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DEHP induced reproductive toxicity and oxidative stress in male rats

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Abstract- DEHP is a well-known chemical of commercial importance present everywhere around us. It has become a chemical of concern due to its high chances of exposure as it is present in quite huge amounts in our environment and its damaging effects reported in various living system. Present study is planned to see how it damages male reproductive system in animal model using Wistar Rat exposed to 1000mg/kg body weight of daily dose for 4, 8, 12 and 16 weeks. This resulted in significant accumulation of the chemical in the testis of rat which showed increase in amount with the increase of period of exposure. This study also showed similar trend as the tissue level accumulation of DEHP, viz. oxidative DNA damage of the testis tissue as shown by the measure of 8-OHdG formed in the DNA of testis tissue due to DEHP exposure was increasing significantly with exposure duration similarly lipid peroxidation level of the serum sample of exposed rat was significantly high in comparisons to that of control group rats and increased significantly with increasing exposure interval. These findings clearly suggest that DEHP by accumulating in the tissue reaches to cells and damages DNA at various levels. Whereas DNA of testis showed high level of oxidative damage, serum of the exposed rat also showed high level of lipid peroxidation, showing it causes oxidative stress in the animal damaging the reproductive system significantly.

Key words: DEHP, Testis, DNA damage, Oxidative Stress, HPLC, rat 8-OHdG

INTRODUCTION

DEHP belongs to a group of compounds known as phthalates, which are used as plasticizers and solvents all around the world. Actually, no part of our ecosystem is left unreached by these chemicals; wherever the plastic is present these chemicals are present. As a large number of damaging effects are associated with DEHP, it has become a threat to the living system. From air, water or soil to which it is released, DEHP finds its way either into food chain or enter living body through inhalation, ingestion or dermal absorption. In living system its concentration builds-ups and causes damage to various system in several different ways. One of the systems prominently affected by DEHP is male reproductive system. Animal models like monkeys, guinea pig, hamsters, rats and mice as well as human male reproductive system are very prone to damages caused by DEHP, therefore it is considered as a reprotoxin. DEHP poses threat to male reproductive system in various ways like cryptorchidism, Testis Dysgenesis Syndrome, hypospadias, decrease in sperm quality and quantity, decreased androgen levels and even testicular cancer.[1,2] Penile length in singleton foetus in pregnant women is reported to be adversely affected due to phthalate exposure.[3] Semen quality and serum testosterone levels have been found inversely associated with serum DEHP

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metabolite levels in men.[4] DEHP has been reported to cause testicular toxicity via JAZF1/TR_4 pathways and oxidative stress in pubertal male rats.[5] In a study performed on MA 10 mouse Leydig tumor cell line as model, cytotoxic and DNA damaging effects of DEHP along with its potent metabolite MEHP was shown.[6] DEHP has been found to cause DNA fragmentation in testes tissue, increased lipid peroxidation, oxidative stress, reduced testosterone hormone, reduced testis and epididymal weight, altered semen characters, decreased mRNA expression of androgen receptors and increased INOS (inducible nitric oxide synthase).[7] DEHP is established as male reproductive toxicant and causes methylation of genes at higher doses around 900mg/kg body weight in case of peripubertal male Sprague Dawley rats.[8] In a study performed on 379 men from an infertility clinic, urinary concentration of phthalate metabolites were measured and sperm DNA damage was analysed suggesting that phthalate exposure may result in population distribution of sperm DNA damage.[9] Maternal exposure to DEHP in mice results in testis dysgenesis syndrome in foetus and pups and epigenetic testicular DNA damage.[10] Reproductive toxicity of DEHP has been studied extensively in rats and mice addressing its role in inhibition of reproductive tract development, suppression of testosterone production and spermatogenesis as well as causing cancer.[11-16]

MATERIALS & METHODS

Animal and Experimental Design: Adult male Wistar Rats of age about 12-14 weeks were selected and kept in polypropylene cages having saw dust bedding at the temperature 28±1°C using air conditioning system for this study. Rats were provided comfortable environment in controlled light (12 hours light and 12 hours dark). As per the ethical guidelines approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) with Registration No. 2088/ PO/Re/S/19/CPCSEA, dated December 18, 2019), Government of India and Institutional Animal Ethics Committee (IAEC) of Anugrah Narayan College, Patna, animals were maintained in ideal conditions. Rats were reared in five groups A, B, C, D and E each having 5 rats. Group A rats were the control one, not exposed to DEHP, group B, C, D and E were the treatment group exposed to 1000mg/kg body weight/day of DEHP dissolved in corn oil for the period of 4 weeks, 8 weeks, 12 weeks and 16 weeks respectively. Dosing was done orally through gavage method. Control group rats were given only corn oil without DEHP.

