**Effect of Di Butyl Phthalate on Reproductive Functions in Pregnant Mouse**

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**Abstract:** Di butyl phthalate (DBP) is an environmental contaminant used in the production of plastics, cosmetics and medical devices. Pregnant women may be exposed to DBP through diet and daily consumer products. In present study, the effects of DBP on reproductive function of pregnant mice were investigated. Pregnant females were given DBP through gastric intubation at 0, 250 and 1000 mg / kg body weight (BW) on days 0 to 7 and 7 to 11 of gestation in two experiments. In first experiment ovarian weight was found significantly (p< 0.001) decreased at higher dose only whereas uterine weight was measured significantly low in both treated groups: DBP 250 (p< 0.01); DBP 1000 (p< 0.001), as compared to control group. Number of implantation was found significantly low (p< 0.001) and percentage of pre-implantation loss were significantly high (p< 0.001) in both DBP treated groups as compared to control. Progesterone level was found significantly (p< 0.01) low in DBP 1000 treated group while estradiol level decreased in both treated groups: DBP 250 (p< 0.01); DBP 1000 (p< 0.001). A significant (p< 0.001) increase post implantation loss along with reduced foetal weight was: DBP 250 (p< 0.05); DBP 1000 (p< 0.001), observed in treated groups in second experiment. Progesterone level was also found significantly low in both treated groups: DBP 250 (p< 0.01); DBP 1000 (p< 0.001), as compare to control group. In conclusion, results suggest that DBP exposure during pregnancy reduces uterine receptivity and slow down embryonic growth.

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**Key words:** DBP; Implantation; Embryo Spacing; Progesterone

**Introduction**

Phthalates are high-production-volume synthetic chemicals, used to impart flexibility in plastic or as a matrix in cosmetic products. Phthalates are not covalently bound to the plastic and thus leach into the environment where they are ubiquitous contaminants. Environmental sources of exposure include dust and water. However, the main sources of human exposure are food and consumer products such as cosmetics (Wittassek et al. 2011). As a result, human exposure to phthalates is widespread (Blount et al. 2000; Calafat & McKee, 2006). Epidemiologic evidence suggests that women have a unique exposure profile to phthalates, which raises concern about the potential health hazards posed by such exposures (Swan & Davis 2003).

Humans exposure to higher phthalate metabolite concentrations documented in patients using medications with phthalate-containing slow release capsules, raises concerns for potential health effects. Furthermore, animal studies suggest that phthalate exposure can modulate circulating hormone concentrations and thus may be able to adversely affect reproductive physiology and the development of estrogens sensitive target tissues (Kay et al. 2013). Phthalates have been reported to leach into plasma from blood storage bags (Jaegar et al. 1972) and haemodialysis units (Gibson et al. 1976). These compounds have also been found in a variety of materials in the environment, including foodstuffs. Thus, pregnant women might be at risk for unintentional exposure to minute amounts of phthalates derived from medical devices and diet.

In present study, the effect of DBP exposure on embryonic development were analysed at a concentration normally present in environment and during medication / cosmetic exposure.

**Materials and Methods**

***Chemicals and Reagents***

DBP was obtained from Merck Chemicals Private Limited and diluted in olive oil. Estradiol and progesterone ELISA kit was purchased from Diametra Italy. 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.

Parke’s strain female mice 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. Female mice were kept with male mice for mating and after detection of vaginal plug, divided into three groups: (i) a control group that received vehicle (Olive oil); (ii) a group treated with 250 mg/kg BW (DBP250); (iii) a group treated with 1000 mg/kg BW (DBP 1000). The day of plug detection was considered day 0 of pregnancy.

***Experiment 1***

This experiment was designed to evaluate the effect of DBP exposure on implantation and early embryo development. In this experiment different groups of mice received DBP from 0 to 7 day of gestation and sacrificed on 8th day to observe number and position of embryos implanted. Ovaries and uterus were collected and weighed. Ovaries were then subjected to histology to count corpora lutea and pre-implantation loss was evaluated. Blood samples were collected and serum was separated to estimate circulating level of progesterone and estradiol.

***Experiment 2***

In this experiment, mice were given DBP from 7- 11day of gestation to observe embryonic development. On day 12 mice were sacrificed, uterus was taken out and put into 10% ammonium sulphide solution to check number of post implantation embryo loss. Viable embryos were taken out and weighed. Blood samples were collected and serum was separated to estimate circulating level of progesterone.

***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**

***Experiment 1***

A significant decrease (p< 0.001) in number of implantation, with decreased embryo spacing was observed in both treated groups (Fig.1D). Pre-implantation loss was also found significantly high (p<0.001) as compared to control group (Fig 1E). Progesterone level was found decreased in treated groups but difference was statistically significant (p< 0.01) at higher dose only (Fig.2A) while estradiol was significantly low in both of the treated groups (p< 0.001) as compared to control (Fig.2B). The ovarian weight was found significantly low (p< 0.001) at higher dose group (Fig.3A) but uterine weight was a significantly low in both treated groups (DBP250, p< 0.01; DBP 1000, p< 0.001) as compared to control (Fig.3B).

***Experiment 2***

A significantly high (p< 0.001) post implantation loss was observed in both treated groups as compared to control (Fig.4). Serum progesterone level was significantly low) in treated groups (DBP 250, p< 0.01; DBP 1000 p<0.001 as compared to control (Fig. 5A). Foetal weight was also significantly low (DBP 250, p< 0.05; DBP 1000, p< 0.001) as compared to control (Fig. 5B).

