



## Teratogenic and Embryotoxic Effects of Clomiphene Citrate in Developing Mice

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**ABSTRACT :** The objective of this study was to assess the teratogenic and embryotoxic effects of clomiphene citrate in mice. The pregnant mice were administered a single dose of clomiphene citrate at different concentrations i.e 1.0, 2.0, 4.0 and 6.0  $\mu\text{g/g}$  BW on day 8 of gestation. Fetuses recovered on day 18 of gestation were analyzed on morphological, morphometric and histological basis. Morphological observations showed defects like open eyelids, anophthalmia, fore and hindlimb micromelia, meromelia, amelia, sacral hygroma, hydrocephaly, hemorrhagic spots, kyphosis and clubbed feet. Morphometric analysis indicated a significant ( $p < 0.001$ ) reduction in fetal body weight, crown rump length, head circumference, eye circumference, forelimb and hindlimb lengths and tail size against controls. The histological observations showed brain defects like hydrocephaly, enlarged ventricles and undifferentiated neuroglial cells in cerebellum. Cleft palate, underdeveloped pharynx and atrophy of jaw muscles were the common anatomical defects of pharyngeal region. It is concluded that the concentrations of clomiphene citrate used during the present study proved teratogenic in mice fetuses. (**Key Words :** Clomiphene Citrate, Teratogenicity, Embryotoxicity, Mice Development)

### INTRODUCTION

Clomiphene citrate is a non steroidal, ovulation stimulant (Csemiczky et al., 2002). It is available under trade name of Clomitab and Serophene, given orally (Kennedy and Adashi, 1987; Macklon et al., 2006). It is a mixture of two stereo isomers (40% En and Zu 60% isomer), which have anti-estrogenic properties. It can regulate the process of ovulation (Clark and Markaverich, 1982). The primary use of clomiphene citrate is to avoid anovulatory infertility associated with polycystic ovary syndrome (PCOS) (ESHRE Capri Workshop Group, 1995). Treatment with clomiphene is also evaluated in male infertility due to spinal cord injury and multiple sclerosis (Brackett et al., 1995). Another use of clomiphene citrate is treatment of gynecomastia in both adolescent and pubertal males (Plourde et al., 1983). This is absorbed in gastrointestinal tract and metabolized in liver and excreted slowly via the bile. Drug and its metabolites remain unchanged and excreted in the faeces. The biological half life is 5 days; although metabolites of this drug can be present in faeces up to six weeks after administration

(Mckenna and Pepperell, 1988). Along with this, clomiphene citrates have a tendency for prolonged occupancy of nuclear receptors on repetitive therapy. This has given rise to speculations about its toxicity and possible teratogenic effects (Dickey and Holtkamp, 1996).

Clomiphene citrate is a synthetic hormone that deceives the hypothalamus into thinking that body's estrogen level is too low. In return, the hypothalamus releases GnRH (gonadotropin-releasing hormone), which activates the pituitary gland to release enough amount of FSH (follicle stimulating hormone). Then increased amount of FSH stimulates development of follicles, ultimately resulting in ovulation (Randal and Templeton, 1991; Martikainen et al., 2006).

Toxic effects of clomiphene citrate range from mild (secondary side effects) to severe (genotoxic effects). There are controversial statements about its toxicity. Some reports and cohort studies showed that it had potential for carcinogenicity, genotoxicity and reproductive toxicity. Deveci et al. (2000) reported that clomiphene citrate given to new born rats showed increased keratinization and irregular hypertrophy in epidermal cells; hyperplasia, dermal fibrosis and lymphohistocystic inflammatory cell infiltration were prominent around the sebaceous glands.

Arriaga-Alba et al. (2001) suggested that clomiphene

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citrate may be adduct forming compound, which is able to inhibit replication if the cell lack DNA polymerase, or it may produce frameshift mutations after replications. So the use of this ovulation inducer is a risk of genotoxic damage and it is advisable to do a risk benefit evaluation in a particular case before its description. A prospective cohort study was conducted in 1,135 women for treatment of infertility in Sweden from 1961-1976. They observed that after use of high doses of clomiphene citrate, patient had an almost 2-fold increased risk of breast cancer (Lerner-Geva et al., 2006; Orgeas et al., 2009).

It is debatable until now to reach conclusion that clomiphene is really a safe drug than others. It shows teratogenic effects like CNS malformations and some types of urinogenital abnormalities (Haring et al., 2005; Harmon et al., 2005). Due to the frequent availability, wide range uses and potential role in female and male infertility and lack of data on reproductive toxicity, it is unavoidable to evaluate this drug for its affects on developing organisms.

