

# Developmental changes in fetal male gonads associated with administration of oral contraceptive pills to their mothers during pregnancy.

Basima M. Al-Juboori<sup>1</sup>, Saad M. Al-Nida<sup>2</sup>, Ban T Saeed<sup>1</sup>

<sup>1</sup>Institute of Embryo Research and Infertility Treatment, Al-Nahrain University

<sup>2</sup>Center for Biotechnology, Al-Nahrain University.

## Abstract:

### Background

The effects of taking oral contraceptive pills that contain steroid hormones (estrogen and progesterone) during pregnancy on some aspects of fetus and newborn have been previously reviewed.

### Objectives

The objectives of this study are to detect the effect of using different doses of progestin and estrogen (contraceptive pills OCPs) by pregnant female mice on the gonadal development of their male fetuses, and on their pregnancy outcome.

### Materials and Methods:

Ninety female mice were divided equally into three major groups: two experimental (G1 and G2) and control group according to the level of the dose. Administration of OCPs (either 0.034 or 0.068 mg/kg b. wt. /d dissolved with 0.1ml distilled water) orally once a day, were started at the first day of gestation and continued till day 14 for the experimental groups G1 and G2 respectively. When the female in each experimental and control groups reach day 17 of gestation, 15 animals of each group were sacrificed, whereas the other 15 animals were left to be sacrificed at birth time. Weight and numbers of fetuses were recorded, in addition, histological study for their testes were done.

### Results

The statistical analysis showed a highly significant ( $P < 0.01$ ) decrease in weights and numbers of fetuses (males and females), and highly significant decrease ( $P < 0.01$ ) in the testes diameter of fetuses belonged to mothers treated with normal and double dose of OCPs at day 17 and at birth time. In addition, fetuses belongs to G2 mothers revealed testicular undescending to its normal position and remained adjacent to the kidneys at the upper part of the abdominal cavity in comparison with control group.

### Conclusion

Administration of steroid hormones orally to pregnant female mice for 14 days causes a highly significant decrease in the weight and number of fetuses at day 17 and at birth time and cause negative effect on the diameter of the fetal testes. The OCPs can inhibit at its high dose, the descending of the testes to its normal location at the pelvic cavity and then to the inguinal canal.

**Key words:** contraceptive pills, pregnancy, fetal testes

## Introduction

Oral contraceptive pills (OCPs) are combined formulations of a progestin and a synthetic estrogen (1). These pills have been widely used all over the world. Recent data indicate that OCPs are annually used by approximately 100 million women worldwide. They are highly effective in preventing pregnancy (2).

In all over the world, it is estimated that between 2 and 5% of

55 to 60 million women who take OCPs continue use during undetected early pregnancy (3), resulting in unintended in utero exposure to reproductive steroidal hormones in the offspring (4). Some studies highlighted the association between estrogen action that widely used in 1970s, and the development of rare types of vaginal tumors in adolescents whose mothers had been exposed to it during the



respective pregnancies and more frequently with vaginal adenosis and cervical pseudopolyps (5). However, prolonged use of OCPs may cause the fetus to develop abnormally, causing problems with the heart, esophagus, kidney, anus and limbs (6). In utero exposure to hormonally- active chemicals increase the incidence of male reproductive tract abnormalities, like cryptorchidism (failure of the testes to descend into the scrotum) and hypospadias (urethral opening along the shaft of the penis) (7), therefore, it is considered as likely markers of endocrine disturbance (8). Therefore, the objectives of this study are detection the effect of using different doses of progesterin and estradiol by pregnant female mice on the gonadal development of the male fetuses, and on pregnancy

## Materials and methods

Ninety female mice (8-10 weeks age) were divided equally into three major groups: two experimental (G1, G2) and control group according to the level of the dose. Each major group was subdivided into two minor groups (15 animals/group) according to different periods for sacrificing during gestation period (day17, and birth time). Vaginal smears were performed to diagnose the stages of estrus cycle, to detect heat stage for mating. Females in the metestrus phase were left with mature healthy males for mating (1male/2female). The occurrence of vaginal plug was considered as the first day of pregnancy (9); the pregnant female was removed into separate cages.

