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EFFECT OF EXPOSURE TO DI-BUTYL PHTHALATE ON REPRODUCTIVE PHYSIOLOGY IN ADULT FEMALE MOUSE

Satendra Singh and Swarn Lata*

Department of Zoology, MMV Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India.

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Correspondence to Author:
Dr. Swarn Lata

Associate Professor,

Department of Zoology, MMV, Banaras Hindu University, Varanasi-22 10 05, UP, India.

E-mail: swarnlatabhu12@gmail.com

ABSTRACT: Di-butyl phthalate (DBP) is an environmental contaminant used in the production of plastics, cosmetics and medical devices. In the present study, the effects of exposure to DBP on reproduction of female mice were investigated. Adult female mice given DBP through gastric intubation at 0 (control), 250 (low dose) and 1000 (high dose) mg / Kg BW/ day for 10 days. After treatment, body weight and ovarian weight of DBP treated mice decreased significantly at high dose while uterine weight decreased significantly both low and high dose as compared to control. Gonadosomatic index (GSI) and uterosomatic index (USI) of these mice were found significantly lower at both doses of DBP. DBP treated mice showed altered estrous cyclicity at both DBP doses as compared to control. Serum estradiol level was found significantly lower but the progesterone level was normal in DBP treated mice as compared to control. In ovary total cholesterol level was almost same in all three groups but activity of 3-β hydroxysteroid dehydrogenase (3-β HSD) was found significantly lower at both DBP doses. In ovary and uterus the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) decreased significantly after treatment at both doses of DBP as compared to control. Histological observation revealed less number of healthy and growing follicles and decrement of lumen size in ovary and uterus respectively. In conclusion, our results substantiate that exposure of DBP compromises the reproduction of female mouse by hampering the growth and antioxidant capacity of reproductive organs.

INTRODUCTION: Phthalates are high-production-volume synthetic chemicals, used in plastic or as a matrix in cosmetic products. DBP is one of the phthalates which are used widely in the industry of plastic piping, various varnishes and lacquers, safety glass, nail polishes, paper coatings, dental materials, pharmaceuticals and plastic food wraps. DBP is a plasticizer which is used to increase flexibility and workability of plastic material.

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DBP is not covalently bound to the plastic and thus leach into the environment where it is a ubiquitous contaminant. Environmental sources of exposure include dust and water. However, the main sources of human exposure are food [1] and consumer products such as cosmetics [2]. Exposure during medical procedures is 100 times higher than those of normal exposure [3, 4]. As a result, human exposure to phthalates is widespread [5, 6].

Phthalates can induce malformations and impairment of reproductive function in experimental animal models and may have similar effects in humans [7, 8, 9]. Epidemiologic evidence suggests that women have a unique exposure profile to phthalates, which raises concern about the potential health hazards posed by such

exposures [10]. DBP is an endocrine disruptor and supposed to have antiandrogenic activity as shown by many studies. Therefore, most of the studies are based on its effects on male reproductive system in mammals [11, 12, 13, 14, 15, 16]. The studies regarding its effects on female reproduction are also reported but these effects are due to long-course or gestational exposure to DBP [17, 18]. There are only few studies regarding short-course or acute exposure to DBP. So present study was undertaken by considering that females are being exposed to DBP at different concentration through diet and cosmetics or when they are undergone certain medical treatment through different medical device and procedures.

MATERIALS AND METHODS:

Chemicals and Reagents:

DBP was obtained from Merck Chemicals Private Limited and diluted in olive oil. Estradiol, progesterone ELISA and cholesterol kits were purchased from Diametra Italy and span diagnostics Ltd, India respectively. Procedures followed in assay were as per manufacturer's instructions. All reagents were of analytical grade.

Animal and treatment:

All the experiments were performed in accordance with institutional practice and within the framework of revised Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA Act of 2007 of Govt. of India on animal welfare. All experimental protocols were approved by Institutional Animal Ethical Committee, Faculty of Science Banaras Hindu University, Varanasi, India (Ref. F.Sc. / IAEC/ 2014-15/ 0334). All surgical processes were performed under mild anaesthesia.

