].

The genotoxic effects of MTX have already been reported in male and female mice including mitotic index study, which was revealed none of the doses of MTX inhibited cell proliferation during the first post-treated cell cycle [12]. Furthermore, in a previous study, it was reported that MTX induced cytotoxic and genotoxic effects in a dose of (5, 10, 20 and 40 mg/kg) in the germ cells of mice [13]. Herman et al.

[14] also reported that the exposure to MTX leads to nucleotide pool imbalance, uracil incorporation into DNA, and genotoxic stress-induced cell death.

The teratogenicity and embryo lethality of MTX at 2, 10 and 20 mg/kg b.w. in different test systems have been studied and reviewed [15, 16]. It was shown in numerous studies to be a potent teratogen in humans as well as in animals [17; 18; 19]. During embryonic development, MTX administration was found to be embryotoxic [20]. Systemic injections of MTX to pregnant rats have embryolethal effects as a function of embryonic development [21]. There is no literatures addressed the effect of low and high doses of methotrexate. So, the present study was undertaken to evaluate the maternal and embryofetal toxicity of methotrexate at doses considerably equivalent to low and high human doses that treat rheumatic arthritis. The oral route of exposure was selected for this study because it is a likely route of human exposure. Folate antagonist drugs have been shown to be well absorbed in animals and humans when given by mouth.

2. Materials and Methods

2.1 Chemicals

Methotrexat® (MTX) oral tablets (2.5 mg), obtained from local pharmacy, manufactured by Orion Corporation, Finland.

Drug solution was prepared by dissolving the MTX tablets in distilled/deionized water.

2.2 Animals

Sexually mature and proven fertility females CD-1 mice with regular estrous cycle (10–12 weeks old; weighing 25 ± 5 g) were obtained from the animal house of faculty of medicine, Alexandria University, Alexandria, Egypt. Animals were housed at 30 x 30 x 40 cm plastic cages with laboratory-grade wood shavings as bedding which were regularly cleaned every day. Mice were acclimatized under controlled environmental conditions at room temperature (23 ± 2 °C) with humidity ($50 \pm 10\%$) and a 12 h light/ 12 h dark cycle. All mice had access to food and provided water ad libitum.

2.3 Mating

After acclimatization period (2 weeks), females were selected for mating. Female mice were cohabited with sexually mature and proven sire male mice from the same strain and supplier in a separate cage with ratio 2/1 (females/male) at 06.00 – 07.00 pm and left overnight. Vaginal plug was examined early in the following morning (06.00 – 07.00 am). Females were considered to have mated if the vaginal plug was observed in the vaginal opening. The day on which evidence of mating occurred was considered as day 0 of gestation. Pregnant females were randomly assigned to experimental dose groups at gestation days (GD) 0-15.

2.4. Dose assessment

Methotrexate tablets is generally administered per oral once weekly for patients with Rheumatoid Arthritis (RA) (average weight 60 kg), with doses ranging from 7.5 to 25 mg/week [22] which equivalent to 0.13 mg/kg bw/week and 0.42 mg/kg bw/week respectively. For human, these dose levels are applied as tablet of 2.5 mg every 12 hr.

2.5 Groups assignment

A total of 75 pregnant female mice were randomly divided into three groups (25 mice per group). The control group was received distilled water by oral gavage. The low dose group (LD-MTX) methotrexate was given orally with 0.13 mg/kg bw/week (GD 0-15) of methotrexate while the high dose group (HD-MTX) methotrexate was administrated orally with 0.42 mg/kg bw/week (GD 0-15).

2.6. Body and organs weight

Maternal body weights were estimated and recorded at intervals as initial and final weights of control and methotrexate-treated groups. At the 18th day of gestation females were sacrificed and dissected. After caesarean section, the gravid uteri and the ovaries were removed, dried and plotted and then the weights were estimated. The liver, kidney, heart and brain were excised from different tissues and then their weights were estimated and recorded.

