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Oxidative stress and male reproductive dysfunctions with reference to orally administered phthalate compound in *Mus musculus*

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Abstract- In order to assess the impact of phthalate diester exposure on oxidative stress and reproductive health of male swiss albino mice *Mus musculus*, it was orally administered for four, six and eight weeks at a dose of 15 mg/kg/b.wt in the experimental groups of mice. Decrease in the testes weight in phthalate treated group III mice in comparison to normal group I and control group II were observed and the values were statistically very significant ($P<0.001$) after four, six and eight weeks of treatment. The sperm count in treated mice of group III decreased after four and six weeks of exposure and statistically significant decrease was observed after eight weeks of treatment. The decreased testosterone level was not significant after four weeks of treatment but showed statistically significant decrease after six and eight weeks of treatment as compared to mice of normal groups I & control group II. Statistically significant increase in serum level of MDA was recorded in phthalate treated group III mice after four and eight eighth weeks of the experimental period. In the present study, phthalate induced reproductive toxicity in male mice have been shown to be associated with decreased sperm count, decreased testicular weight and decreased testosterone level.

Key words: phthalate, swiss albino mice, testosterone, sperm, oxidative stress

INTRODUCTION

Phthalates are widely available in cosmetics and are used as plasticizers in flexible plastics widely used in the food, construction and medical products industries, leading to the human exposure through multiple routes and media.[1] There is growing concern about the signs of an increase male reproductive health problems and their association with exposure to EDCs.[2] The increasing number of hormone-dependent cancers, including prostate cancer, decreased sperm count, poor sperm quality, increased incidence of hypospadias, cryptorchidism, changes in pubertal timing, and testis cancer have all been reported in recent decades and the term testicular dysgenesis syndrome (TDS) is used for this list of male reproductive disorders.[3]

Phthalates are peroxisome proliferators (PPs) and hepatocarcinogens in mice,[4] and target fetal and pubertal testes and lead to alterations in endocrine and spermatogenic functions.[5] Di (2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate derivative, found everywhere in plastics, and the only phthalate currently used in polyvinyl

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chloride (PVC) medical devices. DEHP and its main metabolite, mono (2-ethylhexyl) phthalate (MEHP), have been shown to cause testicular damage in developing and older animals[5-6] and reduction in sperm motility.[7] Their exposure has also been found to lead to a reduction in the production of testicular testosterone (T) in mice,[8] indicating that Leydig, as well as Steroli cells, are their target.[2] Phthalates are negatively related to testosterone (T) levels,[9] decreased sperm count and poor sperm quality,[10] and in combination with other endocrine disorders that can promote reduce fertility.[11] Prenatal exposure leads to anti-androgenic effects such as undescended testes and epididymal agenesis in male mice.[12]

Animal studies have shown adverse effects of phthalate exposure during sexual differentiation in developing male reproductive system.[13] In utero exposure during this critical period of pregnancy to phthalates such as di (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) cause male reproductive tissue abnormalities including anogenital distance (AGD), nipple retention, presence of vaginal pouch, hypospadias, epididymal agenesis, undescended testes and reduced size of the sex glands.[14] Phthalates do not bind to the androgen receptor but are instead antiandrogenic by interfering with testosterone (T) synthesis. Decreased androgen production caused by phthalate exposure during sexual differentiation is linked to phthalate-induced abnormalities.[15]

In human studies, DEHP has been linked to decreased testosterone (TT) levels in children.[16] In addition, prenatal DEHP exposure is reportedly associated with a shorter anogenital distance in boys[17] and lower sperm volume in adolescent males[18] which may be associated with subsequent infertility in adulthood.[19] DEHP can also contribute to the decline in semen quality due to increased production of semen with abnormal heads.[20] High levels of estradiol (E2) and high levels of E2 / TT ratio were also found in male PVC workers exposed to high urinary concentrations of DEHP metabolite.[21]