Assessment of DEHP accumulated in the testis tissue: One of the testes after dissection was used for the study of DEHP accumulation by it on the completion of treatment period with the help of UHPLC (Ultra High-Performance Liquid Chromatography, Dionex Ultima 300, Thermo Fisher Scientific India Pvt Ltd). For sample preparation, completely crushed tissue in acetonitrile was microwaved for 30 seconds 25 to 30 times at 100°C for complete extraction and centrifuged at 3000rpm for 10 minutes, supernatant was collected, filtered and stored at -20°C and further used for UHPLC study. Data were acquired and recorded with the help of Chromeleon 7.2 software provided with the UHPLC.

Assessment of Oxidative DNA Damage in Testis Tissue: Estimation of oxidative DNA damage of the testis tissue was done through measure of 8-OH-dG marker in the testis tissue homogenate with the help of ELISA. For this ELISA kit of Life Technologies (India) Pvt. Ltd (Kit no. LT 21 3002B 2KK BA) was used. One of the testes after dissection on completion of dosing interval was used for DNA damage study. After weighing, washing, mincing and homogenising in ice cold PBS (0.01 M and pH 7.4) tissue sample was put in freeze thaw cycles for complete rupturing of cells and centrifuged at 3000 rpm for 10 minutes to collect supernatant and stored at -20°C for ELISA study. Standard curve was plotted using rat 8-OH-dG (8-Hydroxy-deoxyguanosine) of known concentration. OD of the samples was also taken following all the protocols of the kit under 450nm wavelength.

Estimation of Lipid Peroxidation Levels in Blood Serum: Lipid Peroxidation levels of blood serum were estimated using UV spectrophotometer (Thermo Fisher UV VIS Spectrophotometer). Blood sample was collected in vacutainer tube after sacrificing the rat on completion of dosing interval and centrifuged for 10 minutes at 3000rpm, supernatant serum was collected in ependorff tubes and LPO estimation was performed using 10% TCA (Tri-Chloro Acetic acid) and 0.675% TBA (Thiobarbituric Acid) following standard protocols. OD was read in UV spectrophotometer under 532 nm.

RESULTS

Accumulation of DEHP in Testis Tissue: Among the DEHP treated animals, the highest concentration of DEHP in the testis tissues was observed among the Wistar

rats with 16 weeks of treatment (Group E) followed by 12 weeks (Group D), 8 weeks (Group C), 4 weeks (Group B) (Figure 3.1). There was negligible amount of DEHP observed in the control rats (Group A). The mean±SD of DEHP concentration in group A was found to be 0.0058±0.0044µg/g of testis tissue with 95% confidence interval ranging from 0.0013 to 0.0102µg/g. However, in group B and C, the mean±SD of concentrations of DEHP were 0.0239±0.013µg/g of testis tissue with 95% confidence interval (0.0102-0.037µg/g) and 0.067± 0.046µg/g with 95% confidence interval of 0.11 - 0.021 µg/g of testis tissue respectively. The mean concentration of DEHP in testis tissues increased significantly in group D and E. The mean±SD of DEHP concentrations in testis tissues of group D and E were 0.0848±0.023µg/g with 95% confidence interval of 0.061 - 0.0108 µg/g and 0.0955 ±0.0374µg/g with 95% confidence interval ranging from 0.058 to 0.1329µg/g of testis tissues respectively (Figure 3.2). The one-way ANOVA calculated for the mean values denoted very high significance level (p-value < 0.00046).

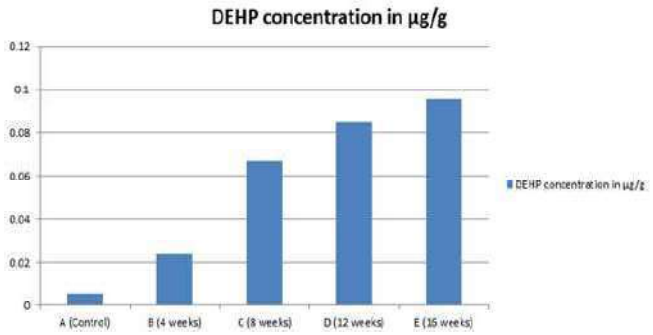

Fig. 3.1: Bar graph representing the level of DEHP in testes tissue of rats in the different groups (A, B, C, D & E).