Administration of OCPs (either 0.034 or 0.068 mg/kg b. wt. /d) were dissolved with 0.1ml distilled water, orally once daily, were started at the first day of gestation and continued till day 14 for the experimental groups G1 and G2 respectively, while the parallel control group was orally administered with distilled water using the same route and dose in the experimental groups. When the female in each experimental and control groups reach (day 17) of gestation, 15 animals of each group were scarified whereas, the other 15 animals sacrificed at birth time. After sacrifice the fetuses were placed in a dish containing warm normal saline 37C for washing. Number of died and live fetuses in each horn were recorded. Each fetus were washed and weighted, then fixed in Bouin's solution for 24 hours, then stored in 70% ethanol alcohol for histological techniques, paraffin sections with 5 microns thickness were prepared and stained with Harris hematoxylin-eosin stain for histological study (10). After that the fetus's gonads were examined under light microscope for detecting the abnormal morphological differences of male gonads.

To calibrate the fetal gonadal diameter, the morphometric analysis was applied by using micro image analyzer system (BEL ver. 2.0). The mean  $\pm$ SD for the different diameters of testes in pixel unit were recorded. The special calibration was done to provide the real world dimensional measurement by using the conversion factor (0.6649) for converting the pixel into micrometer ( $\mu$ m) measurements.

Data from treated and control groups are expressed as mean  $\pm$  standard error (SEM) and analyzed using student t-test to compare values from experimental and control groups at individual time points. Differences between groups were considered significant at  $P < 0.05$  and highly significant at  $P < 0.01$  (11).

## Results

Daily administrations of either normal (0.034 mg/kg b. wt.) or double dose (0.068 mg/kg b. wt.) of OCPs to pregnant female mice until day 14 of gestation cause some changes including:

### A-Weights changes:

The statistical analysis showed a highly significant ( $P < 0.01$ ) decrease in weights of fetuses (males and females) belonged to mothers treated with normal dose of OCPs at day 17 (table 1) and at birth time in comparison with control group. Similar results were noted for fetuses belonged to mothers treated with double dose of OCPs (figure 1). Differences were recorded between the body weight of males and females fetuses at right and left horns in normal dose and double dose administered mothers. These results showed that female's weights were higher than the males. These differences lacked significance. (Table 1)(Figure 1).

### B -Numbers of fetuses:

Results did revealed a highly significant ( $P < 0.01$ ) decrease in the numbers of fetuses belongs to G1 and G2 mothers at day 17 and at birth time in comparison with normal dose and control groups (table 2 and table 3). At early period of gestation, spontaneous stillbirth was recorded with normal dose of COCPs (5.5%) however, higher percentage of stillbirth appeared with the double dose of OCPs (15.58%) in comparison with control group (table 4).

### C-Histological Observations including:

#### 1-Testes diameter:

At day 17 the results noted no significant decrease in testes diameter of fetuses belonged to G1 mothers, while there was highly significant reduction ( $P < 0.01$ ) in testes diameter of fetuses belongs to G2 mothers in comparison with control (table 5). At birth time the results showed a highly significant decrease ( $P < 0.01$ ) in the testes diameter of fetuses belonged to mothers of (G1 or G2) as compared with control group (table 5).

#### 2-Testicular descending:

The histological section of male mice belonged to G1 mothers showed a normal testicular descending to the pelvic cavity at the level of the bladder (Figure 2), fetuses belong to G2 mothers revealed testicular undescending to its normal position at day 17 (Figure 3), it remained adjacent to the kidneys at the upper part of the abdominal cavity. At birth the testes location were adjacent to the kidneys and higher than its normal position in the pelvic region as seen in the control group with greatly reduced size.

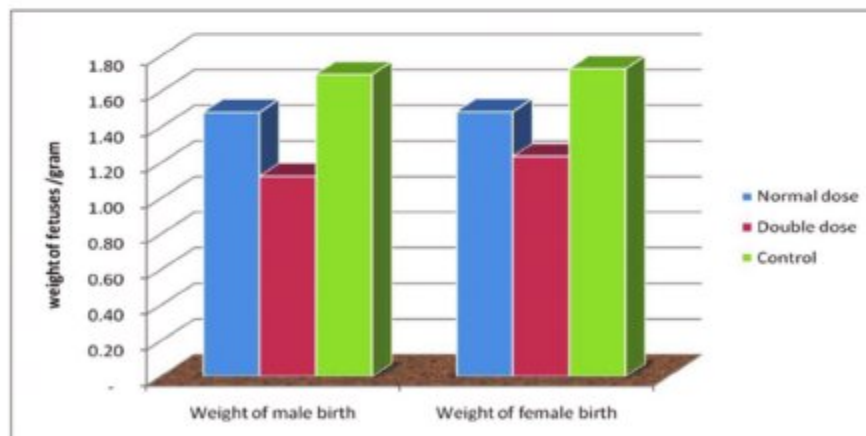
**Table (1):** Changes in fetal body weight (male and female) in right and left horns of pregnant mice administered with 0.034 mg/kg b wt. as (G1) and 0.068 mg/kg B wt. as (G2) of OCPs at day 17 of gestation as compared with control group (Mean± S.E.).