## MATERIAL AND METHODS

Present research work was conducted on Swiss Webster strain of albino mice, *Mus musculus*. The animals were kept under standard protocol of 12 h dark and light cycle, housed in 12"×16" steel cages; under standard room temperature 27±2°C. Tags or cage cards were utilized to determine the date of mating, dosing and dissection of animals. A total number of 50 females of 8-10 weeks old having 28±2 g body weight were used in the experiment, 10 for each dose group. The females in the estrus phase were grouped with the familiar males overnight. Presence of vaginal plug takes it as 0 day of gestation. The above mentioned protocol was used under approved animal treatment condition of medical ethics committee of Punjab University, Lahore.

Clomiphene citrate available in 50 mg tablets, under the trade name of clomitab was used in this experiment. Keeping in view the prescribed dose limits, four doses 1.0, 2.0, 4.0, and 6.0 µg/g BW were used. The doses were prepared by dissolving 50 mg tablet in distilled water in such a way that 0.1 ml solution contains the desired dose. After that each pregnant mouse was treated orally with 0.1 ml of respective dose at day 8 of gestation (organogenetic period). Along with this a control was also maintained, treated with 0.1 ml distilled water.

On 18 day of gestation, treated mice were weighted and anesthetized with anesthetic Ether. Intact gravid uteri were carefully dissected out. After that uteri were opened along the inner side and fetuses were recovered. Then the recovered fetuses were fixed in Bouin's fixative for 48 hours, later on preserved in 70% alcohol. The preserved fetuses were subjected to morphological, morphometric and

histological studies.

Morphological defects of fetal axis, craniofacial region, limbs and trunk were noted and selected fetuses from each dose group were macrophotographed. Morphometric studies involved the fetal body weight, crown rump length, head and eye circumferences, pinna size, snout size, length of forelimb and hindlimb and tail length. All the measurements were made by analytical balance and digital vernier caliper. While the fetal head and eye circumferences were calculated with the help of computer based programme the "Ellipse Circumference Calculator" downloaded from CSG network (CSGN, 2006).

The whole morphometric data were analyzed by a computer based programme SPSS through analysis of variance (ANOVA). The average per litter values were compared for significance by Tukey's test at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ . These values were further subjected to Duncan Multiple Range Test (DMRT) for multiple comparisons (Duncan, 1955).

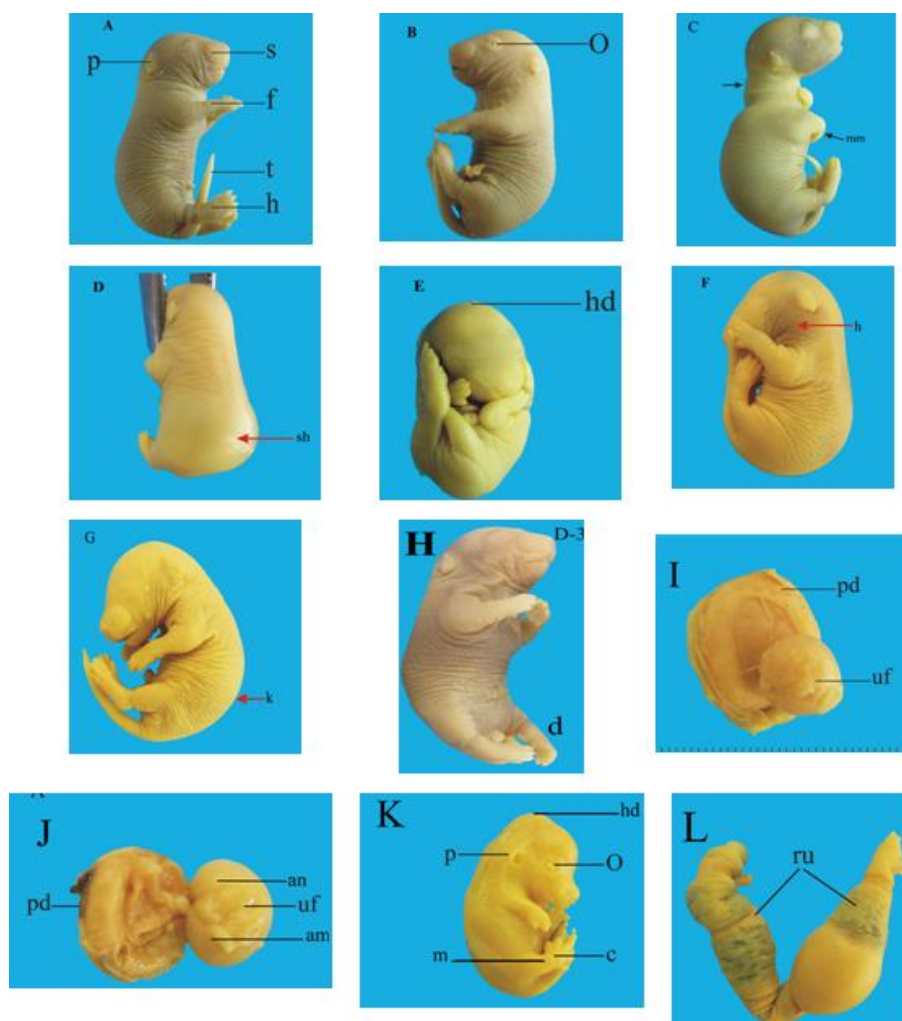
For histological observations selected fetuses from all groups were processed for paraffin sections and for staining hematoxylin and eosin staining procedure was used. Histological defects from CNS and pharyngeal region were studied.

