Original Paper

Assessment of Diethylphthalate Reproductive Toxicity - A Three Generation Chronic Exposure Study in Wistar Rats

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ABSTRACT

The present study was undertaken to understand the reproductive toxic effect of DEP over three generations of male and female Wistar rats. Healthy male and female albino rats of Wistar strain weighing 75-100g were randomly assigned to two groups of six each. Group I (Control) male and female rats were fed on normal diet and water *ad libitum*. Group II (DEP) male and female rats were given DEP dissolved in corn oil mixed with the diet at 50 mg/kg of the diet/day. 100 days after the treatment, females were mated with males for 10 days. Exposure to DEP was continued throughout mating, gestation until termination at weaning, which was 150 days of total treatment period of parental generation.

The F1 and F2 generation pups were then segregated on the basis of their sex, and six male and female pups of both generations were allowed to grow till they were 75-100g in weight. The treatment was then carried out similar to the parental generation but with reduced dose of 25 mg/kg of the diet/day for F1 generation, and 10 mg/kg of the diet/day for F2 generation. 100 days after the treatment, females were mated with males for 10 days. Exposure to DEP was continued throughout mating, gestation (21 days) until termination at weaning (21 days), which was 150 days of total treatment period of the F1 and F2 generation. After 150 days of treatment, the animals were sacrificed.

A significant decline in relative body and testis weight was observed in DEP treated F1 and F2 generation male rats as compared to control rats of all three generations. On the other hand, a significant increase in relative ovary weights was observed in DEP treated parental, F1 and F2 generation female rats, while relative body weights of the female rats were significantly decreased in F1 and F2 generation treated rats. Significant decrease in litter size was observed over generations in the treated group as compared to controls. A significant increase in testicular LDH and glycogen levels were observed in DEP treated F2 generation male rats as compared to control rats. Significant increase in ovarian LDH, cholesterol and glycogen levels was observed in DEP treated F2 generation female rats as compared to control rats. It can be concluded from this study, that continuous exposure over three generations, inspite of dose reduction to DEP, leads to an enhanced reproductive toxic effect in latter generations.

Keywords: Diethylphthalate (DEP), Testis, Ovary, Enzymes

Introduction

Human studies have always focused on health effects associated with exposure to single environmental chemicals, but exploration of interactions among environmental chemicals is an important area of enquiry.[1] Potential routes of phthalates include dietary ingestion of phthalate-containing foods, inhalation of indoor and outdoor air, and dermal exposure through the use of personal care products that contain phthalates.[2] Phthalates have also been detected in pooled breast milk samples of American women.[3] Manufacturers use low molecular weight phthalates such as diethylphthalate (DEP) in various per-

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sonal care products like colognes, deodorants, and fragrance products as denaturing agent and fixative.[4] Diethylphthalate was found in 71% of the personal care products,[5] 57% of the perfumes, and 25% of the deodorants surveyed,[6] and has also been found in different medical devices, dialysis tubing, and intestinal tubing.[7] Releases are expected to be primarily to water or to soil as a result of leaching from land-fills.[8] In India, DEP is used to a large extent as a perfume binder in the incense stick industry; hence workers in this industry are usually occupationally exposed to DEP, as it involves manual rolling of a paste made by mixing DEP with perfumed saw dust using bare hands.[9]

A survey of fragrance manufacturers conducted in 1995-1996 by the Research Institute for Fragrance Materials reported an annual use of approximately 4000 tonnes of DEP in the preparation of fragrance mixtures.[10] Urinary concentrations of monoethylphthalate (MEP), a metabolite of DEP was found to be significantly higher as compared to the metabolites of other phthalates, in males using cologne and aftershaves, indicating DEP as a main ingredient in many colognes, deodorants and fragrance products at concentrations ranging from <0.1% to 28.6% . Current studies carried out on MEP, indicate associations with DNA damage in human sperm,[11] and sperm motility[12] indicating higher usage of DEP in various consumer products as compared to other phthalates. Prenatal exposure to MEP was also associated with shortened anogenital distance (AGD) in male infants.[13]