Day 17	Doses of steroidal hormones (Mg/kg b. w.)	Weight of fetuses(g) Mean± S.E.	P value			
			Among groups	Normal vs. control	Double Vs. control	Normal vs. double
Weigh of male fetuses in left horn	Normal dose	0.584±0.03	≤0.001**	≤0.001**	≤0.001**	0.029*
	Double dose	0.471±0.038				
	Control	0.784±0.009				
Weight of female fetuses in left horn	Normal dose	0.602±0.027	≤0.001**	≤0.001**	≤0.001**	0.010*
	Double dose	0.493±0.029				
	Control	0.828±0.014				
Weight of male fetuses in right horn	Normal dose	0.503±0.025	≤0.001**	≤0.001**	≤0.001**	0.041*
	Double dose	0.421±0.029				
	Control	0.869±0.024				
Weight of female fetuses in right horn	Normal dose	0.505±0.025	≤0.001**	≤0.001**	≤0.001**	0.760 <sup>NS</sup>
	Double dose	0.493±0.032				
	Control	0.822±0.018				

NS= no statistical significant difference.

\*=Statistically significant difference (P<0.05).

\*\*=Highly statistically significant difference (P<0.01).



**Figure (1):** Changes in fetuses weight at birth that born from female mice administered with 0.034 mg/kg B wt. as (G1) and 0.068 mg/kg B wt. as (G2) of OCPs as compared with control group (Mean± S.E.).



**Table (2):** Changes in number of male and female fetuses in left and right horns of uterus associated with the administration of 0.034 mg/kg B wt. as (G1) and 0.068 mg/kg B wt. as (G2) of OCPs to pregnant mice at day 17 of gestation.

Horn	Normal dose				Double dose				Control			
	No. of males		No. of Females		No. of males		No. of Females		No. of males		No. of Females	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Left	19	33.93%	19	35.85%	12	27.27%	10	30.30%	35	51.47%	42	56.00%
Right	37	66.07%	34	64.15%	32	72.73%	23	69.70%	33	48.53%	33	44.00%
Total	56	100.00%	53	100.00%	44	100.00%	33	100.00%	68	100.00%	75	100.00%
Total male & Female fetuses	109				77				143			
P value	0.833				0.771				0.007			

**Table (3):** Changes in number of male and female fetuses belongs to pregnant mice administered with 0.034 mg/kg B wt. as (G1) and 0.068 mg/kg B wt. as (G2) of OCPs at birth time.

Trait group	Number of male at birth		Number of female at birth		Total	
	No.	Percentage	No.	Percentage	No.	Percentage
Normal dose	38	40.43%	56	59.57%	94	100.00%
Double dose	28	39.44%	43	60.56%	71	100.00%
Control	57	46.34%	66	53.66%	123	100.00%
p value	0.005		0.089		0.001	

**Table (4):** Effect of daily administration of 0.034, 0.068 mg/kg b. wt of OCPs to pregnant female mice (G1, G2) at day 17 of gestation on pregnancy outcome as compared with control group.

Trait	Number of stillbirth		Total number at birth	
	Count	Percentage	Count	Percentage
Normal dose	6	5.50%	109	100.00%
Double dose	12	15.58%	77	100.00%
control	3	2.09%	143	100.00%
P value	0.050		0.005	