2.7 Maternal Biochemical Parameters

2.7.1. Collection and preparation of blood samples

After mice were anesthetized, the blood samples of the pregnant females were collected from retro-orbital plexus vein [23] into heparinized tubes, centrifuged at 3600 rpm for 15 min. Plasma were separated, divided into aliquots and kept in a deep freezer at - 40°C till all assays were carried out within a week of collection.

2.7.2. Determination of pituitary and steroid hormones

The estradiol and progesterone levels in the plasma were analyzed by using immulte/immulite 1000, and chemiluminescent enzyme immunoassay. Commercial assay Kit of estradiol and progesterone was used for assessment process according to Bergquist et al. [24]. Determination of LH and FSH concentration in the serum was based on the principle of a solid phase enzyme-linked immune absorbent assay (ELISA) as described by Uotila et al. [25].

2.8 Maternal and Fetal Endpoints

Pregnant dams were anaesthetized and killed by cervical dislocation on gestation day 18. The uterine horns were exteriorized through a midline abdominal incision, opened and examined for number of implantation sites, live, dead fetuses and resorption sites. The number of live and dead fetuses were counted and recorded. The uteri of apparently non-pregnant mice were stained with 10% sodium sulfide and examined for evidence of implantation sites [26]. Each live fetus was weighed, sexed, and given a gross examination for external malformations. Anogenital distance (the distance between anal opening and the genital opening) was recorded. One-half of the fetuses were fixed in Bouin's solution for razor blade sectioning [27], and were evaluated for visceral malformations/variations [28]. The remaining fetuses in each litter were eviscerated, fixed in 90% alcohol and double stained with alizarin red S for ossified bone and Alcian blue for cartilage, and cleared in 2% KOH and glycerin [29; 30] and were assessed for skeletal malformations/variations.

2.9 Histopathology

For histological technique the procedure described by Krause [31] was used. Briefly, fixed ovaries and uteri in 10% v/v buffered formaldehyde were dehydrated through ascending grades of ethanol (70, 90 and 95% v/v). They were cleaned in xylene, impregnated and embedded in paraffin wax (melting point 56 °C); Serial transverse sections were cut at 5 µm on a rotator microtome. The sections were floated out on clean microscope slides, which had previously been albuminized with Mayer's albumin to prevent detachment from slides during staining procedure with hematoxylin and eosin (H&E) and Trichrome staining [32]. After staining, the slides were passed through ascending concentration of alcohol (20 – 100%) for dehydration and then cleaned with xylene. A permanent mounting medium (balsam) was put on the tissue section. A thin glass-covered slip was placed on the covering mounting medium and underlying tissue sections were

allowed to dry. This was later observed using the Olympus CX1 research microscope at x100 and photomicrographs were taken in bright field at x100.

2.10 Data Analysis

Data were analyzed by the one-way analysis of variance (ANOVA) followed by Turkey's multiple comparisons. Differences between dose groups were further tested by the Duncan's test. Statistical calculations were performed using a SPSS program software (Statistical Package for the Social Sciences), and differences were considered as significant when $P < 0.05$.

3. Results

3.1 Maternal Toxicity

Mortalities had occurred during the course of the present study. Clinical observations and measurements indicate evidences of substance related toxicity including hyperactivity, salivation, vaginal bleeding, and tremors were noted during the dosing period among animals in the 0.42 mg/kg bw/week dose group. These signs had been showed in 70% of treated females and appeared immediately after treatment. The deaths of three dams treated with the highest dose (on GD 15 and 17) and one dam treated with the lowest dose (on GD 14) also showed that MTX was maternally toxic at these dose levels. However, no effects were noted on the general appearance of the animals in the 0.13 mg/kg bw/week group.