Recent data also suggest that phthalates are able to produce free radicals in several ways in germ cells including the activation of peroxisome proliferator-activated receptor alpha (PPARα), suggesting the possibility that oxidative stress and mitochondrial dysfunction in germ cells may contribute to phthalate-induced disruption of spermatogenesis. Previous evidence has shown that DEHP has its own anti-androgen effect by suppressing fetal testosterone biosynthesis by activating peroxisome proliferator-activated receptors (PPARs),[22] and subsequent inhibition of anti-oxidant enzymes, leading to free production of radical and oxidative stress, which has contributed to oxidative damage of DNA.[23] After exposure to DEHP during prepuberty and puberty, a significant decrease in glutathione (GSH) / glutathione disulfide (GSSG) redox ratio and a significant increase in activity levels of thiobarbituric acid (TBARS) were observed.[24] Epidemiological analysis has also shown that urinary oxidative marker malondialdehyde (MDA) concentration is strongly associated with DEHP metabolite levels in prepubertal children.[25] Reactive oxygen species (ROS) and DNA-related damage can lead to apoptosis.[26] Oxidative stress is a common pathological condition associated with EDC-induced testicular damage, making oxidative stress monitoring an effective way to investigate the link between multiple toxicants and reproductive effects.[27]

There is growing concern about the reported increase in male reproductive health problems due to exposure to endocrine disrupting chemicals. Oxidative damages are also reported to play an important role in male reproductive toxicity. The present study was undertaken to assess the impact of oral administration of phthalate diester on oxidative stress, alterations in sperm count, hormonal and biochemical parameters of male swiss albino mice *Mus musculus* at a dose of 15 mg/kg/b.wt for the experimental period of four, six and eight weeks.

MATERIALS & METHODS

Chemicals: Phthalate (most common -DEHP) were purchased from Accustandered USA (Marketed by Rankem) and corn oil used as vehicle was procured from Sigma Pvt. Ltd and Neishel Chemical Pvt., Gujarat. All chemicals were used are of Analytical grade.

Experimental Animal: Swiss albino mice *Mus musculus* that were purchased from CDRI, Lucknow were reared in the animal house facility of S. S. Hospital & Research Institute, Patna. Adult male mice weighing 30 - 32 grams were selected for study and were allowed to acclimate for at least two weeks. The mice were housed in the stainless steel wire cages at a temperature of (25±1ºC), humidity (56 ±5%) and lighting (12h light / dark cycle). Food and tap water were provided ad libitum. All animal experiments were carried out as per CPCSEA guidelines. All animal experiments were carried out as per

CPCSEA guidelines (Approval No. 1840/PO/ReBi/S/15/ CPSCEA).

Dosing design: Mice were divided into three groups of six mice each.After acclimatization, mice of group I (normal) received only food and water *ad libitum* ,group II mice(control) were administered corn oil at a dose of 15 ml/kg/b.wt once a day with regular food and water,while mice of group III (treated) were orally administered Phthalate, most commonly used (Di (2-ethyl hexyl) phthalate),using corn oil as vehicle, at a dose of 15 mg/kg/b.wt by gavage in addition to food and water *ad libitum*.

Experimental Design: Assessment of hormonal parameter and oxidative stress were conducted at the time interval of 4, 6 and 8 weeks.

Hormonal Study: It was done by the ELISA (Elisa plate reader - Tulip diagnostic model - lisaquantelisa plate reader) method. After treatment for the time period of 4, 6 and 8 weeks, blood were collected by ocular puncture from control and treated mice. Serum was separated for further analysis and hormonal assay. At the time of blood collection, body weight was recorded and then blood was collected for serum for the estimation of various hormonal parameters.

Sperm count: Epididymis was removed. It was placed in a glass Petri dish containing Pure Sperm Wash with 0.5% bovine serum albumin, and ground with anatomic scissors. The suspension was centrifuged at 800 x g for 10 minutes. The pellet was diluted with Pure Sperm Wash, a 10 µL suspension applied to the Neuberhemocyto meter for sperm count.