Parke's strain female mice of 3 Months old and 26±3 gm weight, used in experiments were housed under controlled temperature and light 12 L: 12 D with free access to mice feed and water *ad libitum*. Vaginal smears of each mouse were recorded daily and only mice showing at least 2 consecutive 4-day cycles were used in the experiments. Adult female mice of same age and body weight (BW) were divided into three groups: (i) a control group that received vehicle (Olive oil); (ii) a group treated with 250 mg/kg BW of DBP; (iii) a group treated with 1000 mg/kg BW of DBP. Treatment doses of DBP were selected on the basis LD50 of DBP for mice which is in a range of 5-16gm/ Kg BW [19]. We had selected lower range of LD50 i.e. 5 gm/ Kg BW. The low dose 250 mg / Kg BW was 1/ 20 and high dose 1000 mg /Kg BW was 1/4 of LD50. The rationale for taking these doses with very high difference was to monitor dose dependent changes in female reproductive physiology at low range of exposure in human female through diet and cosmetics or high range of exposure during medical treatment respectively.

Weight of mice was taken before and after treatment. There were 6 mice per treatment group. Mice were treated once a day for 10 days orally. Some of mice from all groups sacrificed to collect blood samples and reproductive organs (ovary and uterus). Fresh tissues were soaked on blotting sheet, weighed and organ to body weight ratios,

GSI and USI, were calculated by given formula:

$$\frac{\text{Organ weight}}{\text{Body Weight}} \times \mathbf{100}$$

Remaining mice of all groups were used for estrous cycle check for next 15 days.

Estrous cycle monitoring:

After completion of treatment mice were checked for cytology of vaginal smear daily in the morning between 8-10 am. After 15 days of observation, diestrus index was calculated by following formula:

$$\text{Diestrus Index} = \frac{\text{Number of days with clear diestrus smear}}{\text{Total duration of observation}} \times 100$$

Serum hormone assay:

To determine the circulating level of progesterone and estradiol, blood was collected by heart puncturing method. Serum was isolated and stored at-80 °C for further analysis. Serum levels of progesterone and estradiol were measured by using respective ELISA kit (Dia Metra, Italy).

Estimation of 3β-HSD activity and total Cholesterol Level in ovary:

Ovaries of animals were pooled and homogenised to prepare 10 % homogenate in PBS to access the 3β-HSD activity. Assay procedure followed was colorimetric method as described by T.

Shivandappa, 1998. Ovarian cholesterol level was checked by biochemical kit (cholesterol kit, Span diagnostic).

Estimation of antioxidant enzymes activity in reproductive organs:

In ovarian and uterine samples (10% homogenate) SOD, CAT and GPx activities were checked with commercially available diagnostic kits (Sigma-Aldrich, New Delhi, India)**.**

Histology of Ovary and Uterus:

Ovaries and uteruses of 5 animals of each group were taken and fixed in Bouin's fluid, embedded in paraffin, serially sectioned at 5 µm thicknesses and stained with hematoxylin and eosin. Serial sections were observed under light microscope to check number of healthy or atretic follicles, lumen size and proliferation of uterine glands in ovary and uterus respectively.

Statistical analysis:

The results were expressed as mean± standard error mean (SEM) for six animals in each group. Differences between the groups were assessed by one way analysis of variance (ANOVA) using the SPSS 16.0 software for Windows, followed by Dunnett's test to test for significance of data of different groups. A value of $p< 0.05$ was considered to be statistically significant.

RESULTS:

Body weight and reproductive organ weight:

A significant decrease ($p< 0.05$) in BW was recorded after treatment in mice at high dose of DBP only (**Fig. 1A, B**). Ovarian weight was also decreased significantly ($p< 0.001$) at high dose only (**Fig.2A**) while uterine weight was decreased significantly at both low ($p< 0.01$) and high (0.001) doses (**Fig.2B**). GSI was calculated significantly low ($p< 0.001$) at both doses (**Fig.2C**). USI was also estimated significantly low at both low ($p< 0.01$) and high ($p< 0.001$) doses (**Fig.2D**).