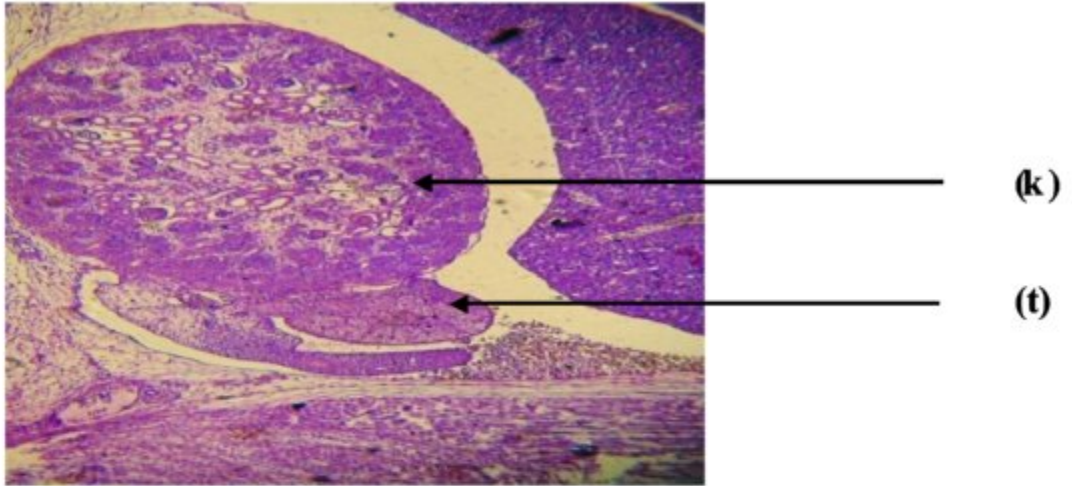
**Table (5):** Changes in testicular diameter ( $\mu\text{m}$ ) of male mice fetuses belong to female mice administered with 0.034mg/kg B wt. as (G1) and 0.068 mg/kg B wt. as (G2) of OCPs at day 17 and at birth time as compared with control group (Mean $\pm$ S.E.).

	Groups	Testes diameter ( $\mu\text{m}$ )	P value			
			Among groups	Normal vs. control	Double vs. control	Normal vs. double
Day 17	Normal dose	83.524 $\pm$ 0.193	0.305 <sup>NS</sup>	0.215 <sup>NS</sup>	$\leq$ 0.001 <sup>**</sup>	$\leq$ 0.01 <sup>*</sup>
	Double dose	78.079 $\pm$ 7.752				
	Control	88.081 $\pm$ 0.662				
Birth	Normal dose	126.571 $\pm$ 0.466	$\leq$ 0.001 <sup>**</sup>	$\leq$ 0.001 <sup>**</sup>	$\leq$ 0.001 <sup>**</sup>	$\leq$ 0.001 <sup>**</sup>
	Double dose	120.567 $\pm$ 0.599				
	Control	142.143 $\pm$ 0.506				

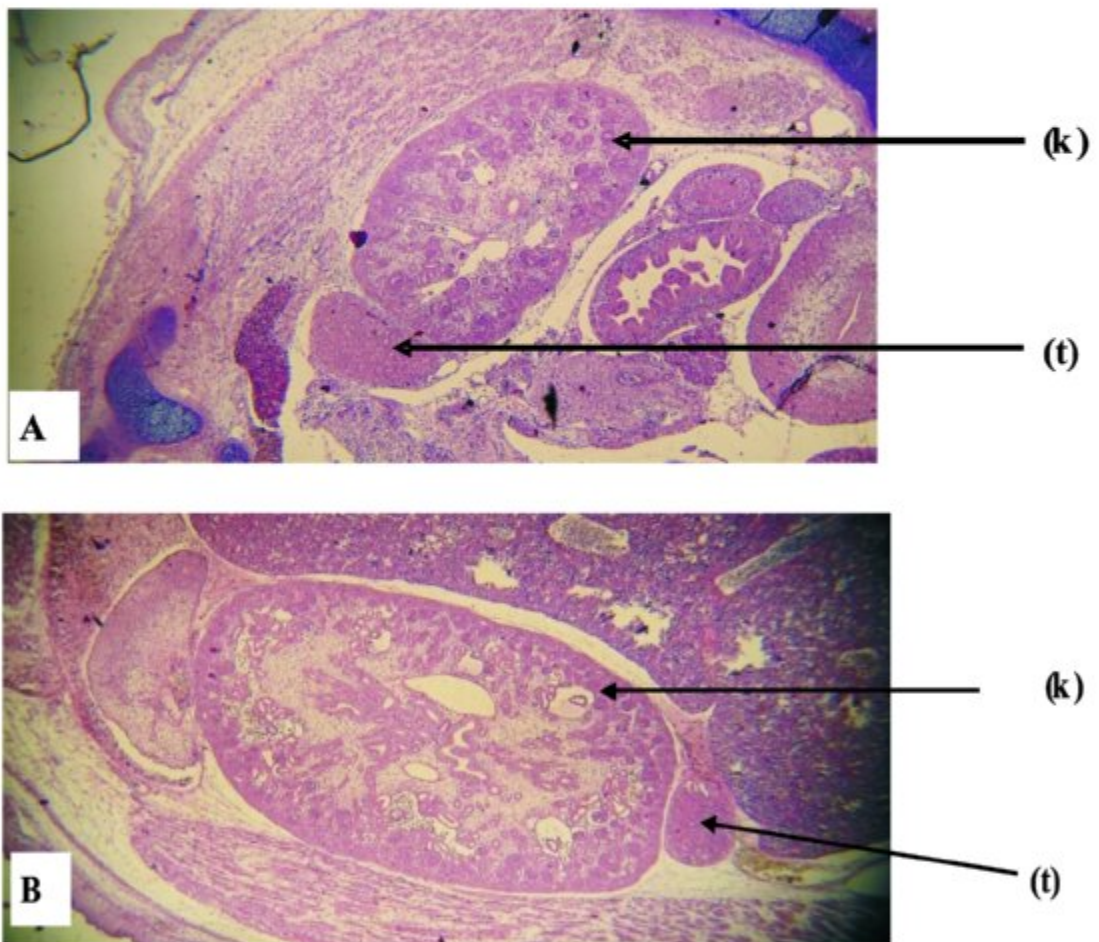
NS= no statistical significant difference.