## RESULTS

Morphological observations showed that fetuses recovered from control were well developed. They had normal size and well formed organs (Figure 1A). The fetuses from dose group 1.0 µg/g BW showed morphological anomalies like open eyelid (11.8%) (Figure 1B), micromelia of forelimb (2.7%) (Figure 1C), Extradactyly (4.2%), and distorted axis (2.4%). Whereas in dose group 2.0 µg/g BW defects observed were sacral hygroma (2.7%) (Figure 1D), hydrocephaly (6.5) (Figure 1E), forelimb micromelia (3.8%), Microcephaly (4.6%) and hemorrhagic spots (8.3%) (Figure 1F). The dose group 4.0 µg/g BW included abnormalities kyphosis (7.9%) (Figure 1G), hydrocephaly (5.3%), hemorrhagic spots (2.6%), underdeveloped eyes (3.8%) and hindlimb dysplasia (7.2%) (Figure 1H). Deformities observed in dose group 6.0 µg/g BW were kyphosis (2.7%), hindlimb dysplasia (2.4%), agnathia (5.7), open eyelid (2.4%), Clubbed feet (7.0%), hydrocephaly (4.9%). Various anomalies and their percentages increased by increasing dose concentrations (Figures I-L; Table 1).

Morphometric analysis showed a significant ( $p < 0.001$ ) dose dependent decrease in fetal body weight, crown rump length, head and eye circumference, forelimb and hindlimb length and tail size as compared to control (Table 2).

Histological studies through head and pharyngeal region



**Figure 1.** Macrophotographs of 18 days old mice fetuses recovered from mothers treated with different doses of clomiphene citrate on day 8<sup>th</sup> of gestation, showing a wide range of morphological defects. A: 0.0 µg/g BW; B and C: 1.0 µg/g BW; D, E and F: 2.0 µg/g BW; G and H: 4.0 µg/g BW; I: 4.0 µg/g BW; J, K and L: 6.0 µg/g BW. Snout (s); pinna (p); forelimb (f); hindlimb (h); tail (t); open eyelid (O); forerlimb micromelia (mm); sacral hygroma (sh); hydrocephaly (hd); hemorrhagic spots (h); kyphosis (k) and hindlimb dysplasia (d); Placental disc (pd); underdeveloped fetus (uf); open eyelid (O); clubbed feet (c); abnormal pinna (p) and resorbed fetuses in both horns of uterus (ru); meromelia (m); anophthalmia (an); amelia (am).

**Table 1.** Morphological abnormalities in 18 days old mice fetuses recovered from mothers treated orally with different doses of Clomiphene Citrate on day 8 of gestation

Parameters Dose µg/g BW	Total No. of fetuses recovered	Resorptions (% age)	Body axis (% age)	Head (% age)	Eye (% age)	Snout (% age)	Limb (% age)	Claws (% age)	Hemorrhagic spots (% age)	Tail (% age)
0.00	91	1.09	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	85	2.35	Distorted axis (2.4)	Microcephaly (4.6)	Open eyelid (11.8)	0.0	Forelimb micromelia (2.7)	Extradactyly (4.2)	0.0	Kinky tail (11.2)
2.0	83	3.61	0.0	Hydrocephaly (6.5)	Open eyelid (6.2)	Micrognathia (2.7)	Forelimb micromelia (3.8)	0.0	Sacral hygroma (2.7)	0.0
4.0	69	5.79	Kyphosis (7.9)	Hydrocephaly (5.3)	Underdeveloped (3.8)	Agnathia (3.4)	Hindlimb dysplasia (7.2)	Clubbed feet (3.8)	Hemorrhagic spots (2.6)	Degenerate (3.9)
6.0	71	5.63	Kyphosis (2.7)	Hydrocephaly (4.9)	Open eyelid (2.4)	Agnathia (5.7)	Hindlimb micromelia (2.4)	Webbed feet (7.0)	0.0	0.0