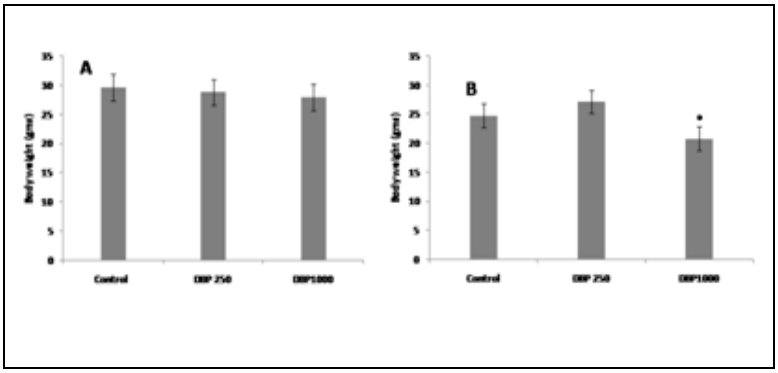

FIG.1: EFFECT OF DBP ON BODY WEIGHT: INITIAL BODY WEIGHT (A), FINAL BODY WEIGHT (B).
*p <0.05 Vs control, N= 6

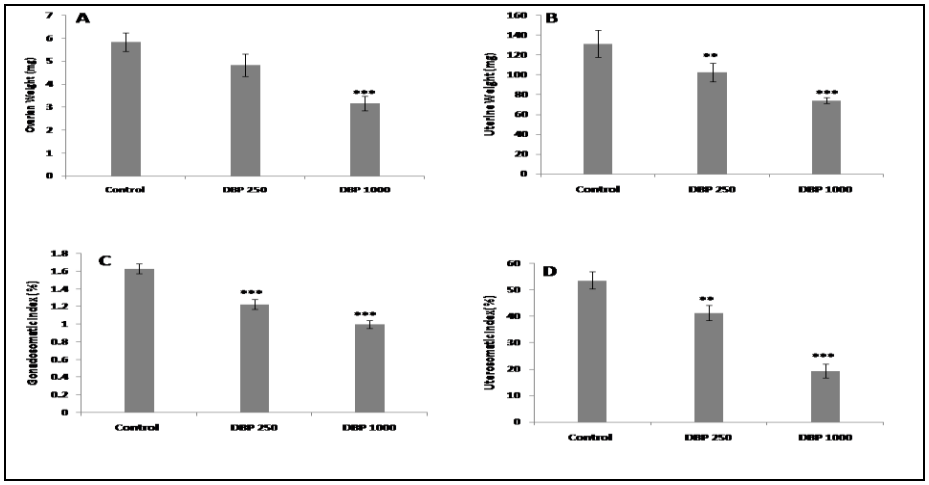

FIG.2: EFFECT OF DBP ON OVARIAN WEIGHT (A), UTERINE WEIGHT (B), GSI (C) AND UTERINE SOMATIC INDEX (D).
** p< 0.01,*** p< 0.001 Vs control ,N= 6

Serum hormone assay: Serum estradiol level was found significantly low at both low (p< 0.01) and high (p< 0.001) doses (**Fig. 3A**) but the progesterone level was not changed at any dose of DBP as compared to control (**Fig. 3B**).

Estimation of 3β-HSD activity and total Cholesterol Level in ovary: Ovarian 3β-HSD activity was evaluated significantly low at both low (p< 0.01) and high (p< 0.01) doses **(Fig. 3C**) but the total cholesterol level was not changed in DBP treated groups as compared to control (**Fig. 3 D**).