\*=Statistically significant difference (P&lt;0.05).

\*\*=Highly statistically significant difference (P&lt;0.01).



**Figure (2):** Longitudinal section of mice fetus at day 17 belong to mothers treated with 0.034 mg/kg B wt. of COCPs as normal dose (G1), showing the persistence of the testes(t) adjacent to the kidneys (k) (metanephron) in the abdominal cavity (10X).



**Figure (3):** Longitudinal section of mouse fetus, A:at day 17,B: at birth time belongs to mothers treated with 0.068 mg/kg B wt. COCPs (G2), showing the persistence of the testes (t) adjacent to the kidneys (k)(metanephron)in the abdominal cavity(10X).



## Discussion

In the present study, the daily administration of contraceptive pills which contain estrogen and progesterone as normal dose (0.034 mg/kg b. wt.) or double dose (0.068 mg/kg b. wt.) orally to pregnant female mice showed a significant reduction in fetal weight, these result agreed with that found by Evans et al., (1980) (12) who proved that infants born to mothers with prenatal exposure to steroids had an increased risk of low birth weight. The mechanism by which the fetal body weight reduced may be due to decreased fetal growth because progesterone has cytoplasmic and membrane receptors in a variety of reproductive and non reproductive tissues including the brain and is a potent inhibitor of GnRH for that exogenous progesterone may disturb the normal hormone work and interfere with normal genital development in fetal reproductive tract (13).

In experimental groups G1 and G2, there were a highly significant decrease in fetuses number at day 17 of gestation and at birth time, these results may be related mainly to the high percentage of failure of implantation compared to that of control group, this result may be explained on the bases that OCPs prevent implantation of an embryo into the mother's endometrium, which is the inner lining of the uterus by making the endometrium thinner, less vascular and less glandular. Even so, the progesterone make it harder for the fertilized egg to become attached to the walls of the uterus, making it difficult to become pregnant (14), and exposure to estrogen during pregnancy decreased the number of the pups at the highest estrogen doses, the dose-dependent decrease in number of implantation scars suggests that estrogen treatment induced early post implantation pregnancy loss, because dosing began after fertilization (15), and early implantation does not result in implantation scars, thus decreases in early implantation cause reduction the number of new pups (16). Moreover, the results of this study showed some differences in the numbers of female fetuses which was higher in comparison with the numbers of male fetuses in (G1) and (G2), these results are due to that sex hormones strongly influence the anatomical and functional organization of the central nervous system (CNS), sex organs, and skeletal and muscular structure, thus playing a key role in sexual differentiation (17). In particular, the presence of estradiol in the prenatal period, from conception to puberty, determines the female organization of the CNS, whereas its absence results in male organization (18). The female is protected from the masculinizing action of estrogens by Alfa -fetoproteins, which reduce the amount of active estrogen (19). In litter mammals, even small physiological changes of the hormonal milieu during pregnancy can affect adult morphological reproductive parameters of the offspring (20). This was reflected by the significant decrease in the diameter of testes of (G1) and (G2) at 17

and 20 dpc since the COCPs contain estrogen, which cause significant reductions in the testis weight (21).