3.2 Maternal body weights

The effects of MTX on pregnancy weight gain or weight change and on the weight of maternal organs are shown in **Table 1**. At the two doses tested 0.13 and 0.42 mg/kg bw/week, MTX markedly decreased maternal weight gain. The reduction of total weight gain GD (0–18) resulted, to a great extent, from a harmful effect on the mother because it was even more evident when gravid uterus weight at term was subtracted from the weight gain during whole pregnancy (**Table 1**). Maternal toxicity of the two doses of MTX was additionally demonstrated by a pronounced decrease of liver, kidney, and brain weights (**Table 1**).

3.3 Biochemical Parameters

3.3.1. Pituitary and Steroid hormones

As demonstrated in **Figure 1**, female mice treated orally with 0.13 and 0.42 mg/kg bw/week methotrexate had a significant inhibition in plasma estradiol and progesterone. On the contrary, significant elevation in levels of plasma FSH and LH hormones was recorded in 0.13 and 0.42 mg/kg bw/week treated groups

3.5 Embryofetotoxicity Findings

Females treated individually with each of 0.13 and 0.42 mg/kg bw/week had no changes in number of corpora lutea/dam significant versus control as depicted in **Table 2**. The number of implantation sites per dam detected by the method of Salewski [26] was not altered by MTX (**Table 2**). Since in the mouse implantation takes place on GD 4.5–6 [33], these findings indicated that MTX administered from GD 0-6 on did not induce implantation embryo losses. Nonetheless, an increased occurrence of resorptions as well as a decreased number of live fetuses demonstrated that the highest dose 0.42 mg/kg bw/week was embryolethal (**Table 2**). MTX produced increases in the frequency of early resorptions (**Table 2**), therefore indicating that most embryos died soon after implantation, during the first days of treatment. This observation was indicative of development which suggest that MTX play a protocol on the rapid growth of the epiblast, the extraembryonic ectoderm (derived from the trophoctoderm) and the overlying visceral endoderm (derived from the primitive endoderm) leads to the formation and elongation of an egg-cylinder-shaped embryo [34]. Therefore, Most of the defects observed result from disturbance in the initiation of gastrulation [35]. Similarly, a statistically significant increase in the number of post-implantation loss was shown in the group treated with 0.42 mg/ kg bw/week. Fetal sex ratios for treated groups did not differ from that of the control group, a finding indicative that the lethal effect of MTX affected to the same extent male and female embryos (**Table 2**). Near term fetal body weight was reduced at the highest dose, a result suggestive that, at dose higher than 0.13 mg/kg bw/week, MTX retarded the growth of the surviving embryos (**Figure 2; Table 2**). Except for an increased occurrence of edema at the highest dose group (**Figure, 2B**), there is significant difference was found between control and 0.13 mg/kg bw/week treated females regarding the frequency of externally-visible abnormalities (**Figure 2; Table 3**). Nevertheless, visceral examinations revealed a markedly higher frequency of uterus malposition and internal hemorrhage in MTX-treated females, particularly in those of the highest dose group (**Table 3**). Noteworthy, in the groups exposed to doses of MTX equal to 0.13 mg/kg bw/week, some fetuses exhibited with apparently liver disfigured and internal hemorrhage (**Table 3**). Increased occurrences of misshapened liver, malpositioned testes and malpositioned uterus were also noted in fetuses exposed to doses of 0.42 mg/kg bw/week MTX (**Table 3**). The occurrence of skeletal abnormalities in fetuses exposed to MTX on GD 0–15 is shown in **Table 3**. Fetal skeleton examination revealed that MTX caused a marked and dose dependent increase in the incidence of poorly ossified skull bones. MTX produced a dose-related increase in the occurrence of rib anomalies and sterna as well (**Table 3**). Poor ossification of some forelimb bones (e.g. ulna, humerus, and radius) was also noted among 0.13 mg/kg bw/week exposed fetuses but, in all these cases, the increased occurrence related to the dose of the compound (**Table 4**).