**Table 2.** Morphometric analysis of 18 days old mice fetuses recovered from mothers treated with different doses of clomiphene citrate on day 8 of gestation

Dose µg/g BW	Parameters	Fetal body weight (mg±SE)	Crown rump length (mm±SE)	Head circumference (mm <sup>2</sup> ±SE)	Fetal eye circumference (mm <sup>2</sup> ±SE)	Forelimb size (mm±SE)	Hindlimb size (mm±SE)	Tail length (mm±SE)
0.00		1,536.20±28.93 <sup>A</sup>	24.66±0.26 <sup>A</sup>	25.68±0.25 <sup>A</sup>	7.30±0.12 <sup>A</sup>	8.17±0.07 <sup>A</sup>	9.05±0.0 <sup>A</sup>	11.85±0.06 <sup>A</sup>
1.0		1,446.77±30.91 <sup>B</sup>	24.40±0.23 <sup>A</sup>	24.35±0.39 <sup>B</sup>	6.94±0.13 <sup>A</sup>	7.15±0.12 <sup>BC</sup>	8.56±0.12 <sup>B</sup>	11.93±0.11 <sup>A</sup>
2.0		1,270.62±17.44 <sup>C</sup>	22.96±0.23 <sup>B</sup>	23.04±0.19 <sup>C</sup>	6.01±0.04 <sup>B</sup>	7.47±0.12 <sup>B</sup>	8.27±0.05 <sup>B</sup>	11.15±0.25 <sup>BC</sup>
4.0		1,223.74±29.44 <sup>CD</sup>	21.33±0.56 <sup>C</sup>	22.20±0.38 <sup>D</sup>	5.78±0.14 <sup>B</sup>	6.93±0.20 <sup>C</sup>	8.26±0.27 <sup>B</sup>	11.22±0.28 <sup>B</sup>
6.0		1,153.74±16.46 <sup>D</sup>	20.14±0.14 <sup>D</sup>	21.60±0.16 <sup>D</sup>	5.40±0.16 <sup>C</sup>	6.28±0.17 <sup>D</sup>	7.41±0.16 <sup>C</sup>	10.60±0.16 <sup>C</sup>
ANOVA (Intergroup comparison)		***	***	***	***	***	***	***

Entries key: parameter size±standard error.

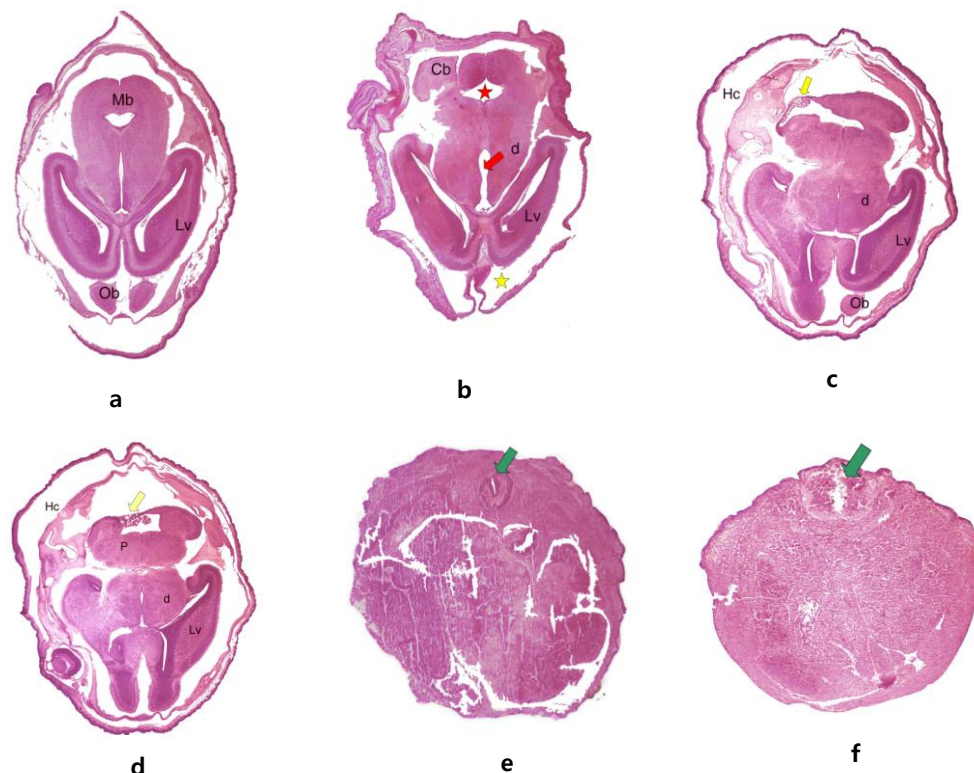
DMRT comparison of the different dose groups (intergroup); sharing the same alphabet show their means lie in the same range.