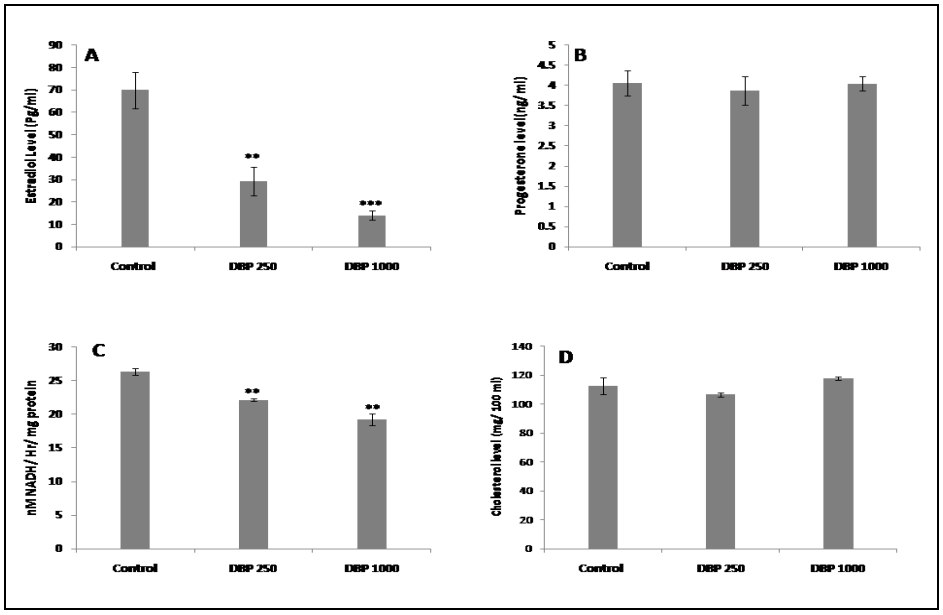

FIG.3: EFFECT OF DBP ON SERUM HORMONE LEVEL, ESTRADIOL (A), PROGESTERONE (B), 3β- HSD ACTIVITY (C) AND OVARIAN CHOLESTEROL LEVEL (D).
**p< 0.01, ***p< 0.001 Vs control, N=6

Estrous cycle monitoring:
DBP treated mice had shown altered estrous cycle. The number of estrous cycles in these mice decreased with significantly lower duration of estrus at both low (p< 0.01) and high (p< 0.01) doses (**Fig.4 A**). Duration of diestrus and diestrus index were found significantly higher at both low (p< 0.05; p< 0.05) and high (p< 0.01; p< 0.01) doses (**Fig.4 B, C**) respectively.

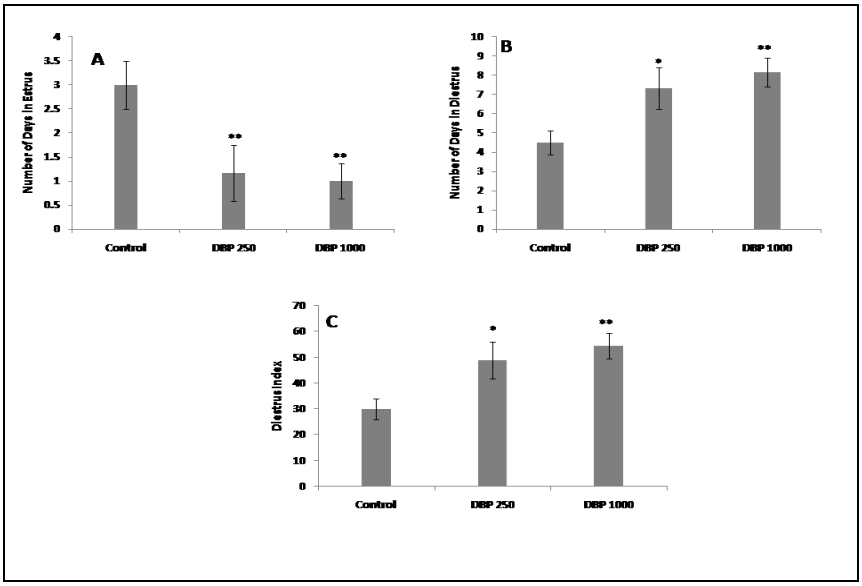

FIG.4: EFFECT OF DBP ON ESTROUS CYCLE, NUMBER OF DAYS IN ESTRUS PHASE (A), DIESTRUS PHASE (B) AND DIESTRUS INDEX (C).
*p< 0.05, **p<0.01 Vs control, N=6