Sharpe and Skakkebaek (22) had developed an "estrogen hypothesis" in which they argued that estrogen-like molecules could impair adult male fertility by acting during gonadal development and that inappropriate exposure to estrogens during fetal or neonatal life could affect adult reproductive functions. Our results also support the "estrogen hypothesis".

The relatively estrogenic potencies of suspected endocrine disrupters suggest that estrogenic activity of these hormones can produce adverse effects during fetal development and endocrine disrupters have been shown to interact not only with the estrogen receptor (ER) but also with the androgen receptor (AR) or to interfere with steroid hormone synthesis or metabolism (23) and estrogen can induce abnormal male reproductive functions in the presence of large concentrations of maternal circulating estrogen, estradiol, estrone, and estriol. One explanation for this observation could be that the fetal estrogen level is very low in spite of high maternal levels of estrogen (24). During the gestational phase, estrogen target cells respond to estrogen in a manner that leads to an abnormal or cancerous response from subsequent exposure to estrogen when the animals reach sexual maturity. Since differentiation of the reproductive tracts starts about days 12 to 13 in these species, the ER is no longer distributed randomly, which appears to be the case in the very early embryo at the blastocyst stage (gestation days 4 to 5) (25). Thus, even a small change in the estrogen level during this fetal period may have a significant effect, and our findings support this possibility.

Undescending of the testes to its normal position was observed in G1 at day 17 and at birth time, it remained adjacent to the kidneys at the upper part of the abdominal cavity while it was normally relocating at the base of the abdominal cavity as it appeared in the control group at 17 dpc and descent into inguinal canal at 20 dpc (26). Transfer of the male gonad from its site of origin at the urogenital ridge, opposed to the kidney, into the scrotum is critical event in male sexual differentiation (27). Sexual differentiation is a complicated, hormone-dependent sequence of molecular and cellular processes that must occur with precision and coordination to develop the male reproductive system and associated secondary sexual characteristics. Sexual differentiation, organogenesis and development of the endocrine system are sensitive to exogenous hormones during prenatal development (27). This study show that experimental animals that exposed to steroid hormones can disrupt interdependent developmental processes and cause persistent functional and structural changes, This finding is in a good agreement with Beral and Colwell (1981) (28), they reported that some reproductive tract toxicants specifically, interfere with



normal endocrine function and cause endocrine disruption, the theory of endocrine disruption posits that low-dose exposure to exogenous steroid hormones that interact with hormone receptors can interfere with reproduction, development, and other hormonally mediated processes (29). The administration of estrogen during fetal and neonatal development has been reported to be associated with a series of male reproductive disturbances, such as cryptorchidism (30). Estrogen receptors (ERs) and aromatase are found at all stages of testicular development in the rodents (31), Leydig cells within the rodent fetal testes contain ER alpha until birth, several studies have suggested that estrogen exposure may interfere with fetal hypothalamo-pituitary-testes axis, leading to an inhibition of fetal Leydig cells androgen production, thus interfering with testicular descent (32), however, it is well known that in utero exposure of male fetuses to high levels of estrogen can interfere with testicular, furthermore estrogen deprivation during fetal development is also associated with this process (33).