3.6 Histopathology

Histopathology of ovaries is shown in the photomicrographic panels in **Figure 3**. The ovaries from control and low dosed animals appeared normal. They contained numerous corpora lutea as well as follicles at various stages of development or atresia. The cortical stroma was composed of strands of loose fibrous tissue supporting scattered interstitial cells. The follicles illustrating a well defined zonal granulosa surrounding the oocyte and compact theca folliculi and the presence of some primordial follicles (**panels A, B Fig. 3**). Ovaries from 0.42 mg/kg bw/week treated groups distinguished by vacuoles and hemorrhage evident in the luteinized cells of corpora lutea (**CL, panel C Fig.3**). Also, cytoplasmic clumping of oocytes which created an edematous space between the cytoplasm and zona pellucida in most of the follicles was evident (**Asterisk, Fig. 3**) and many pyknotic granulosa cells were visible in atretic follicles. The ovaries showed some cellular hypertrophy of the theca folliculi, complete distortion/destruction of the basement membrane separating the theca folliculi from the zona granulosa. Degenerative and atrophic changes were observed in the oocyte and zona granulosa; these were more pronounced in those that received 0.42 mg/kg bw/week of MTX. There were marked vacuolations appearing in the stroma cells.

4. Discussion

Results from the present study revealed that MTX was toxic to pregnant mice at low and high oral doses. The fetotoxic effects of MTX, however, were observed at 0.42 mg/kg bw/week that is higher dose. Similar data have been reported for other folate antagonists drugs [36; 37]. These results indicate that teratogenic and fetotoxic effects of MTX are not attributable to maternal toxicity and are consistent with the previous published results [38]. An explanation for the weight reduction is that MTX as a dihydrofolate reductase (DHFR) inhibitor alter folate coenzyme pattern, which in turn may alter cell differentiation and tissue growth [39].

Indeed, the biologic effects of methotrexate are attributed to inhibition of dihydrofolate reductase (DHFR) essential for DNA precursors and suppression of transmethylation reactions with accumulation of polyamines [40]. Intracellularly, methotrexate is biotransformed to methotrexate-Glutamate which is more active than MTX in inhibiting folate-dependent enzymes [41]. Further, methotrexate has been proven to accumulate in the tissues where it persisted for weeks even after oral administration was stopped [42]. Therefore, the maternal toxic effects of MTX can be attributed to combination of its intracellular accumulation and subsequent DHFR inhibition.

A higher frequency of resorptions and a lower ratio of live fetuses per implantations and fetal growth could be disturbed by inadequate gene and amino acid methylation [43]. It seems that disturbances in the methylation cycle may result in fetal anomalies or defects [44]. Previous data have already suggested that the disruption of the cell cycle caused by high

dose MTX treatment may be the initial step of the apoptotic sequence of dying cells and may explain the antiproliferative effects [45]. Also, experimental studies in a number of animal species demonstrated that folate deficiency causes intrauterine death, growth retardation and various congenital malformations [46; 47]. Disturbances in folate metabolism are also thought to play a crucial role in cell division and differentiation and then birth defects [48]. Methotrexate and its metabolites are found in the blood and may transfer through the placenta to the fetus [49].

Embryotoxicity of methotrexate was also revealed by an increased occurrence of a variety of external, visceral, and skeletal alterations at the maternally toxic dose of 0.42 mg/kg per week. These findings support the data reported that when administered several folate antagonists to pregnant rats and rabbits by gavage during the organogenesis period, they were able to produce external and visceral defects [50; 51]. The cytotoxic and/or the direct inhibitory effects of MTX on rapidly proliferative tissues which require DNA synthesis [52] and the induction of apoptosis represent interpretation of fetotoxicity [53].

Such a decrease in estradiol and progesterone levels reflects greater ovarian follicular damage and is in agreement with the reports of Manna et al. [54] and Karri and Vanithakumari [55] that MTX exposure inhibited steroidogenesis and ovarian cell function through down regulation of StAR protein and P450SCC gene expression levels. Methylation steps also play an important role in the metabolism of neurotransmitters [56]. Subsequent increase in serum FSH and LH suggests that both smaller and larger follicles are equally important in the regulation of serum gonadotropins [57].