Oxidative stress study: The oxidative stress in testes was evaluated by malondialdehyde (MDA) level after 4, 6 and 8 weeks of treatment in all the experimental groups with the help of Double beam UV-vis spectrophotometer.

Statistical Analysis: Data was analysed using the SPSS version software 11.0 of statistical software package. All values were expressed in mean ± SEM. The effects of treatment over time were compared between the control and management groups by covariance analysis. P values of less than 0.05 were considered statistically significant.

RESULTS & DISCUSSION

The present study was undertaken to assess the impact of oral administration of phthalate diester on oxidative stress, alterations in sperm count, hormonal and biochemical parameters of male swiss albino mice *Mus musculus* at a dose of 15 mg/kg/b.wt for the experimental period of four, six and eight weeks. Oxidative stress level is reported to play an important role in inducing testicular toxicity due to exposure to plasticizer pollutant and was assessed. About 9-10% mortality was recorded among the treated mice during the experimental period of eight weeks. Almost negligible deaths were recorded during the experimental period of eight weeks in the control groups, which proved that corn oil at the dose of 15mg/kg/ body weight can be used as a vehicle for toxicological studies. A progressive reduction in the mean values of testes weight in treated group III in comparison to normal group I and control group II was observed after four weeks of oral administration of phthalate at a dose of 15mg/kg/b. wt/ day. The changes in testes weight were statistically not significant ($P>0.05$) in four weeks while it was statistically very significant ($P<0.01$) in six and eight weeks in treated group III (Table 1). Decreased sperm count was recorded after oral administration of phthalate in mice of treated group III after for four, six and eight weeks of treatment.

In the present study a decrease in serum testosterone level in mice of treated group III was recorded after four, six and eight of experimental time period (Fig. 2) and the values were statistically not very significant after four weeks but very significant after six and eight weeks of treatment as compared to normal group I and control group II. An increase in serum level of MDA in mice of treated group III was observed after four and eight weeks of the experimental time period (Fig. 3).

A variety of natural / xenobiotic chemicals including phthalates have been shown to cause oxidative stress, which may target the endocrine system and cause reproductive imbalances.[28] Phthalates are one of the categories of environmental endocrine disruptors (EDs), used as plasticizers in polyvinyl chloride plastics. More recently, these chemicals have been considered because prenatal exposure to rats can cause testicular dysgenesis (TDS) in the male offspring postnatally.[29]

In the present study phthalate induced reproductive toxicity in male mice have been shown to be associated with decreased sperm count, decreased testicular weight and decreased testosterone level. The results of the present study (Table 1) were in agreement with the study of which reported a statistically significant reduction in testicular

weight in male rats treated with DEHP at a dose of 1000mg/ kg body weight.[9]

In the present study, the reproductive toxicity induced by DEHP in male mice has been shown to be associated with decreased sperm count, testicular weight and decreased testosterone level. The present study showed a significant decrease in relative testis weight in DEHP-treated mice groups as compared with control mice groups. The results of the present study (Table 1) were in agreement with a study who reported a statistically significant reduction in testicular weight in male rats treated with DEHP at a dose of 1000mg/ kg body weight.[9]

Phthalates are also commonly studied as potential EDC. The effects of di (2-ethylhexyl) phthalate (DEHP) have been studied in mice and shown to cause a reduction in daily sperm production[30] which is clearly shown in our study as there is reduction in sperm count (Fig. 1). A worker suggested that the reduction in the weight of accessory reproductive organs indicates reduced availability of androgens.[31] It has been reported that the adverse effects of DEHP in male rats exposed during the pre and early postnatal periods showed that the testosterone levels were significantly lower.[32] These results coincided with present studies that showed decreased serum testosterone levels (Fig. 2). Several studies have reported the adverse effects of DEHP on the development of the male reproductive tract when animals were perinatally exposed to DEHP.[33] These effects include reduced testes size, decreased sperm production, cryptorchidism, and reduced reproductive organ weights as testosterone is the most important sex hormone in males and plays a critical role in testis development, spermatogenesis, and the maintenance of normal masculinization.