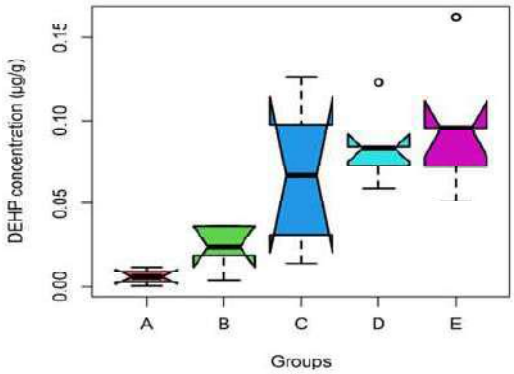

Fig. 3.2: Boxplot representing the median, interquartile, min, max and outliers for DEHP concentration in testis of rats in the groups A, B, C, D & E.

Analysis of Oxidative DNA Damage (8-OH-dG DNA): The levels of 8-OH-dG DNA in testis tissues were observed to be significantly higher in DEHP treated groups as compared to control group (Figure 3.3). The mean±SD of 8-OH-dG DNA level in the testis tissues of group A rats was computed to be 6.9±1.01 ng/L with standard error of 0.45 and confidence of interval ranging from 7.91 to 5.88 ng/L, which was observed to be the lower than the DEHP treated groups. The highest mean±SD of oxidative DNA damage in rats treated with DEHP was found as 11.116±1.407 ng/L in the rats with DEHP exposure for 16 weeks (Group E) with standard error 0.62 and confidence of interval of 9.708 – 12.52 ng/L followed by group D 10.69 ± 1.405 ng/L with standard error of 0.628 and confidence of interval ranging from 9.2899 to 12.1 ng/L, group C 10.27±1.109 ng/L with standard error 0.49 and confidence of interval (9.46 – 12.38 ng/L) and group B 7.32±1.17 ng/L with standard error of 0.524 and confidence of interval from 6.15 to 8.499 ng/L(Figure 3.4). The one-way ANOVA for the difference in the mean values of the different groups were calculated providing p-value < 0.0001showing high level of significance.

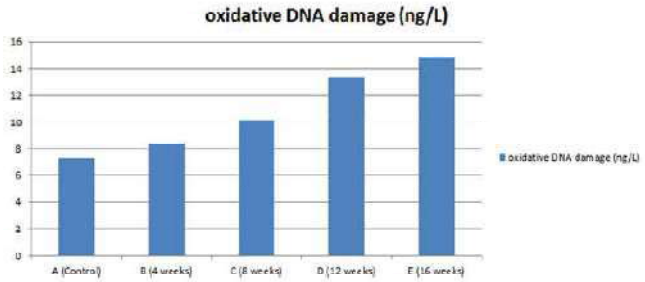

Fig. 3.3: Bar graph representing the level of oxidative DNA damage in testis tissue of rats in the different groups (A, B, C, D & E).

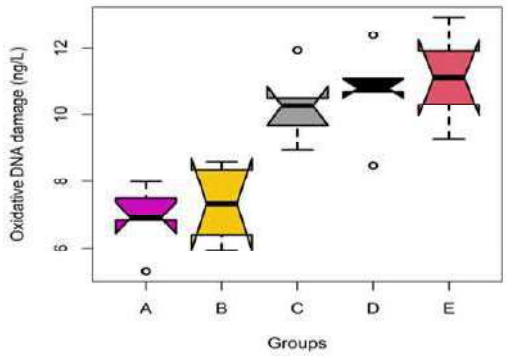

Fig. 3.4: Boxplot representing the median, interquartile, min, max and outliers for oxidative DNA damage levels in testis of rats in the groups A, B, C, D & E.

Estimation of Lipid Peroxidation levels in blood serum: The levels of lipid peroxidation in blood serum were found to be significantly higher in DEHP treated groups as compared to control group (Figure 3.5). The mean±SD of LPO level in the blood serum of group A rats was computed to be 7.34±0.58 nMol/ml with standard error of 0.26 and confidence of interval ranging from 6.76 to 7.92 nMol/ml, which was observed to be the lower than the DEHP treated groups. The highest mean±SD of LPO in rats treated with DEHP was found as 14.76±1.522 nMol/ml in the rats of Group E with standard error 0.68 and confidence of interval of 13.24 – 16.28 nMol/ml followed by group D 13.37 ± 1.808 nMol/ml with standard error of 0.808 and confidence of interval ranging from 11.56 to 15.18 nMol/ml, group C 10.23±1.48 n/ml with standard error 0.66 and confidence of interval (8.75 – 11.71 nMol/ml) and group B 8.14±0.72 nMol/ml with standard error of 0.32 and confidence of interval from 7.42 to 8.87 nMol/ml (Figure 3.6). The one-way ANOVA for the difference in the mean values of the different groups were calculated showing highly significant outcome with p-value < 0.0001.