The present study showed that HD-MTX causes significant destruction and vacuolation of ovary, and haemorrhage of uterus. Follicle atresia by HD-MTX is evident in the histopathological analysis of ovary, and disruption of both granulosa and thecal layers was noticed dose dependently. In accordance with these findings, Padmanabhan et al. [13] reported that gonadal toxicity induced by MTX was clearly evident from the genotoxic evaluation. Or by interference in the gametogenesis process that disturbs follicular development [58]. It is possible that the effect of MTX treatment affects the hypothalamic-pituitary control system, uterus, and/or oviduct as well as in the ovary. Therefore, it is possible that MTX during pregnancy affects the ovary directly, and thus leads to adult reproductive dysfunction [55].

On the other hand, methotrexate is an endocrine disruptor, which elicits its reproductive and development toxicity through pathways involving androgen and androgen receptor dependent mechanisms [59; 60] and also to induce disruption of microtubules or meiotic disturbance [41]. In fact, agents that interfere with mitosis have been shown to have a teratogenic effect [61].

In conclusion, the present data provide a mechanistic foundation on the maternal toxicity and embryofetal toxicity

of MTX. These data collectively increase confidence in the conclusion that MTX represents a heightened risk to the developing fetus. It seems likely that marked maternal toxicity contributed to the observed alterations in fetal growth retardation and skeletal development. The NOAEL for developmental toxicity was observed at 0.13 mg/kg bw/week.

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6. Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Table 1: Effects of MTX dosed orally by gavage to mice on gestation days 0–15 on maternal weight and weight gain

Parameters	MTX (mg/kg bw/week)		
	Control	LD-MTX (0.13)	HD-MTX (0.42)
Treated females	25	25	25
Mortality %	0	0	0
Maternal body weight (g)			
GD0	31.98±3.23	31.47±3.12	30.95±3.03
GD6	35.26±3.60	36.38±3.02	35.25±3.00
GD15	49.55±3.60	43.82±4.09	38.74±3.78
GD18	61.32±4.08	48.28±4.07	39.93±3.55
[GD0-18] - gravid uterus wt	40.63±3.89	32.87±3.42	31.28±3.23
Maternal weight gain			
[GD0-18] - gravid uterus wt	8.65±0.24	1.40±0.15*	0.33±0.04*

Data shown as means ± SD

GD: Gestation day

*: Significantly different from control (p<0.05)

Maternal toxicity was estimated from maternal body weights, weight gain and organ weights.

Table 2: Effects of MTX dosed orally by gavage to CD-1 female mice on gestation days 0–15 on embryonic development

Parameters	MTX Dosages (mg/kg bw/week)		
	Control	LD-MTX (0.13)	HD-MTX (0.42)
Pregnant females	25	24	22
Implant sites/dam	10.94 ± 1.09	10.49 ± 1.02	10.18 ± 1.07
Live fetuses /dam	10.84± 1.18	10.37 ± 0.87	6.61 ± 0.70*
Dead fetuses /dam	0	0	0
Early resorptions /dam	0.03±0.03	0.03 ± 0.04	1.07 ± 0.11*
Late resorptions /dam	0.07±0.03	0.09 ± 0.03	2.50 ± 0.28*
Resorptions /dam	0.10±0.04	0.12 ± 0.05	3.57 ± 0.57*
Corpora lutea	11.32±0.57	11.06±0.87	11.99±0.74
Pre-implantation loss %	3.36±0.57	5.15±0.70	15.09±0.97
Post-implantation loss %	0.91±0.07	1.14± 0.11	35.07 ± 3.27*
Fetal weight (g)			
i- Male	0.99 ± 0.09	0.95 ± 0.08	0.53 ± 0.05*
ii- Female	0.92 ± 0.10	0.90 ± 0.08	0.48 ± 0.05*
Crown Rump Length(CRL) (mm)			
i- Male	1.96 ± 0.17	1.92 ± 0.14	1.11 ± 0.12*
ii- Female	1.52 ± 0.14	1.42 ± 0.10	0.92 ± 0.08*
Sex ratio [M/F]	6.5/4.4	5.9/4.5	3.4/3.2