## References

1. Skegg DCG. Third generation oral contraceptives. *J Br. Med.* 2000;7255:190-191.
2. Darroch JE, Haas T and Ranjit N. Contraceptive failure rates: new estimates from the 1995 National Survey of Family Growth 1999; 31: Pp56-63.
3. Smithells RW. Oral contraceptives and birth defects. *Dev. Med. Child. Neurol.* 1981; 23: Pp369-372.
4. Pickrell JO. Oral contraception linked to prostate deformities. *Health-New Scientist* 2005; 33:13-44.
5. Ness RB, Grisso JA and Klapper J. Risk of ovarian cancer in relation to estrogen and progestin dose and use characteristics of oral contraceptives. *Am. J. Epidemiol* 2000; 152: Pp233-241.
6. Wilson JG and Brent RL. Are female sex hormones teratogenic? *Am. J. Obstet. Gynecol.* 1981; 141: Pp56-60.
7. Robaire B and Hales BF. *Advances in Male Meditated Development Toxicity.* Kluwer Academic, Plenum Press, New York 2003; 13: Pp441-445.
8. Skakkebaek A. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum. Reprod.* 2001; 5: 972-978.
9. Roberts RV. The mouse: its reproduction and development. *Anim. Sci.* 1968: 54-60.
10. Bancroft JD and Steven A. *Theory and practice of histological techniques.* Churchill, Livingston, London. 1982: Pp 110-111.
11. Daniel WW. Multiple regression and correlation, In: *Biostatistics, A foundation for analysis in the health science* 1988; 18: Pp 13-22.
12. Evans AN, Brooke OG and West RJ. The investigation by pregnant women of substances toxic to the fetus. *Practitioner* 1980; 224: Pp315-319.
13. Mari S, Golub D, Farla L, Kaufman D, Marissa A, Campbell A and Ling-Hong L. Natural progesterone: information on fetal effects. *Birth defects research part B: Rep. Toxicol.* 2006; 455-470.
14. Nagel SC. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity," *Proceedings of the Society for Experimental Biology in Medicine* 1998; Pp: 217, 300-309.
15. Garside DA, Chariton A and Heath KJ. Establishing the timing of implantation in the Harlan Porcellus Dutch and New Zealand White rabbit and the Han Wistar rat. *Regul. Toxicol. Pharmacol* 1996; Pp: 23, 69-73.
16. National Toxicology Program. Multigenerational reproductive toxicology study of ethinyl estradiol in sprague-dawley rats (feed studies). Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences, National Toxicology Program, Research Triangle Park, NC 2007; 57-63.
17. Morris JA, Jordan CL and Breedlove SM. Sexual differentiation of the vertebrate nervous system. *Nat Neurosci* 2004; 7: 1034-1039.
18. McEwen BS and Alves SE. Estrogen actions in the central nervous system. *Endocrinol Rev* 1999; 20: 279-307.
19. Bakker J, De Mees C, Douhard Q, Balthazart J, Gabant P, Szpirer J and Szpirer C. Fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. *Nat. Neuro.Sci* 2006; 9: 220-226.
20. Vom Saal FS. Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. *J Anim Sci* 1989. 67: 1824-1840.
21. Mathews E. Estrogen during pregnancy. *Neurotoxicol. Teratol* 2009; 26: 591-661.
22. Sharpe RM and Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 1993; Pp: 341: 1392-1395.
23. LeBlanc GA, Bain LJ and Wilson VS. Pesticides: multiple mechanisms of demasculinization. *Mol. Cell. Endocrinol.* 1997; 126: 1-5.
24. Gibori G and Sridaran R. Sites of androgen and estradiol production in the second half of pregnancy in the rat. *Biol Reprod* 1981; 24: 249-256.
25. Bern A. Diethylstilbestrol (DES) syndrome: present studies of animal and human. In: *Hormonal Carcinogenesis.* New York: Springer-Verlag 1992; 1-8.
26. Metzler M. Metabolic activation of xenobiotic stilbene estrogens. *Fed. Proc.* 1987; 46: 1855-1857.
27. Bernstein L, Pike MC, Depue RH, Ross RK, Moore JW and Henderson BE. Maternal hormone levels in early gestation of cryptorchid males: a case-control study. *Br. J. Cancer* 1988; 58: 379-381.
28. Beral V and Colwell L. Randomized trial of high doses of stilboestrol and ethisterone therapy in pregnancy: long-term follow-up of the children. *J. Epidemiol. Commun. Health.* 1981; 35: 155-60.
29. Nagel A. "Developmental effects of estrogenic chemicals are predicted by an in Vitro assay incorporating modification of cell uptake by serum," *Steroid Bioch. Mol. Bio.* 1999; 69: 343-357.
30. Carmichael SL, Shaw GM and Laurent C. Maternal progestin intake and risk of hypospadias. *Arch. Pediat. Adolesc.* 2005; 159: 957-962.
31. Storgaard L, Bonde LP and Olsen J. Male reproductive disorders in humans and prenatal indicators of estrogen exposure. A review of published epidemiological studies. *Reprod Toxicol* 2006; 21: 4-15.
32. Rommerts FF, de Jong FH, Brinkmann AO, Vander M and Molen HJ. Development and cellular localization of rat testicular aromatase activity. *J. Reprod. Fertil.* 1982; 65: 281-288.
33. Abney TO. The potential roles of estrogens in regulating Leydig cell development and function. *Steroids* 1999; 64: 610-617.