\*\*\* Significant difference (p<0.001).

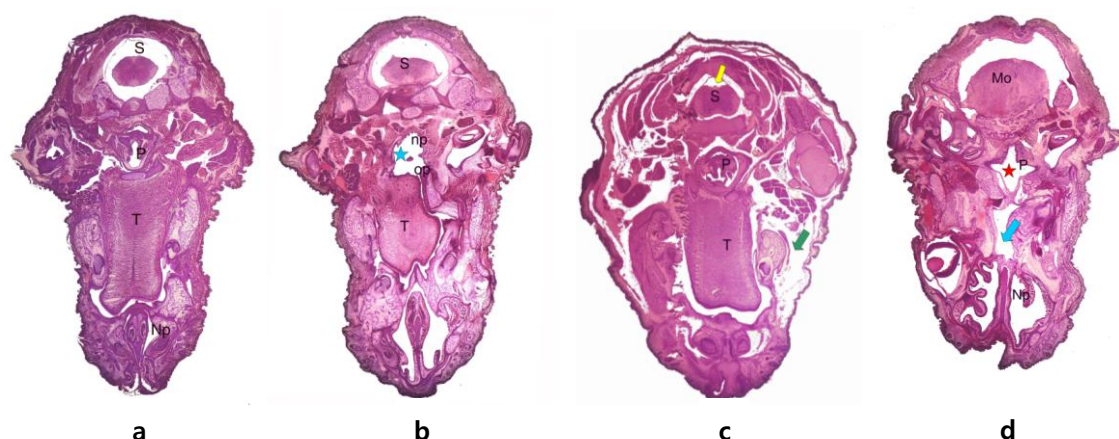
were carried out to determine the histological defects. The selected section from vehicle control showed well developed lateral ventricles, olfactory process, pones, cerebral hemispheres and diencephalons (Figure 2a). Sections from dose group, 1.0 µg/g BW showed enlarged third and fourth ventricles, lack of olfactory process underdeveloped cerebellum (Figure 2b) In dose group 2.0 µg/g BW showed abnormalities underdeveloped lateral part

of cerebellum, hydrocephaly and enlarged 4<sup>th</sup> ventricle (Figure 2c and 2d). Histological defects observed in dose group 4.0 and 6.0 µg/g BW included spina bifida (patent neural tube) and incomplete organogenesis (Figures 2e and 2f respectively).

Serial sections through the pharyngeal region in vehicle control showed well formed tongue, oropharynx and nasopharynx (Figure 3a). In dose group in 1.0 µg/g BW



**Figure 2.** Microphotographs of sections through eye and brain region of the mice fetuses recovered from mothers treated with different doses of clomiphene citrate on day 8 of gestation. a: 0.0; b: (1.0 µg/g BW); c and d: (2.0 µg/g BW); e: (4.0 µg/g BW); f: (6.0 µg/g BW). Mb: midbrain; Cb: cerebellum; d: diencephalon; P: pones; Np: nasal pouches; Lv: lateral ventricles; Ob: olfactory bulbs; Hc: hydrocephaly; yellow arrows: underdeveloped lateral part of cerebellum; red star: enlarged (herniated) fourth ventricle; Green arrows: open neural tubes; yellow star: absence of olfactory process; red arrow: enlargement of 3<sup>rd</sup> ventricle.