Data shown as means ± SD

*: Significantly different from control. (p<0.05)

M/F: Male/female

Table 3: Fetal anomalies after maternal treatment with MTX on GD 0-15

Parameters	MTX Dosages (mg/kg bw/week)		
	Control	LD-MTX (0.13)	HD-MTX (0.42)
No. of fetuses/litters examined	271/25	250/24	145/22
External anomalies^a			
Hematoma F/L (%) ^b	0/0	1/1(0.4/4.2)	5/4 (3.4/18.2)
Tail short F/L (%) ^b	0/0	0/0	1/1(0.69/4.5)
Tail Kinky F/L (%) ^b	0/0	0/0	1/1(0.69/5.6)
Edema F/L (%) ^b	0/0	1/1(0.4/4.2)	15/8 (10.3/36.4)
Exophthalmia F/L (%) ^b	1/1(0.4/4)	0/0	11/7(7.6/31.8)
Limb defects F/L (%) ^b	0/0	0/0	1/1(0.69/4.5)
Visceral anomalies^a			
Eye (microphthalmia) F/L (%) ^b	0/0	0/0	2/2(1.4/9.1)
Liver disfigured F/L (%) ^b	1/1(0.4/4)	1/1(0.4/4.2)	3/2 (2.1/9.1)
Genital organs(testes/ovaries) F/L (%) ^b	0/0	0/0	10/8(6.9/36.4)
Uterus malpositioned F/L (%) ^b	0/0	0/0	17/13(11.7/59.1)
Internal hemorrhage F/L (%) ^b	1/1(0.4/4)	1/1(0.69/4.2)	14/11(9.7/50)
Kidneys malpositioned F/L (%) ^b	0/0	0/0	8/5(5.5/22.7)
Skeletal alterations^a			
Wavy ribs F/L (%) ^b	1/1(0.4/4)	1/1(0.4/4.2)	6/4(4.1/18.2)
Sternebrae F/L (%) ^b	1/1(0.4/4)	1/1(0.4/4.2)	4/2(2.8/9.1)
Caudal vertebrae (absent) F/L (%) ^b	0/0	1/1(0.4/4.2)	1/1(0.69/4.5)
limbs poorly ossified F/L (%) ^b	0/0	1/1(0.4/4.2)	2/1(1.4/4.5)
Skull poorly ossified F/L (%) ^b	1/1(0.4/4)	1/1(0.4/4.2)	3/2(2.1/9.1)

Data shown as means \pm SD

a: Fetus may be represented more than once in listing of malformations.

b: F/L fetus/litter

*: Significantly different from control ($p < 0.05$)

