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Protection of male reproductive toxicity in rats exposed to di-*n*-butyl phthalate during embryonic development by testosterone



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ABSTRACT

Di-*n*-butyl phthalate (DBP) widely spread industrial chemical that made drastic alteration in male reproductive system. The present study elucidates the protective role of testosterone on reproductive toxicity in prenatal DBP exposed adult male rats. Pregnant rats were injected with corn oil or 100 and 500 mg/kg body weight of DBP on gestation day (GD) 1, 7 and 14. F1 male rats were weaned, injected with either testosterone or vehicle. On postnatal day (PND) 100 F1 adult male rats were cohabited with untreated female rats. Then rats were sacrificed and analyzed for other reproductive end points. Prenatal DBP exposed male rat testes, seminal vesicle weight, sperm count, motility, viability and HOS tail coiled sperm were significantly decreased with increased sperm morphological abnormalities. The levels of testicular 3 β , 17 β HSD, serum testosterone were significantly decreased with increased FSH, LH levels in experimental rats. The fertility studies revealed that increased pre, post-implantation losses and resorptions in normal females cohabited with experimental rats. Higher testicular LPO with lower SOD, CAT and GPx activity levels in experimental rats. Administration of testosterone to prenatal DBP treated male rats showed significant protection in above all parameters. In conclusions, testosterone deteriorates prenatal DBP induced reproductive and fertility toxicity by decreased oxidative stress and increased testicular antioxidant enzymes.

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1. Introduction

During the past few decades, there had been increased in number of occurrences of impairment of reproductive functions in humans and wildlife by exposure to environmental pollutants [38]. DBP was popular industrial chemical that had been adversely effects on male reproduction by acting as an endocrine-disrupter [35]. DBP was used as plasticizers and also widely used in personal care products, food packaging, adhesives, printing inks, aerosols, nail polish, perfumes and cosmetics [16]. Moreover, DBP was not covalent members of plastic polymer, they can easily leach into the environment, consequently humans are exposed [47]. DBP was detected in various human body fluids including urine, serum, seminal fluid, amniotic fluid, breast milk and saliva [28].

In-utero exposure of DBP was capable to reach fetus via placental transfer [45] and it interference with reproductive tract development and causes deformities like hypospadias, cryptorchidism, reduced anogenital distance (AGD), retention of nipples,

alteration in testicular marker enzymes and disturbed spermatogenesis [3,48]. A number of studies had reported an association between increased DBP concentration with decreased sperm concentration, motile sperm, serum testosterone and increased sperm DNA damage [23,44]. DBP directly effect on Leydig cells or Sertoli cells and alters the steroidogenic enzymes, it leads to reduction of testosterone production [21]. Numerous studies investigated that prenatal exposure of DBP on window period [(GD6–15 GD 12–21; GD 14– PND 3; GD 16–19 or GD 17– postnatal day (PND 3)] causes marked male reproductive abnormalities and teratogenic anomalies [12,31]. Moreover, our previous study elucidate that prenatal exposure of DBP on GD 1, 7 and 14 affected male reproductive function by decrease spermatogenesis, steroidogenesis, fertility and testosterone levels [14].

Testosterone was crucial for primary and secondary sexual characters and it controls the formation of reproductive tract, sperm production, sexual drive and sexual performance in men [40]. Impairment of testosterone production may be initiating factor for testicular toxicity with sexual complications [30]. In earlier studies described that co-administration of zinc, vitamin A & C, and resveratrol failed to protect phthalate-induced testicular toxicity [7,41].

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Numerous experiments suggest that testosterone plays vital role in promoting the recovery from reproductive toxicity [8,18]. Moreover, co-administration of testosterone to di (2-ethylhexyl) phthalate (DEHP) exposed male rats prevents the decreased sperm count and alterations in testicular enzymes [34]. Therefore, we hypothesised that testosterone protection against di-*n*-butyl phthalate reproductive toxicity. In the present study evaluate the protective role of testosterone depot (4.16 mg/kg/b.w) on PND 35, 55 and 75 days against *in-utero* (GD 1, 7 and 14) exposed DBP at 100 and 500 mg/kg b.w induced reproductive toxicity in male rats.

2. Materials and methods

2.1. Chemicals

DBP (Cat. No. 84-74-2, 99% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and testosterone depot manufactured by German Remedies, Goa, India was purchased from local drugstore and all other chemicals were analytical grade.

2.2. Animals

Healthy adult male and female Wistar rats weighing 170 ± 20 g were obtained from the Sri Venkateswara Traders, Bangalore, India. Rats were housed in polypropylene cages (43 cm \times 30 cm \times 15 cm) covering sterile paddy husk and maintained in an air-conditional room at a temperature of $25^\circ\text{C} \pm 2^\circ\text{C}$ and $50 \pm 5\%$ humidity with well-regulated 12 h light-dark cycle. Animals were fed with standard rat pellets (Godrej Agrovet Ltd., Mumbai, India) and filter tap water was provided *ad libitum*. The animal experiments were conducted with the guidelines of Indian Council of Medical Research (ICMR), Hyderabad, India, also this study was approved by an Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No: 10/(I)/a/CPCSEA/IAEC/SVU/PSR/NG).

