

REPRODUCTIVE EFFICACY IN FEMALE RAT EXPOSED TO BISPHENOL A DURING GESTATION PERIOD.

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ABSTRACT

The study was conduct to determine the effect of exposure to Bisphenol A (BPA) during gestation on serum reproductive hormones levels (Estradiol" E2", Luteinizing Hormone "LH" and follicle- stimulating Hormone" FSH"), age and weight at vaginal opening onset as well as reproductive efficacy in F1 female offspring.

Thirty six pregnant female rats (F0) were gavage three doses of BPA suspended in corn oil (50 µg, 50 mg, 250 mg/kg/BW) or corn oil only as control group from gestational day (GD) 6 till gestation day (GD) 21. After delivery, twelve female pups of each group were hold for three months; However, Female rats' offspring, after weaning, were weighed and checked for vaginal opening (VO) every day until completion, then at postnatal day (PND) 90, blood samples were collected from six F1 female of each group to perform hormonal tests and other six females were mated with untreated male rat in a separate cage for 14 days in order to evaluate fertility efficacy. The results showed significant increased ($P<0.05$) in serum E2 levels, and decrease in LH level, but FSH levels were unchanged in all treated groups compared with control group. The results also revealed significant decrease age at VO onset in all treated groups compared with control group , while body weight at age of VO onset was non-significantly differ between all groups.

Fertility rate, number of birth and implantation sites were reduced and resorption sites were elevated in F1 female rats that gestationally exposed to deferent levels of BPA in comparison with control group. From the present study it has been revealed that the BPA exposures during pregnancy adversely affect F1 female reproduction and caused early puberty onset.

INTRODUCTION

The increasing incidence of reproductive disorders observed over the past few decades has raised concern about the role of substances known as endocrine disrupters (EDs) that are capable of modulating or disrupting the function of the endocrine system(1 & 2). Numerous studies indicate that female reproduction efficiency has deteriorated over the past half century. A clear declining trend in conception rate has been registered. Bisphenol A (BPA) is an estrogenic chemical produced in large quantities for use primarily in the production of polycarbonate and epoxy resins. In 2004, the estimated production volume of BPA in the United States was approximately 2.3 billion pounds. Of the 1.9 billion pounds of BPA used in 2003, nearly three quarters was used to manufacture various consumer products including polycarbonate containers for storage of foods and beverages (3).

Exposure occurs because when BPA molecules are polymerized, they are linked by ester bonds that are subject to hydrolysis, which is accelerated as temperature increases and in response to contact with acidic or basic substances. The consequence is that as polycarbonate products are repeatedly washed, or polycarbonate plastic or metal cans are exposed to heat and/or acidic or basic conditions, significant leaching of BPA due to hydrolysis of the ester bond occurs (4 & 5).

BPA was first synthesized by A.P. Dianin in 1891 and was later investigated in the 1930s during the search for synthetic estrogens. At that time, it was tested for its estrogenic properties but abandoned for pharmaceutical use when diethylstilbestrol (DES) was determined to be much more potent .Thus, until recently; BPA was considered a weak environmental estrogen because of its relatively low affinity for estrogen receptors compared to estradiol (6 & 7). However, results from recent studies have revealed a variety of pathways through which BPA can stimulate cellular responses at very low concentrations, below the levels where BPA is expected to bind to estrogen receptors (8).

Curiously, while the association between exposure to environmental factors and the reproductive capacity of males has been deeply investigated and has raised also public interest (9), a similar attention has not been dedicated to females. Nevertheless, the ability of synthetic chemicals to alter reproductive function and health in females has been described for the first time more than forty years ago, when the effects of diethylstilbestrol (DES) on the daughters of women given treatment with DES were firstly reported (10).