DEP studies carried out elsewhere at a very high dose levels for a short period of time, has failed to show any reproductive toxic effects.[10] A continuous breeding study on Swiss CD-1 mice conducted by National Toxicology Program at higher doses (340, 1770 and 3640 mg/kg body weight) of DEP for 14 weeks also showed lower reproductive effects.[14]

The assessment of permanent effects of xenobiotics on adult organ functions and tissue metabolisms, after perinatal exposure, is a specialized field in toxicology.[15] Taking all these facts into consideration, an experiment was designed to study the reproductive toxic effects of DEP through the diet over three generations on male and female Wistar rats.

Materials and Methods

Chemicals

Diethylphthalate (DEP) (CAS No. 84-66-2) 99.9% was purchased from E. Merck, Mumbai, India. All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India and were of analytical grade (99.99%).

Animals and Treatments

Healthy male and female albino rats of Wistar strain weighing 70-100 g (6-7 weeks old) were obtained from Haffkine Institute, Mumbai, India and the maintenance of the animals were as per national guidelines laid down by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) no.373/01/ab/CPCSEA. One week after arrival, the male and female albino rats were randomly assigned to two groups of six each. Group I male and female rats were fed on normal diet and water *ad libitum*. Group II male and female rats were given DEP dissolved in corn oil mixed with the diet at 50 mg/kg of the diet/day (2.85 mg/kg body wt/day). 100 days after the treatment, females were mated with males for 10 days. Exposure to DEP was continued throughout mating, gestation (21 days) until termination at weaning (21 days), which was 150 days of total treatment period of the parental generation.

The F1 and F2 generation pups were then segregated on the basis of their sex, and six male and female F1 and F2 generation pups were allowed to grow till they were 75-100 g in weight. The treatment for the F1 generation rats was then carried out similar to the parental generation but with doses reduced to 25mg/kg of the diet/day (1.425 mg/kg body wt/day), and to the F2 generation 10 mg/kg of the diet/day (0.57 mg/kg body wt/day). 100 days after the treatment, the F1 generation females were mated with males for 10 days. Exposure to DEP was continued throughout mating, gestation (21 days) until termination at weaning (21 days). The total treatment period of parental, F1 and F2 generation adult male and female rats was 150 days.

Minimal amount of corn oil was used as a vehicle in all generations, as the test material was lipophilic in nature. The diet was prepared fresh daily and with the increase in weight of the animals; the diet was proportionately increased on weekly basis to maintain the concentration of the dose. During the treatment animals were weighed thrice a week and food consumption was monitored daily

for dose calculations. The rats were anesthetized with diethyl ether.

Enzyme and Biochemical Parameters Assayed in Testis and Ovary for Parental, and F1 Generation

Testes from male rats and ovaries from female rats were dissected out, washed in ice-cold saline, blotted with dry filter paper and quickly weighed on a chilled watch glass. Ten percent of testicular and ovary homogenate was prepared in ice-cold saline and used for the estimation of enzyme activity. The enzymes assayed were acid phosphatase (ACP)[16] and lactate dehydrogenase (LDH),[17] while biochemical parameters estimated were total cholesterol[18] and glycogen.[19] Protein in the testis and ovary homogenate was estimated by Lowry method,[20] and specific activity of enzyme was calculated.

Statistical Analysis

Data was analyzed using a two-way ANOVA. Several tests of the data were performed to ensure that the data conformed to the assumptions of the two-way ANOVA. Mean value given as ± S.D with significance given at P ? 0.05.

Results

A significant decrease in litter size was observed in case of F0 generation DEP treated rats as compared to the controls (**Table 1**). Reduction in litter number was also observed in F1 generation DEP treated rats which was much more significant than F0 generation DEP treated rats. Normal breeding was observed in case of the F0 and F1 generation control rats.

A significant decrease in relative body weights was observed in F1 and F2 generation DEP treated male rats as compared to controls of all generations (**Table 2**). Significant decrease in relative testes weights was also observed in F0, F1 and F2 generation DEP treated rats as compared to three-generation control rats (**Table 2**). Reduction in testis weight was much more significant in F1 and F2 generation DEP treated rats as compared to F0 generation DEP treated rats.