Estimation of antioxidant enzymes activity in reproductive organs:

In ovary activity of SOD and CAT decreased significantly at both low ($p< 0.05$; $p< 0.05$) and high ($p< 0.01$; $p< 0.05$) doses respectively (**Fig. 5-A, B)**. But GPx activity retarded significantly at high ($p< 0.001$) dose only (**Fig. 5C**).

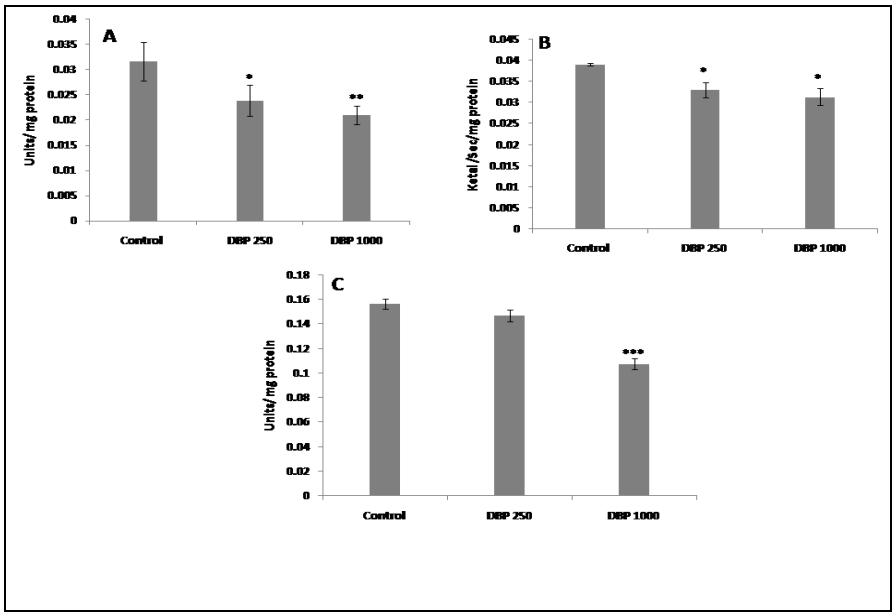

FIG.5: EFFECT OF DBP ON ACTIVITY OF ANTIOXIDANT ENZYMES IN OVARY, SOD (A), CATALASE (B) AND GPx(C).
* $p< 0.05$,**$p< 0.01$,***$p< 0.001$, Vs control N=6

In uterus activity of SOD, CAT and GPx decreased significantly at both lower ($p< 0.001$) and higher ($p< 0.001$) doses as compared to control (**Fig. 6 A, B and C**)