MDA levels indicated that accumulated lipid peroxidation occurs in the testes of pubertal rats post-exposure to DEHP[34]. Under normal circumstances, oxidant and antioxidant systems in testes maintain an equilibrium state, by which spermatozoa are equipped with antioxidant defense mechanisms, thereby quenching ROS and protecting gonadal cells and mature spermatozoa from oxidative damage.[35] However, excessive production of ROS overtakes the antioxidant capacity of the seminal plasma and causes oxidative stress.[35] Oxidative stress is a condition representing an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage,[36] considered to be an important cause of male infertility. Although oxidant-caused damage can be repaired in most situations, spermatozoa are highly susceptible to oxidative stress. The mitochondria-mediated apoptosis pathway can be activated via the elevation of MDA levels.[37] Results from the present study are consistent with the findings (Fig. 3) and suggest that DEHP might induce oxidative stress and impair testicular function.[38]

In the present study of dose-dependent reproductive toxicity of phthalate in male mice after oral exposure of phthalate for four, six and eight weeks, reduced sperm count, showed decreased testes weight decreased serum testosterone level and increased MDA level in comparison to normal and control groups of mice indicating the potential toxicity of phthalate on reproductive system of experimental mice model at a particular dose.

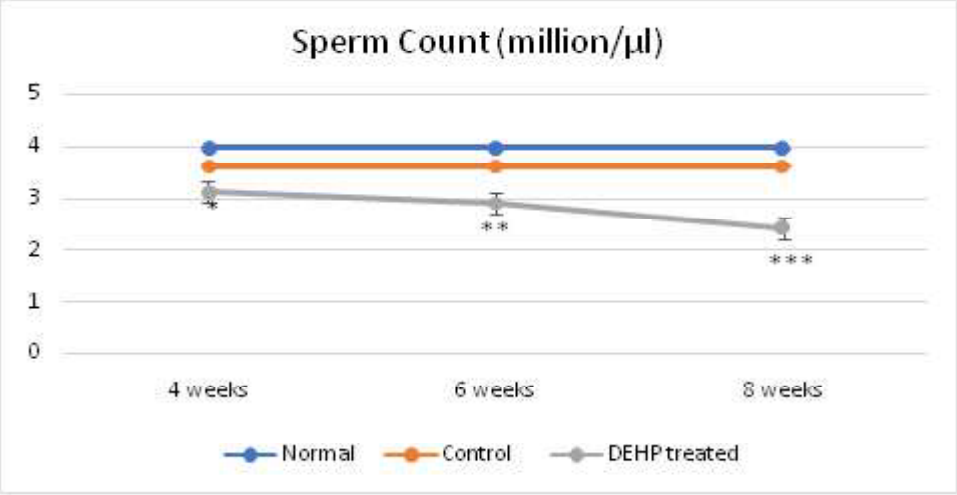

Fig 1: Showing sperm count after 4, 6 and 8 weeks of experimental period in normal, control and treated groups of mice.(*considered significant, **considered very significant and * considered very significant)**