Several experimental studies have reported that endocrine disruptors can affect at very low doses the endocrine system and the development of mammalian and nonmammalian

species (11 & 12). Moreover, exposure to these environmental chemicals has been proposed to contribute to several gynaecologic pathologies, especially when exposures occur during critical periods of development (13, 14, 15, 16 and 17). Nevertheless, it has been proposed in recent studies that hormonal perturbations during embryo-foetal or neonatal development may predispose individuals to numerous diseases and/or dysfunction later in life. These include increased incidence of tumours such as uterine adenocarcinomas (18) and breast cancers (19). Most of these reported effects of endocrine disruptors in mammals are caused through alteration of estrogen signalling, since it is crucial for proper ontogeny and function of all the female reproductive system (20). The current study aimed to investigate effects of BPA treatment during prenatal periods on reproductive performance of female albino rat to better establish the association between endocrine disruptors exposure and female reproductive health.

MATERIALS AND METHODS

Chemicals:

Bisphenol A (BPA, CAS 80-05-7, > 99% pure) was purchased from Sigma Aldrich Company(USA) via OMA company (Iraq). Tocopherol-stripped corn oil (ICN Biomedicals Inc., Aurora, OH) served as the vehicle and control substance. Appropriate amounts of BPA were mixed with corn oil to achieve the desired concentrations. Fresh solutions were prepared weekly for each concentration and stored in glass containers. Based on the body weight of pregnant rats dose was administered to each one.

Animals of the study:

The present study included 36 mature female and 12 mature male albino rats were obtained from the Laboratory Animal Unit, College of Medicine, Baghdad University, Iraq. They were 14 to 16 weeks old with an average body weight (200-250gm) for females and (250-300gm) for males. The animals are clinically healthy, kept under hygienic conditions, housed in metal cages to avoid bisphenol exposure from old polycarbonate cages. Tap water were provided via glass bottles, and feed were giving *ad libitum* throughout the experimental period. The animals were accommodating to the laboratory conditions for 30 days before beginning of experiment. The light system was 12/12 hrs light/dark cycle.

Experimental Design:

Females were examined daily using vaginal smear technique to ensure that they were in regular estrous cycle (21). Female proved to be in estrous phase were mated with mature male rat in a separate cage. After mating a vaginal smear was taken. The presence of sperms indicated zero day of gestation (22). The pregnant female albino rats (36 females) were separated from the stud then divided into four main groups:

Group1 : Nine pregnant female rats served as control group which received corn oil only as vehicle.

Group 2: Nine pregnant female albino rats administered BPA (50 µg/kg BW. /day) dissolved in corn oil via gavage as Tolerable Daily Intake dose (TDI) for human according to (23).

Group 3: Nine pregnant female albino rats administered BPA (50 mg/kg B.W /day) dissolved in corn oil via gavage as Lowest Observed Adverse Effect Level (LOAEL) according to (23).

Group 4: Nine pregnant female albino rats, orally administer BPA 250mg/kg BW. /day (1/20 LD50) dissolved in corn oil via gavage as high dose (24).

The pregnant females (dams) dosed BPA daily according to their groups from Day six of pregnancy, till GD 21. Then separated without further treatment till PND 90 however, twelve female rats' offspring, after weaning, were weighed and checked for (VO) every day until completion, then at postnatal day (PND) 90

At the end of experiment (PND) 90 six mature F1 female rats offspring of each group were weighted and then sacrificed, followed by collection of blood to perform hormonal investigation and other six female were mated with untreated male in separate cage for 14 days to evaluate reproductive efficacy .

Parameters of study:

1. Hormonal assay: Estimation of serum estradiol, luteinizing hormone (LH) and Follicles-stimulating hormone (FSH) levels by follow manufacture instructions of kit. All kits used for hormone assay were (Monobind Inc. lake forest CA 92630, USA).
2. Puberty in the female rat corresponds with vaginal opening (25). Female rats' offspring at PND 25 were weighed and checked for VO every other day until completion. The appearance of complete vaginal opening was recorded on the days they were observed.