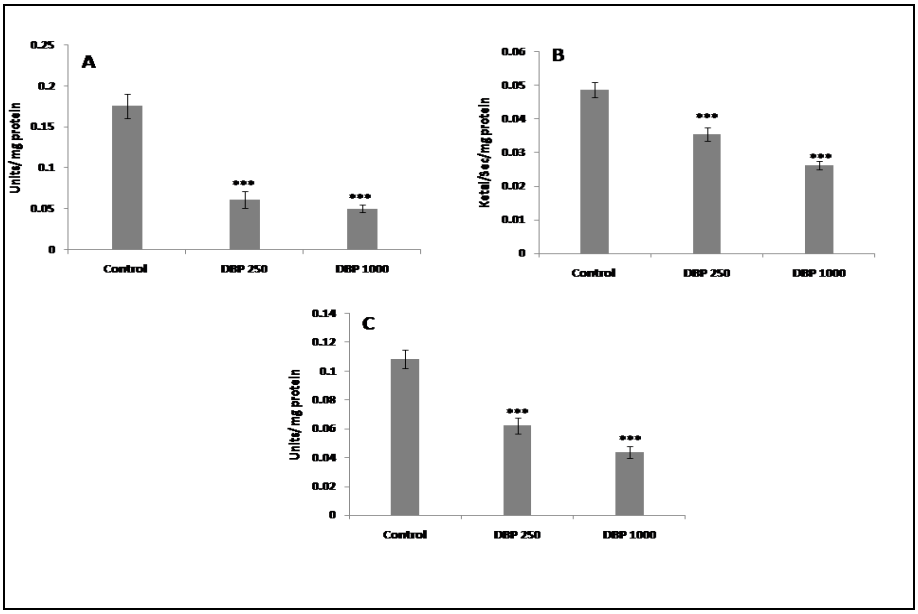

FIG.6: EFFECT OF DBP ON ACTIVITY OF ANTIOXIDANT ENZYMES IN UTERUS, SOD (A), CATALASE (B) AND GPx(C).
*** $p < 0.001$ Vs control, N= 6

Histology of Ovary and Uterus:

Histological observation of ovary revealed presence of less number of healthy and growing follicles in DBP treated mice as compared to control (**Fig. 7 A, B** and **C**). In the sections of uterus lumen size found decreased in a dose dependent manner in DBP treated mice as compared to control. Proliferation of uterine gland

was also found decreased in DBP treated mice as compared to control mice (**Fig. 7 D, E** and **F**).

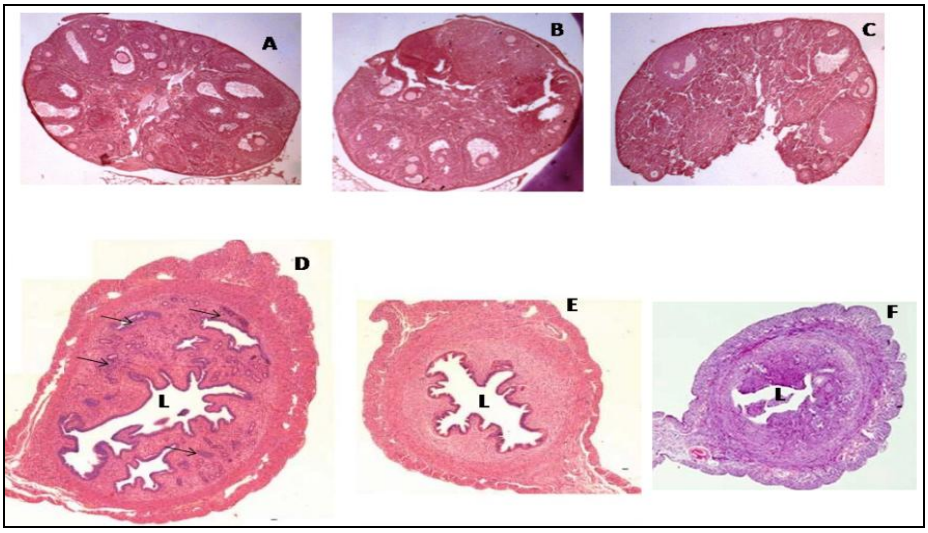

FIG.7: TRANSVERSE SECTIONS OF OVARY AND UTERUS, H&E, 40X, L: LUMEN SIZE, ARROW MARK: UTERINE GLAND. IN OVARY OF CONTROL MICE THERE WERE SO MANY GROWING FOLLICLES SEEN (A) BUT NUMBER OF GROWING FOLLICLES DECREASED IN TREATED MICE DBP 250 (B) AND DBP 1000(C). IN UTERUS OF CONTROL MICE LUMEN SIZE AND UTERINE GLAND PROLIFERATION FOUND PROMINENT (D) WHILE IN TREATED MICE, DBP 250(E) AND DBP 1000(F), THESE PARAMETERS DECREASED IN A DOSE DEPENDENT MANNER.