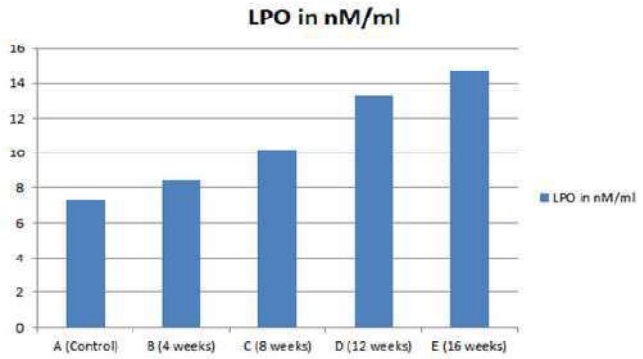

Fig. 3.5- Bar graph representing the level of LPO in all the rats in the different groups (A, B, C, D & E).

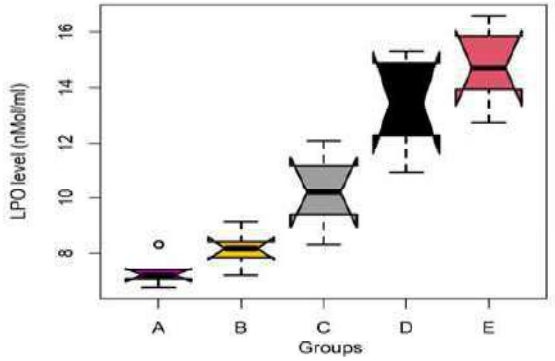

Fig. 3.6- Boxplot representing the median, interquartile, min, max and outliers for LPO levels in serum of rats in the groups A, B, C, D & E.

DISCUSSION

The present study chiefly focuses on the toxic effect of DEHP on male reproductive system of Wistar rats. It presents the critical findings and quantity of toxicity data, which provides adequate evidence towards the impact of DEHP on male reproductive system of rat.

Accumulation in Testis Tissue: The level of DEHP deposited in testis was observed to be significantly high in male Wistar rats exposed to DEHP. A steady rise in the concentration of DEHP in the testis tissues was noted according to increasing length of duration of treatment (Figure 3.1). Studies have reported high levels of phthalate metabolites in other organs of animal models. A study reported high levels of mono-iso-butyl phthalate, mono-n-butyl phthalate, MEHP and DEHP in in the liver of fish roach from Orge River, France.[17] In another study on methods developed to use HPLC to detect DEHP and its metabolite in different organs of rat after single dose treatment, suggested their deposition in kidney, liver, brain, blood plasma and testis.[18]

Oxidative DNA damage (8-OH-dG): The level of oxidative DNA damage in testis tissues was estimated and found to increase significantly with the increasing duration of treatment with DEHP (Figure 3.3). The highest oxidative (8-OH-dG) DNA damage in testis tissues was found in the groups E, followed by D and C (Figure 3.4). It can be observed that group A and B had lower oxidative DNA damage among all the DEHP treated groups. Deoxyguanosine (dG) in DNA is attacked by superoxides due to increased oxidative stress, which lead to transformation of dG into 8-hydroxy-2'-deoxyguanosine (8-OH-dG). The 8-OH-dG is one the most important biomarkers for the determination of oxidative stress which makes the genes to express unusually.[19] The oxidative DNA damage has also been associated with the testicular carcinoma development.[20]

Lipid Peroxidation (LPO) Levels in Blood Serum: The findings of the level of lipid peroxidation in the blood serum for DEHP treated rats for different durations of exposure suggests that level of lipid peroxidation increases with the increase in the duration of exposure (Figure 3.5). It has been hypothesized that oxidative damage to biomolecules, such as proteins, lipids or DNA can significantly induce and accelerate the ageing as well as disturbances in normal developmental process by modulating the genes regulating such phenomena,[21] as well

as inducing various pathological conditions, including varicocele, cryptorchidism, torsion in testis, these could be deleterious to male fertility.[22]

CONCLUSION

As discussed above based on results obtained, it can be concluded that daily exposure to 1000 mg/kg body weight, DEHP plays a very significant role in causing oxidative stress at the level of serum, tissue, and DNA in the testis of rats. Along with these effects, accumulation of DEHP in testis tissue proves it as a potent toxicant that shows gradually increasing deleterious effects with the increase in exposure duration causing damage to the reproductive system targeting it in various ways. Human population is at high risk of exposure to this chemical because of enormous use of plastic and plasticizer in modern life style. So, this study needs to be elaborated further to address the problems related to reproductive system and infertility.

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