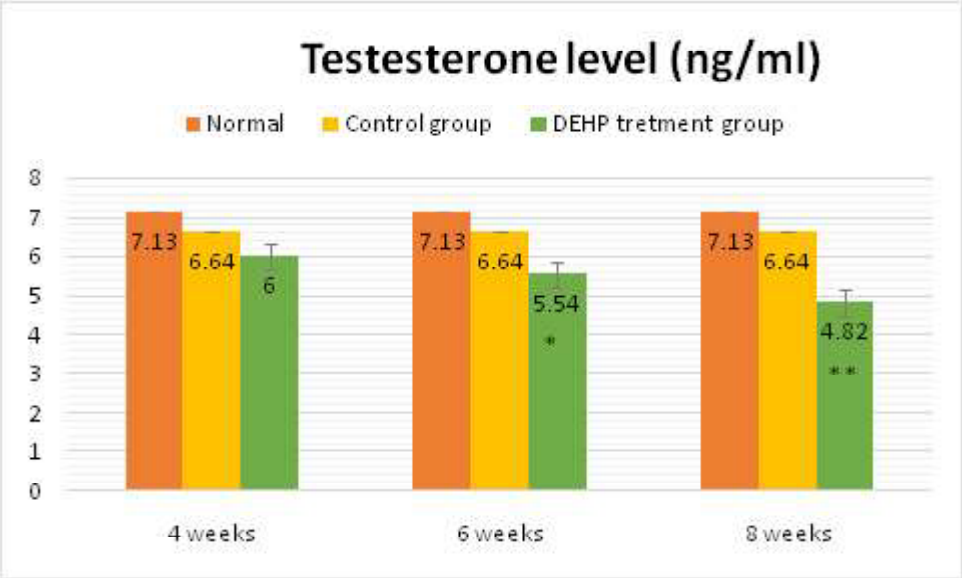

Fig 2: Showing serum level of hormone Testosterone after 4, 6 and 8 weeks of experimental time period in control and treated groups of mice (*considered significant and ** considered very significant)

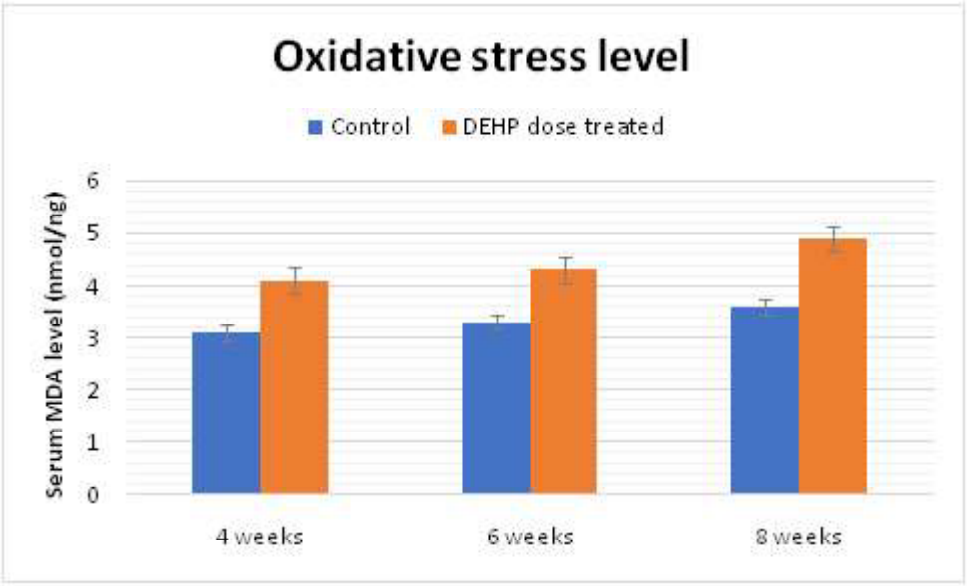

Fig 3: Showing serum level of MDA after 4, 6 and 8 weeks of experimental time period in normal control and treated group of mice (considered very significant)**

Table 1. Changes in testes weight in normal, control and treated groups of mice

Sl No.	Parameter in (g)	Normal male mice Group I	Control Group II (Corn oil treated male mice)	Group III DEHP-treated male mice		
				4 weeks	6 weeks	8 weeks
1.	Testes Weight	12.98 ± 0.310	12.137 ±0.714	11.09 ± 1.32*	10.6 ± 0.512**	9.8 ± 1.42***

Values expressed as mean ±SEM. P>0.05 was considered statistically significant; P>0.01*, P>0.001**, P>0.0001***

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