2.3. Experimental design

On the evening of pro-oestrus, virgin female rats left to mate overnight with fertile male rats. Successful mating was checked by the presence of vaginal plug or presence of spermatozoa at the vaginal orifice by using 0.9% saline. Pregnant female rats were randomly separated into 3 groups of 8 rats each. Pregnant animals in group I considered as controls. Rats in groups II and III received intra-peritoneal injection of DBP (vehicle as corn oil) at a dose of 100 and 500 mg/kg b.w respectively on GD 1, 7 and 14 days in 50 μl volumes. The control group received corn oil in equal amounts as in the experimental groups.

After delivery, all pups were allowed to grow with dams for 1 month. Then, male pups were separated and housed 4 per cage, and allowed free access to the standard rat chow and tap water *ad libitum*. Male rats were injected intra-peritoneally with 4.16 mg/kg b.w testosterone depot in 100 μl of corn oil and benzyl benzoate (4:1) on PND 35, 55, 75 ($n=8$), while its vehicle group or control group received 100 μl solvent and rats were maintained up to PND 100. The dosage selection and exposure period of DBP was based on our earlier reports in which adverse effects on male reproductive system arise between 100 mg and 500 mg DBP/kg b.w without systemic toxicity [14]. The dosage of testosterone and their injection time were chosen according to previous studies in our laboratory [18].

2.4. Fertility assessment

At 100 days of age, eight males each control and experimental groups (two male offspring from each dam) were mated 1:2 with

untreated pro-oestrus, virgin female rats. The pre-coital sexual behavior of the paired rats was observed 1–2 h later. The day that noticed a vaginal plug and/or sperm in vagina was considered as day 0 of gestation (GD 0). On the 6th day of gestation (GD 6), half of the females (females per male from control and DBP groups) were anesthetized with ethyl ether, followed by sacrificed and subjected to laparotomy and observed uterine horn for the number of *corpora lutea* and the number of implantations. On the 18th day of gestation (GD 18), another half of females were laparotomized then number of live, dead fetus and resorptions were recorded. From these results the following parameters were calculated.

$$\text{Mating index(\%)} = \frac{\text{number of sperm positive female}}{\text{number of pairing}} \times 100$$

$$\text{Fertility index(\%)} = \frac{\text{number of pregnant females}}{\text{number of sperm positive}} \times 100$$

Pre – implantation loss(%)

$$= \frac{\text{number of corporalutea} - \text{number of implantations}}{\text{number of corporalutea}} \times 100$$

Post – implantation loss(%)

$$= \frac{\text{number of implantations} - \text{number of live fetuses}}{\text{number of implantations}} \times 100$$

2.5. Euthanasia, body and organ weights and serum collection

When copulation was confirmed, the prenatal DBP exposed male rats body weight was recorded and they were anesthetized with ethyl ether and sacrificed with cervical dislocation. The blood was collected from cardiac puncture. The blood was centrifuged at $2,000 \times g$ for 15 min after overnight storage at 4°C , then serum was separated into sample bottles and stored at -40°C until analysis. Testes, epididymis, prostate gland, vas deferens and seminal vesicle (without the coagulating gland) were dissected out immediately and weighed on nearest mg electronic balance (Shimadzu, model no: BL-220H). The tissue somatic index was calculated by using following formula:

$$\text{Tissue somatic index} = \frac{\text{Weight of the tissue in grams}}{\text{Weight of the body in grams}} \times 100$$

2.6. Evaluation of sperm parameters

Immediately after sacrifice, cauda epididymis was minced by using anatomical scissors in 5.0 ml physiological saline (0.9%, NaCl) in a petri dish. One drop of sperm suspension was placed on a slide, covered by cover slip, and evaluated for motile or immotile sperms in 10 microscopic fields under a phase contrast microscope at $200\times$ magnifications by the method of Belsey et al. [4]. The epididymal sperms were counted using the Neubauer chamber as described earlier [4]. Briefly, a 10 μl of an aliquot of sperm sample was taken into the Neubauer chamber (Deep 1/10 mm, LAMBART, Darmstadt, Germany) of haemocytometer and allowed to stand for 5 min in a humid chamber and then count the sperms which are sedimented. The viability of epididymal sperm was determined by staining with 1% trypan blue solution [39]. The hypo-osmotic swelling (HOS) test was performed by exposing 100 μl of sperm sample to 1.0 ml of hypo-osmotic solution and observed for tail coiled sperms under microscope by the method described by Jayendran et al. [20]. To analyse the sperm morphological

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