1- Each one F1female of were mated with one F1 male rat of control group in a separate cage for 14 days. Vaginal smears were examined on the every following

morning for the presence of spermatozoa in the vaginal smears, this was considered indicative of pregnancy, and this day was counted as day 0 of pregnancy. Sperm-positive females were housed separately and killed after delivery; the two-horned uteri were removed and opened by cutting longitudinally to expose the bluish implantation sites visually inspected and to identify resorption sites. The number of implantation sites was defined as the result of the total number of bluish implantation sites plus the total number of resorptions sites (26).

Statistical Analysis

The results were expressed as mean \pm SD. The comparisons between groups were performed with analysis of variance (ANOVA) by using computerized SPSS program (Statistical Program for Social Sciences). $P < 0.05$ was considered to be least limit of significance. Least significant different test (LSD) was calculated to test difference between means (groups) for (ANOVA) (27)

RESULTS AND DISCUSSION

In F1 female rats, exposure to BPA during, *In Utero* stage in the present study lead to significant increase in serum E2 level in all three doses in compare with control group (table 1). During critical periods of embryonic and postnatal development, the hormonal milieu is crucial for the correct organization of neuroendocrine circuits that coordinate sex-specific physiology so, the altered expression levels of hormones at the hypothalamus and pituitary levels may be the cause and/or the consequence of the changes in gonadal steroidogenesis and sex hormone production (28). There are possible mechanistic effects of BPA on the local regulatory circuits of hypothalamus and pituitary (29) , BPA produces its effects by interfering with one or both of the primary forms of the estrogen receptor (ER; ESR1 or ESR2, formerly known as ER α and ER β) within the hypothalamic-pituitary-gonadal (HPG) axis (30).

Taken together, these finding suggest that the increase of serum E2 level in the present study may be resulted from interference of BPA with developmental mechanisms of local regulatory circuits of hypothalamus and pituitary which occur during gestational and neonatal period and seems to be a critical “exposure window” for BPA to affect reproductive neural circuits in hypothalami of both male and female rat pups. LH levels were significantly decreased in all treated groups of the present

study compared with control groups (table 1). The decrease of serum LH level in females could be resulted from BPA-induced reduction in Luteinizing hormone releasing hormone(LHRH) biosynthesis in the hypothalamus or/ and from direct effect of BPA on LH secretion from pituitary due to decrease stimulation of gonadotropes by GnRH as a result of impairing IP3/inositol system as mentioned by (28) Moreover, the decrease serum LH level by BPA may be due to the consequence of the increase in GnRH pulse frequency, leading to desensitization of the pituitary, as also suggested by others (31). Additionally, decrease of serum LH might cause by estrogenic action of BPA at the level of the HPG axis, mimicking the estrogenic suppression of LH secretion but did not affect FSH secretion, (FSH level in female was unaffected by BPA in the present study) while it inhibited LH secretion, suggesting that the effect of BPA on gonadotropes is specific to the mechanism that underlies the control of LH secretion. The reason why BPA has no effect on the FSH secretion is uncertain in the present study, but the control of FSH secretion differs from that of LH secretion (32). This selectivity of BPA on gonadotropin secretion may be due to, that the estrogen regulates LH mRNA more robust than that of FSH as mentioned by Furuta, *et al.* (33).

Table (1) The Effect of *In Utero* Exposure to BPA on Reproductive Serum Hormones Levels in F1 Mature Female Rats (Means \pm SE) .

Parameter Groups	Estradiol (pg/ml)	LH μ IU/ml	FSH μ IU/ml
Group 1 Control	B 51.55 \pm 0.37	A 3.390 \pm 0.174	A 4.754 \pm 0.264
Group 2 (50 μ g/kg B.W.)	AB 53.75 \pm 0.44	B 2.505 \pm 0.166	A 4.161 \pm 0.280
Group 3 (50mg/kg B.W.)	B 53.23 \pm 0.68	C 1.520 \pm 0.180	A 4.367 \pm 0.177
Group 4 (250mg/Kg.B.W.)	A 55.75 \pm 1.19	C 1.104 \pm 0.185	A 4.134 \pm 0.298
LSD	2.2013	0.5211	0.7651

N=6

Different letters represent a significant difference at ($p \leq 0.05$)

All doses of BPA that used the present experiment affected puberty onset of female rats, by advancing age at vaginal opening onset (VO). Advanced puberty was not associated with increased body weight, as shown in table (2) which represents effect of BPA exposure during, *In Utero* stage.