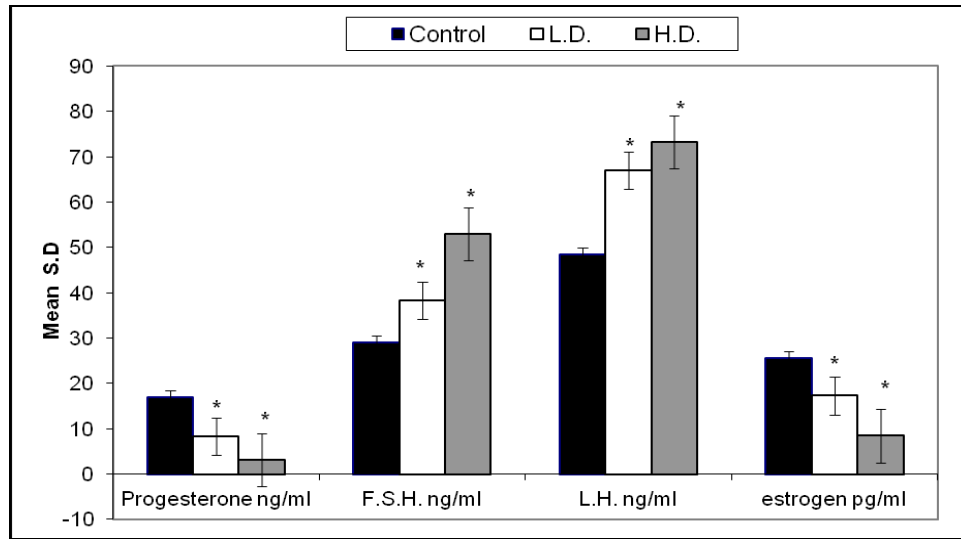


Figure 1: Showing the effect of MTX doses low and high on the level of estrogen ,progestrone, FSH and LH comparable to control group.

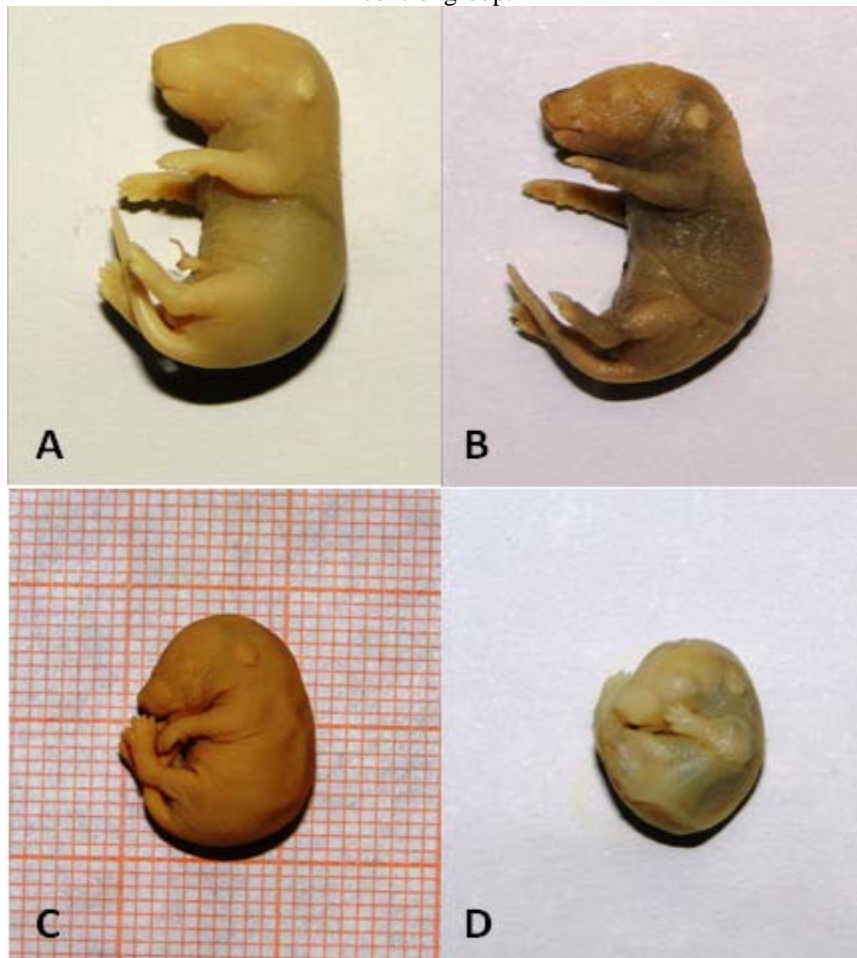


Figure (2): Showing mice fetuses from control (A) and HD-MTX (B, C and D) groups. A. Fetus from control group revealing normal external features. Note retardation in length of mice fetu. B, hepatoma and external edema; C, rounded craniofacial morphology; D, abnormal body curvature