Significant decrease was also observed in relative body weights of F1 and F2 generation DEP treated female rats as compared to controls (**Table 3**). A significant increase in ovary weight was observed in F0, F1 and F2 generation DEP treated rats, as compared to three-generation control rats.

Table 1 Number of Dams in F1 and F2 generations to number of bred mothers of DEP treated rats for three generations

Groups	Sex	Control	Oil control
No of dams (F1)/ No of bred mothers	Males Females	24/6 30/6	28/6 26/6
No of dams (F2)/ No of bred mothers	Males Females	24/6 26/6	25/6 22/6

Table 2 Relative body weight and testis weight of all treated groups for three generations

Groups / Tissue Wts (gms)	Control	Oil control	DEP
Body weight	369.33 ± 26.85	408.66 ± 16.553	423 ± 15.27
Testis weight (F0)	3.25 ± 0.05	3.26 ± 0.0235	3.148 ± 0.0151^{+}
Body weight (F1)	381 ± 20.132	400.12 ± 10.215	248.5 ± 5.219 *
Testis weight (F1)	3.166 ± 0.1154	3.201 ± 0.0045	2.9 ± 0.12^{++}
Body weight (F2)	311.01 ± 6.645	325.6 ± 10.245	262.60 ± 8.5906*
Testis weight (F2)	3.254 ± 0.082	3.231 ± 0.051	2.67 ± 0.12041^{++}

Table 3 Relative body weight and ovary weight of all treated groups for three generations

Groups / Tissue Wts (gms)	Control	Oil control	DEP
Body weight	281.33 ± 42.015	270 ± 43.5889	306.66 ± 61.1010
Ovary weight (F0)	0.1005 ± 0.0072	0.101 ± 0.0035	0.14 ± 0.0032*
Body weight (F1)	265.5 ± 5.021	272.365 ± 10.21	213.35 ± 15.27+
Ovary weight (F1)	0.112 ± 0.008	0.1005 ± 0.00552	0.138 ± 0.004*
Body weight (F2)	280.3 ± 3.545	292.25 ± 1.2468	176.33 ±10.504+
Ovary weight (F2)	0.11 ± 0.0045	0.0982 ± 0.01	0.129 ± 0.003*

Testicular ACP activity was significantly increased in F2 generation DEP treated rats as compared to controls of three generations, but levels were lower as compared to F0 and F1 generation DEP treated rats. ACP activity in F0 generation was significantly higher than as compared to F1 and F2 generation DEP treated rats.

Testicular LDH activity was significantly increased in F0 and F2 generation DEP treated rats as compared to controls three generations and F1 generation DEP treated rats. Levels were significantly high in the F1 generation DEP treated rats compared to controls, but were lower than as compared to F0 and F2 generation DEP treated rats. No significant difference was observed in LDH activity between F0 and F2 generation DEP treated rats. Cholesterol levels in testes were significantly increased in F0 and F1 generation DEP treated rats as compared to controls of three generations and F2 generation DEP treated rats. A significant increase in cholesterol levels in testes was observed in F0 generation DEP treated rats as compared to F1 generation DEP treated rats. Compared to the F0 and F1 generation DEP treated rats, cholesterol level in the testes was significantly low in F2 generation DEP treated rats, but not significant as compared to controls.

On the other hand, testes glycogen levels were significantly increased in F1 and F2 generation DEP treated rats as compared to controls and F0 generation DEP treated rats. No significant difference was observed between testes glycogen levels of F1 and F2 generation DEP treated rats. Testes glycogen level was significantly low in F0 generation DEP treated rats as compared to F1 and F2 generation DEP treated rats, but not significant as compared to controls.

Ovary ACP activity was significantly increased in F0 and F1 generation DEP treated rats as compared to control rats and F2 generation DEP treated rats. ACP activity in the ovary of F0 generation DEP treated rats was significantly increased as compared to F1 generation DEP treated rats, where as in F2 generation DEP treated rats, ovary ACP activity did not show any significant change as compared to controls, but significantly lower than F0 generation DEP treated rats.