**Discussion**

Phthalates have been identified as reproductive and developmental toxicant in several animal species (Ema et al. 1998 & 2000). It is also known to be embryotoxic and teratogenic in rats following maternal administration of large oral doses during organogenesis (Ema et al. 1997). Irrespective to period of treatment (throughout or during part of organogenesis), DBP and its metabolite MBP caused marked dose related increase in post implantation loss. Ema et al have reported that administration of 500or 625 mg MBP/ kg by gastric intubation to wistar rats from day 7 to 15 resulted in increased in post-implantation loss and foetus at term Ema et al 1995a. An increase in resorptions was also noted when MBP was administered from day 7 to 9 of pregnancy.

Our study confirmed that DBP led to compromised embryo implantation, which is a critical stage of pregnancy. The number of implantation sites was significantly reduced in the both DBP 250 and DBP 1000 groups compared with that of the control group. We had also found that administration of DBP during post implantation stage, led to increase in post- implantation loss of embryos and retarded embryo growth.

Synchronization of embryo development to the blastocyst stage with uterine receptivity a prime requirement for successful implantation. Indeed, implantation failure due to inappropriate uterine receptivity is one of major causes of infertility in humans, particularly in case of embryo transfer following in vitro fertilization (Nikas et al. 1999). Uterine receptivity is defined as the window of limited time when the uterine environment is conducive to support blastocyst growth, attachment reaction and subsequent events of implantation. Molecular and cellular events leading to uterine receptivity for blastocyt implantation in mice depend on ovarian steroid (Dey et al. 1999). Various local factors including growth factors, cytokines, transcription factors and prostaglandins, concerned with molecular events of implantation are regulated by Progesterone and estradiol (Das et al. 1995; Song et al. 2004). Progesterone plays an important role in the maintenance of pregnancy and well being of conceptus. Adequate level of progesterone is required for normal uterine decidulization and normal uterine function. A reduced level of progesterone in treated dams is resulted in lower rate of embryonic growth as shown by low fetal weight in our study. During the early phases of pregnancy, when estrogen receptors (ER) and progesterone receptors (PR) are plentiful, the endometrium differentiates into a secretory tissue, in response to both estradiol and progesterone (Somkuti et al. 1997). But at the midsecretory phase, ER abundance falls in all compartments and the endometrium becomes dependent of progesterone action only. This loss of estrogen action may determine which proteins are expressed in the epithelium (Kurihara et al. 2007) and progesterone-induced paracrine factors from the stroma also dictate epithelial gene expression (Lessey BA 2003). These events ultimately convert prereceptive endometrium into receptive endometrium which is a specialized structure that is both secretory and differentiated. Cell adhesion molecules (CAMs) are increased at the apical surface and loosened at the lateral attachments. Underlying stroma becomes "epithelialized" in preparation for trophoblast invasion. Specialized changes in the luminal epithelium provide an opportunity for embryo–endometrial interactions. None of these changes occur in the absence of progesterone (Svechnikova et al. 2007).

Phthalate esters, like DEHP, were reported to reduce uterine receptivity in mouse (Li et al. 2012). It might be due low progesterone level in phthalate treated groups because a declining progesterone level is a critical factor that restricts uterine receptivity (Song et al. 2007). DBP administration is related with suppression of uterine decidualization which led to abnormal implantation and reduction of embryo spacing as found in our results. Therefore, pre and post embryonic loss due to DBP may correlate to the suppression of the responsiveness of the uterus to decidudoma formation. The ovarian weight is regulated by gonadotrophin whereas uterine weight is by estradiol (Chattopadhyay et al. 2003). A significant low level of estradiol level corresponds to decrease in uterine weight. Decreased in ovarian weight and its hormone levels may be due to change in activity of hypothalamic-pituitary and ovarian axis in DBP treated groups (Svechnikova et al. 2007).

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Figure 1- Number of Implantation sites in Control (A), DBP 250(B) and DBP 1000 (C). In control group implantations are proper and there is no reabsorption while in treated group number of implantations is higher with abnormally less inter embryo space. Statistical analysis of the number of implanted embryos (D) and (E) % Pre-implantation loss, N= 10

The ability of mouse embryos to survive and develop in low concentrations of progesterone has been documented in several studies. Blastocyt can survive up to 4 day of pregnancy in ovarectimised mouse (Weitlay and Greenwald 1968). Progesterone plays an important role in endometrial development and its sensitivity to blastocyst and other decidual stimulations. It suggests that progesterone regulate uterine receptivity but does not have direct effect on embryo development before implantation. In contrast low concentration of progesterone in post implantation stages have been associated with reduced embryo survival (Wilmut et al 1986). Early post-implantation development requires a high level of progesterone as decidual reaction start at this time and it undergoes a rapid and massive development accompanied by a greatly increased blood flow during first few days after implantation.

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Figure 2- Circulating level of progesterone (A) was found significantly low in DBP 1000 (p< 0.01) while estradiol level (B) was found significantly low in both DBP 250 (p< 0.01) and DBP 1000 group (p< 0.001) as compare to control group, N = 6.

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Figure 3- Ovarian weight (A) was significantly low in DBP 1000 group (p< 0.001) and uterine weight in both treated group was significantly low, DBP 250 (p< 0.01) and DBP 1000 (p<0.001), N= 6

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Figure 4- Post implantation loss was found higher in treated group as compared to control group. Control group (A), DBP 250 group (B), DBP 1000 group (C). Statistical analysis of post-implantation loss (D)

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Figure 5- Progesterone level (A) was found significantly, DBP 250 (p< 0.01) and DBP 1000

(p< 0.001), low as compare to control. Fetal weight (B) at 12th day of gestation was found significantly low in both groups DBP 250 (p<0.05) and DBP 1000 (p< 0.001) as compared to control group N= 6.

**Conclusion**

The result of present study demonstrates that exposure of DBP during early stages of pregnancy reduces uterine receptivity and slow down post implantation developmental events by decreasing progesterone level in treated mice.

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