**Figure 3.** Microphotographs of sections through Pharyngeal region of the mice fetuses recovered from mothers treated with different doses of clomiphene citrate on day 8 of gestation. a: 0.0; b: (1.0 µg/g BW); c: (2.0 µg/g BW); d: (6.0 µg/g BW). T: tongue; P: pharynx; S: spinal cord; Np: nasal pouches; np: nasopharynx; op: oropharynx; green arrow: Atrophy of jaw muscles; blue star: degenerated septum between the oropharynx and nasopharynx; blue arrow: cleft palate; red star: poorly formed pharynx; yellow arrow: hollowed piamater.

undifferentiated pharynx with degeneration of some jaw muscles were noted (Figure 3b). Atrophy of septum between oropharynx and nasopharynx were observed in dose group 2.0 µg/g BW (Figure 3c). Dose groups 4.0 µg/g BW and 6.0 µg/g BW showed histological defects including cleft palate, poorly formed pharynx and under developed nasal pouches (Figure 3d).

## DISCUSSION

Clomiphene citrate has been the principal drug used for induction of ovulation in women with polycystic ovarian syndrome (PCOS). The drug is associated with adverse effects such as low pregnancy rates attributed to long-lasting estrogen receptor depletion, spontaneous pregnancy loss, multiple pregnancy rates and congenital anomalies (Casper, 2007). It has long half life and its metabolites have been found in faeces up to 6 weeks after administration (Adashi, 1993).

Unfortunately there is limited information available on this drug and only few studies and some case reports are available about its teratogenicity. The data available on this drug are controversial and not conclusive. According to some researchers it is teratogenic (Elizur and Tulandi, 2008) while others take it as a safe drug (Asch and Greenblatt, 1976). Keeping in view these situations it is unavoidable to work on this drug to find its safety and teratogenicity.

Observations made during the present study indicate clomiphene citrate induced embryotoxicity and teratogenicity in mice. The litter size was decreased and embryonic resorptions were increased by increasing the dose concentration and exposure time (Table 4.1). These findings are in accordance with the studies by Motta and Hutchinson, (1991). They gave a dose of 2 mg/kg BW of

clomiphene citrate to guinea pigs on days 5, 9 and 20 of pregnancy. Seventy five percent females showed either pregnancy with embryos undergoing resorptions or no sign of pregnancy.

Current study showed the decrease in fetal body weight and CR length by increasing the dose concentration. These results are in conformity with the studies of Dziadek (2005). He observed that preovulatory administration of clomiphene citrate caused dose dependent decrease of implantations and also caused growth retardation of surviving fetuses.

The anomalies including microcephaly, hydrocephaly and anencephaly were noted in different dose groups. In 1980, Schardein reported the association of CNS malformation and clomiphene citrate. Eye abnormalities observed during the present study ranges from open eyelids to microphthalmia and anophthalmia. These observations are supported by the findings of Aker and Bulay (1985) that some eye abnormalities, like cataract can be induced in neonates by clomiphene.

Furthermore, limb abnormalities found during the current study included micromelia, meromelia, Phocomelia, limb dysplasia as well as clubbed feet. Alatas et al. (1995) has reported a case of phocomelia after treatment with clomiphene citrate. Treated mother gave birth to male infant with abnormal limbs. The arms were defective the distal parts of upper extremities were missing and the fingers and hands were deformed.

Along with a number of fetal morphological defects found during the present study histopathological anomalies attributable to developmental exposure of clomiphene citrate included meningomyelocele, hydrocephaly, microcephaly, underdeveloped cerebellum, dilated lateral ventricles, defective eye lens, corneal and retinal defects. A well pronounced defect noted was spina bifida. Banhidy

and Czeizel (2008) found the possible association of clomiphene treatment and risk of neural tube abnormalities. They treated mothers with clomiphene during the first and second month of pregnancy and they found association with clomiphene citrate only for neural tube defects. In another study Elizur and Tulandi (2008) also claimed that repeated clomiphene administration was associated with slightly higher risk of hypospadias and neural tube defects. Sections through nasopharyngeal region showed abnormalities like, fused oropharynx and nasopharynx, cleft palate and muscles atrophy. Which are supported by some earlier studies on rats and rabbits where clomiphene citrate was associated with gastroschisis, stunted limb, cleft palate and hydrocephaly (Bernustein, 1970; Morris, 1970).

On the basis of these findings it is concluded that oral administration of clomiphene citrate is teratogenic in mice within the dose limits prescribed to patients by the doctors particularly if such exposure occurs during organogenesis. This study will provide awareness about this drug particularly from the stand point of view for its teratogenic and embryotoxic effects. A well planned and controlled epidemiological study is needed to find the chain of abnormalities; because this drug is continuously being used by anovulatory women and also during a rapidly expanding technique IVF.

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