DISCUSSION: In the present study, we have found that exposure of DBP caused decrease in body weight and reproductive organs weight which is clear sign of gross toxicity of DBP. We also found that DBP affects steroidogenic pathways and antioxidant defence system in reproductive tissues.
DBP is an endocrine disruptor and has ability to disrupt hormone dependant processes. Harmful effects of DBP can be explained in adult individual on the basis of steroidogenesis and antioxidant mechanisms.

Steroidogenesis is the fundamental process for essential physiological functions such as blood salt balance, carbohydrate metabolism and reproduction. The rate limiting step in all steroid production is the delivery of substrate cholesterol from outer mitochondrial membrane to inner part. Pregnenolone is the next to be metabolised into progesterone and androgen by 3-β HSD. 3β-HSD is required for the biosynthesis of all classes of steroid hormones, such as glucocorticoids, mineralocorticoids, progesterone, androgens and estrogens [20]. Antioxidant defence system is an important reactive oxygen (RO) scavenging system in organisms, and plays a crucial role in the protective defence response reaction occurring in an organism in response to toxicant. SOD is first enzyme of this system to react with the reactive oxygen i.e. superoxide ion (O_2^-) to disproportionally form H_2O_2 and O_2. Subsequently, H_2O_2 is decomposed by CAT and GPx into H_2O and O_2. Any disruption of this defence system will cause accumulation of RO and lead to oxidative damage [21].

A significant decrease in activity of 3β-HSD may be responsible for lower production of estradiol in DBP treated mice. Estradiol is essential for normal reproductive physiology in mouse and human. A significant low level of estradiol in DBP treated mice may be responsible for altered estrous cycle i.e. decrease in estrus and increase in diestrus phases and low uterine weight in these mice as estrous cyclicity and uterine weight is regulated by estradiol [22, 23]. A low level of estradiol resulted in imbalance of estrogen progesterone ratio which is responsible for cyclic change during estrous cycle i.e. follicular development, ovulation and uterine endometrium proliferation.

Increase in diestrous index in DBP treated mice indicates that DBP have antiestrogeinc activity [24]. Low ovarian weight in treated mice at higher dose may be due to low rate of folliculogenesis. Follicle Stimulating Hormone (FSH) from pituitary regulates development of follicles in ovary. At higher dose it might be possible that DBP decreases

activity of HPG axis and thus retard secretion of FSH resulting in lower folliculogenesis and lower ovarian weight. Lower development of follicles also results in decrease in number of antral follicle which is responsible for estrogen production in ovary.

This is also explaining significantly lower level of estradiol in higher dose group. In present study it is also found that DBP inhibits the activity of SOD, CAT and GPx in ovary and uterus. One Possible mechanism for that oxidatative DBP may antagonise metal ions such as (Cu_2^+, Zn_2^+, Fe_2^+, and Mg_2^+) that are essential for the activity of antioxidant enzymes resulting loss in activity of SOD, CAT and GPx. Disruption of antioxidant enzymes in ovary may be another mechanism for decrease in follicular growth resulting lower ovarian weight and lower GSI in DBP treated group, as it is already established that phthalates induce oxidative stress in antral follicles and oxidative stress is one of the risk factors to disrupt normal cell proliferation and cause apoptosis [20, 24]. In uterus oxidative stress is generated due low activity of antioxidant enzymes which may be responsible for decrease in uterine weight and USI in addition to lower estradiol level.

CONCLUSION: Results of the present study demonstrate that exposure to DBP generates oxidative stress in reproductive organs and reduce the estrogen production by steroidogenesis in ovary of mouse by affecting the activity of 3β-HSD. A continuous disruption of steroidogensis and antioxidant capacity may further lead to early reproductive senescence and infertility. Further study is also needed.

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