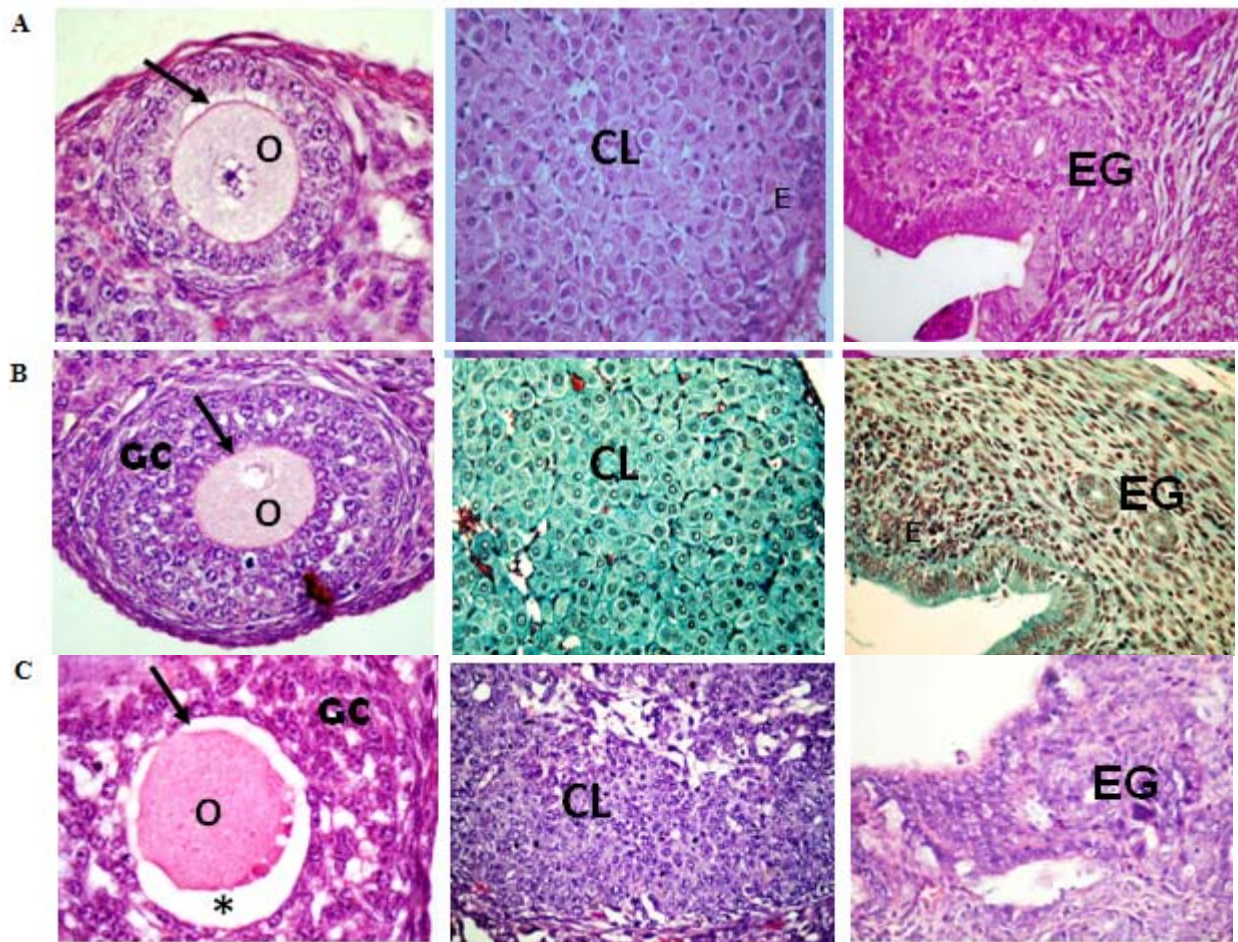


Figure (3): Photomicrograph shows histopathology of ovary of control and methotrexate (MTX) treated female mice, H & E. Controls (panel A), MTX 0.13 mg/kg bw/week (panel B) and MTX 0.42 mg/kg bw/week (panel C). Arrow= zona pellucida; O= oocyte; CL= corpus luteum; GC= granulosa cells; * = edema. Original magnifications 200X.