A similar advance in puberty onset has also been described previously by (13and 15) Puberty can be divided into two subdivisions, central and the peripheral puberty. Central puberty consists of the onset of GnRH and gonadotropin secretions as the HP axis matures, the gonads are stimulated, the HP axis stimulates gonadal secretion of sex steroid hormones, and the steroids send feedback to the HP axis. Peripheral puberty consists of processes other than the activation of the hypothalamic-pituitary-gonadal axis. In the female, it includes secondary sex characteristics such as the development of mammary glands, the vaginal opening, and uterine hypertrophy (34). Though, the onset of puberty is a genetically driven event (35), it can be changed by environmental factors (36).

Table (2) The Effect of *In Utero* Exposure to BPA on the age at Vaginal opening in F1 Mature Female and Male Rats(Means \pm SE)

Parameters Groups	Age at (VO) (Day)	Weight at (VO) (g)
Group 1 Control	A 44.833 \pm 1.137	A 135.352 \pm 5.35
Group 2 (50 μ g/kg BW)	B 40.333 \pm 0.557	A 139.711 \pm 11.32
Group 3 (50mg/kg BW)	B 39.833 \pm 0.703	A 144.439 \pm 16.33
Group 4 (250mg/kg BW)	B 40.333 \pm 1.145	A 141.873 \pm 9.28
LSD	3.667	11.217

N=6

Different letters represent a significant difference at ($p \leq 0.05$)

Puberty changes occur as a consequence of the activation of the hypothalamic-pituitary-gonadal axis .The HPG axis is under the control of both inhibitory and

stimulatory mechanisms (37). As discussed previously, BPA, when administered to immature rats, induced acceleration in GnRH pulsatility (28). Signaling pathways on GnRH neurons may be altered by developmental exposure to BPA and that steroid-negative (rather than positive) feedback may be impaired (30)

Precocious puberty in female rats could be due to precocious hypothalamic–pituitary maturation caused by BPA and inducing or may be due to decrease of LH during pre-pubertal stage. Although decrease of LH level in the present study was detecting during adulthood, but we suspect presence of this reduction earlier according to previous studies (38 and 39) they recorded BPA caused decrease in LH in pre pubertal stage.

After two weeks of mating period of female rats treated with 50 µg, 50 mg and 250 mg / kg B.W. BPA to study the reproductive efficiency. It seems that the BPA effect negatively on fertility percentage of treated females exposed to BPA during *In Utero* stage (table 3) showed reductions in fertility rates, number of implantation sites, number of birth, while the number of resorption sites. The present data suggest that exposure to BPA caused subfertility. Similarly, BPA exposure results in a reduction in pregnancy rate and litter size (40 and 41). In humans, exposure to BPA has been associated with recurrent miscarriage (42). *In utero* exposure appears to have an effect on the uterus that persists in the adult (16). Several *in vitro* and *in vivo* experimentations have highlighted the molecular mechanisms through which BPA are able to interfere with normal development of the reproductive tract. In fact, it has been demonstrated that BPA exposure alters Hox gene expression in the developing mullerian system (43, 44 and 26).

Table (3) The Effect of *In Utero* Exposure to BPA on fertility outcome of F1 male and female rat offspring.

Doses of BPA	Parameters		No. of females delivered	Fertility rate %	Total No. of Implantation sites (mean±SE)	No. of birth(mean±SE)	No. of Resorption Site (mean±SE)
	Groups						
Control	Group 1	CM×CF	6	6	100%	A	A
50µg/kg B.W.	Group 2	CM×TF	6	5	83.33%	B	B
50mg/Kg B.W.	Group3	CM×TF	6	3	50.00%	B	C
250mg/Kg B.W.	Group 4	CM×TF	6	2	33.33%	B	C

TF: female from BPA-treated groups. CM: Male from control group.
CF: Female from control group.