Ovary LDH activity was significantly increased in F2 generation DEP treated rats as compared to controls of three generations, but levels were lower than as compared to F0 and F1 generation DEP treated rats. Ovary LDH levels were significantly higher in F0 generation DEP treated rats as compared to controls, F1 and F2 generation DEP treated rats.

Cholesterol levels in ovary were highly significant in F2 generation DEP treated rats as compared to controls of three generations and F0 and F1 generation DEP treated rats. Cholesterol levels in ovary of F0 generation DEP treated rats were significantly higher than controls, but significantly lower than F1 and F2 generation DEP treated rats. Ovary glycogen levels were significantly increased in F0, F1 and F2 generation DEP treated rats as compared to control rats of three generations. No significant difference in ovary glycogen levels was observed between F0, F1 and F2 generation DEP treated rats.

Discussion

General population exposure to DEP occurs by daily use of various consumer products that contain DEP at low as well as high concentrations, hence usage would be several times a day for a prolonged duration. MEP urinary concentrations in human populations have been increasing in the past few years,[2] indicating a concern for

DEP, as it is being used extensively and has been confirmed to be least reproductive toxic at very high doses.[10] DEP is also known to be transported through the placenta,[21] making it more susceptible to the future generations.

In a recent study, DEP has been found to be least toxic over two generations at concentrations ranging from 600 to 15000 ppm through dietary exposure.[22] It is evident from the present study that continuous administration of DEP over three generations leads to enhanced toxic effect in latter generations in male and female rats. This is evident from the decreased relative testis weight, increased relative ovary weight, remarkably decreased ACP activity of testis and ovary in F2 generation DEP treated rats as compared to the F0 and F1 generation, indicating inhibitory effect of DEP on this enzyme due to continuous exposure to DEP. Increased LDH activity in the testes of F2 generation DEP treated rats indicates tissue injury, which could be due to the continuous toxicant insult to the organs at smaller doses over three generations. On the other hand, LDH activity in the ovary showed steady decrease over the generations, indicating inhibitory effect of DEP on the enzyme due to continuous exposure.

In addition, ovary cholesterol and glycogen metabolism were also severely impaired in F2 generation DEP treated rats, which was quite different in testes of F2 generation rats. Significant accumulation of cholesterol in the ovarian tissue of F2 generation DEP treated rats indicates that DEP impairs cholesterol metabolism in the ovary leading to its steady accumulation over the generations due to continuous exposure to DEP. This may lead to reproductive failure or reduced reproductive potential. From the litter number obtained from breeding F1 generation DEP treated individuals, it is evident that DEP does affect reproductive potential, hence the reduced litter number.

On the other hand, the cholesterol levels in testes of F2 generation DEP treated rats were remarkably low, indicating that continuous exposure to DEP leads to depletion of cholesterol reserves in testes. This may lead to impaired function of the testes and reduced levels of testosterone, because cholesterol acts as a precursor of testosterone synthesis.

It is also possible that cytochrome P450 side-chain cleavage enzyme (P450scc), which is located on the matrix side of the inner mitochondrial membrane involved in transport of cholesterol and its metabolites for steroidogenesis,[23,24] might be affected by the DEP toxicity, leading to increased cholesterol accumulation in the ovary, which needs to be looked into.

Significant accumulation of glycogen in the testis and ovary due to continuous exposure to DEP over three generations indicates that DEP impairs glycogen metabolism by leading the carbohydrate metabolism towards anaerobiosis (glycolysis) rather than oxidative phosphorylation. This could be one of the reasons for reduced reproductive potential in DEP treated rats over successive generations.

It can be concluded from this study that continuous exposure to DEP over more than one generation because of transplacental transfer, and transfer through mother's milk, as well as further exposure at lower concentrations through the diet leads to a significant reproductive toxic effect in the latter generations of both sexes. Further studies could help in understanding the risk associated with such pollutants, as well as mechanistic aspects of reproductive toxicity.

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