Hoxa10 is a gene that is expressed in the developing urogenital tract during embryogenesis and in the adult uterus during early pregnancy (45). Hox genes are essential mediators of the correct axial development of the primitive Mullerian duct in the fallopian tubes, uterus, cervix, and upper vagina. (46); early alteration in *Hoxa10* gene expression might affect functional differentiation of the uterus during pregnancy as part of an altered endocrine signal transduction pathway (47). The pathways may be explaining the influence of BPA on the reproductive disorders in the present study. a direct effect of BPA on gene expression induction of Hox genes because *in utero* BPA exposure brought about unmethylation of HOXA10 gene(43 and 48).

الكفاءة التكاثرية في إناث الجرذان المعرضة للبسفينول أ خلال فترة الحمل

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الخلاصة

اجريت الدراسة الحالية لدراسة مدى التأثير السمي نتيجة التعرض لمادة البسفينول أ اثناء فترة الحمل على خصوبة اناث الجرذان المولوده وذلك من خلال دراسة مستويات هرمونات التكاثر(الاستراديول،

الهرمون اللوتيني و الهرمون المحفز للجريب) وكذلك من خلال عمر الاناث عند البلوغ الجنسي , بالاضافة الى دراسة الكفاءة التناسلية عند عمر 90 يوم.

استخدمت في هذه الدراسة (36) انثى جرد حامل قسمت بالتساوي الى اربعة مجاميع, المجموعة الاولى اعطيت زيت الذره النقي فقط عن طريق الفم واعتبرت كمجموعة سيطره اما المجاميع الثلاث الاخرى جرعت عن طريق الفم مادة البسفينول – أ المعلقة بمحلول زيت الذره وبالجرع الاتية 50 مايكروغرم, 50 ملغرام و 250 ملغم لكل كغم من وزن الجسم ابتداء من اليوم السادس من الحمل وصولا الى اليوم 21 منه . بعد الولاده تم رعاية 12 من الاناث المولوده من كل مجموعة لغاية عمر 90 يوم . عموما تم متابعة ظهور الفتحة المهبليه بعد عمر 25 يوم وسجل يوم ظهور الفتحة المهبليه ووزن الجسم عند ظهور هذه الفتحة كمؤشر للوصول للبلوغ الجنسي وعند عمر 90 يوم تمت التضحية ب 6 حيوانات للحصول على الدم كل مجموعة وعزلت الاناث ال 6 المتبقية في اقصاف منفردة مع ذكور بالغة غير معرضة للبسفينول لمدة 14 يو لغرض الجماع و تقييم الكفاءة التناسليه للاناث.

وقد اظهرت النتائج وجود ارتفاع معنوي في مستوى هرمون الاستراديول وانخفاض معنوي في مستوى الهرمون اللوتيني في المجاميع المعامله مقارنة بمجموعة السيطره غير ان الهرمون المحفز للجريب لم يتأثر بالتعرض لمادة البسفينول. كما بينت نتائج الدراسة ايضا وجود انخفاض معنوي عمر الاناث عند حدوث الفتحة المهبليه من دون ان يتغير وزن الجسم معنويا عند حدوث الفتحة المهبليه.

كما بينت نتائج دراسة الكفاءة التناسلية وجود انخفاض في كل من معدلات الخصوبة وعدد المواليد وعدد مواقع الغرس في اناث المجاميع المعرض للبسفينول خلال المرحلة الجنينية مقارنة بالاناث غير المعرضة للبسفينول. وقد خلصت الدراسة الحالية الى مادة البسفينول – أ لها تأثير ضار على الكفاءة التناسلية لاناث الجيل الاول التي سبق لها التعرض لهذه المادة خلال مرحلة الحمل اضافة الى تسببه في حدوث الفتحة المهبليه بعمر مبكر كدليل للوصول المبكر للبلوغ الجنسي.

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