THE MALE REPRODUCTIVE EFFECTS OF LOW-DOSE EXPOSURE OF DEHP IN RATS

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Introduction

Di(2-ethylhexyl) phthalate (DEHP), a well-known high molecular weight phthalate, is used as a plasticizer in the manufacture of flexible polyvinyl chloride (PVC). Experimental animal studies have demonstrated that several phthalates are related to anti-androgenic activity and male reproductive toxicity[1, 2]. Several epidemiological studies have shown that phthalates may have adverse effects on human conventional semen quality[3-5]. We recently reported semen quality data that demonstrated an association between DEHP concentration in ambient air and adverse effects on sperm motility and potential chromatin DNA damage in male workers employed at PVC pellet plants[6]. However, little research has been conducted to investigate the male reproductive effects of low-dose exposure to DEHP in rats. The aim of this study is to examine male reproductive effects of low-dose exposure to DEHP and evaluate the lowest-observed-effect level (LOEL) for sperm function in male rats.

Materials and methods

We designed a study to investigate the effects of DEHP exposure of DEHP on male reproductive function. DEHP (99.9%) was obtained from Aldrich-Chemie. A total number of 75 six-week old male Sprague-Dawley rats were randomly divided into test and control groups, and gavaged with 30, 100, 300 and 1000 μg/kg of DEHP and corn oil per day for 9 weeks. On Day 105, body, liver and reproductive organ weights, cauda epididymal sperm count, motility, morphology, chromatin DNA structure, and oxidative stress were investigated. Body and reproductive organ weight: The animals were sacrificed by CO_2 and the paired testes, paired epididymis and seminal vesicles were removed and weighed. Relative organ weight was measured by calculating the ratio between organ weight and body weight. The left cauda epididymis was used to perform sperm chromatin structure analysis (SCSA). The extent of DNA denaturation per cell was quantified as alpha-T (αT), and expressed as the tendency of sperm chromatin DNA damage. Spermatozoa with chromatin DNA damage were represented by DNA fragmentation index (DFI), and expressed as the percentage of sperm with chromatin DNA damage. Sperm SCSA and reactive oxygen species (ROS) generation were measured using a FACScan flow cytometry (FCM) (BD Immunocytometry Systems, San Jose, CA, USA). A total of 10,000 spermatozoa were collected and analyzed at a flow rate of 100~200 cells/s.

Results and discussion

Body and reproductive organ weights:

There were no significant differences in relative weights of body, testis, kidney, epididymis, cauda epididymis, and seminal vesicles among the four groups exposed to DEHP exposed and the controls (Table 1). Liver weight was found to be significantly more decreased in the groups gavaged with 1000 μg/kg of DEHP than in control ($p < 0.05$) (Table 1).

Table 1. Body and tissue weight between rats postnatally exposed to di(2-ethylhexyl) phthalate (DEHP) (30, 100, 300 and 1000 μg/kg) and unexposed controls.

Parameter	Treatment of DEHP (μg/kg)				
	Control (n=15)	30 (n=15)	100 (n=15)	300 (n=15)	1000 (n=15)
Body weight (kg)	0.5±0.1	0.5±0.1	0.5±0.04	0.5±0.1	0.5±0.1
Left testis (g/kg B.W.)	2.9±0.6	3.3±0.4	3.1±0.5	3.1±0.3	3.2±0.4
Right testis (g/kg B.W.)	2.9±0.6	3.2±0.4	3.2±0.4	3.1±0.3	3.2±0.4
Left epididymis (g/kg B.W.)	1.0±0.3	1.0±0.2	1.0±0.2	0.9±0.1	1.1±0.6
Right epididymis(g/ kg B.W.)	1.0±0.3	1.0±0.2	1.0±0.2	1.0±0.1	1.0±0.2
Left cauda(g/ kg B.W.)	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
Right cauda(g/ kg B.W.)	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
Liver(g/ kg B.W.)	38.9±2.9	40.5±2.5	38.1±3.3	37.7±2.1	35.9±1.9*
Left kidney(g/ kg B.W.)	4.0±0.5	4.1±0.4	4.1±0.4	4.0±0.3	4.1±0.2
Right kidney(g/ kg B.W.)	4.1±0.5	4.1±0.5	6.7±10.1	4.6±2.4	4.1±0.2
Seminal vesicle(g/ kg B.W.)	3.0±0.5	3.0±0.6	3.1±0.4	3.1±0.5	3.1±0.4

Data was expressed mean ± standard deviation. * $p < 0.05$ as compared with control group.

Sperm quality:

There were also no significant differences in sperm count, motility, percentage of sperm with excessive superoxide anion (O_2 -) generation, and intensity of sperm with excessive O_2 - generation between the groups exposed to DEHP and the controls (Table 2). We found a significant increase in percentage of DFI in the 100, 300 and 1000 μg/kg groups compared to that of the control group. Meanwhile, the intensity of Sperm αT was found to be significantly more increased in the 30, 100, 300 and 1000 μg/kg groups than in controls ($p < 0.05$). In addition, the group exposed to 1000 μg/kg was found to have a significantly higher percentage and intensity of sperm hydrogen peroxide (H_2O_2) generation than control group (Table 2).

Table 2. Sperm quality, epididymal sperm chromatin structure assay (SCSA), mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) generation in rats exposed to di(2-ethylhexyl) phthalate (DEHP) and unexposed control group.

Parameter	Treatment of DEHP (μg/kg)				
	Control (n=15)	30 (n=15)	100 (n=15)	300 (n=15)	1000 (n=15)
Sperm motility (%)	21.5±11.4	29.5±11.7	19.9±5.9	25.1±14.0	18.6±11.8
Sperm count (10^6/mL)	10.1±5.0	11.0±3.7	13.1±4.0	11.1±4.6	11.1±4.6
DFI (%)[a]	2.1±1.0	3.8±2.1	4.8±2.1*	5.7±2.2*	6.4±2.1* [#]
αT (AU)[b]	339.2±8.3	351.7±6.1*	358.1±11.2*	360.6±10.8*	362.3±9.3* [#]
Sperm $O_2^{-}\cdot$ (%)[c]	16.8±17.0	12.6±14.0	12.9±15.1	15.6±14.6	13.8±13.8
Sperm $O_2^{-}\cdot$ (AU)[d]	10.0±5.2	8.5±1.8	8.5±2.0	8.7±2.3	8.4±1.7
Sperm H_2O_2 (%)[c]	16.2±6.6	25.9±6.4	35.5±6.6	30.0±6.4	45.5±6.9*
Sperm H_2O_2 (AU)[d]	6.6±2.9	8.6±2.2	9.6±3.5	9.2±2.5	10.4±2.7*

Data was expressed mean ± standard deviation. * $p < 0.05$ as compared with control group. [#] $p < 0.05$ as compared with 30μg/kg group.

[a] the percentage of cells outside the main population (% DFI)

[b] αT expressed in fluorescence channel units.

[c] percentage of sperm with excessive H_2O_2 or O_2 - generation.

[d] fluorescence intensity.

Relationships among sperm DNA damage and ROS generation:
We performed a regression analysis of SCSA and H_2O_2 generation in Figure 1. The αT was positively and significantly associated with percentage of sperm with excessive H_2O_2 generation ($p = 0.025$) and the intensity of sperm with H_2O_2 generation ($p = 0.01$).

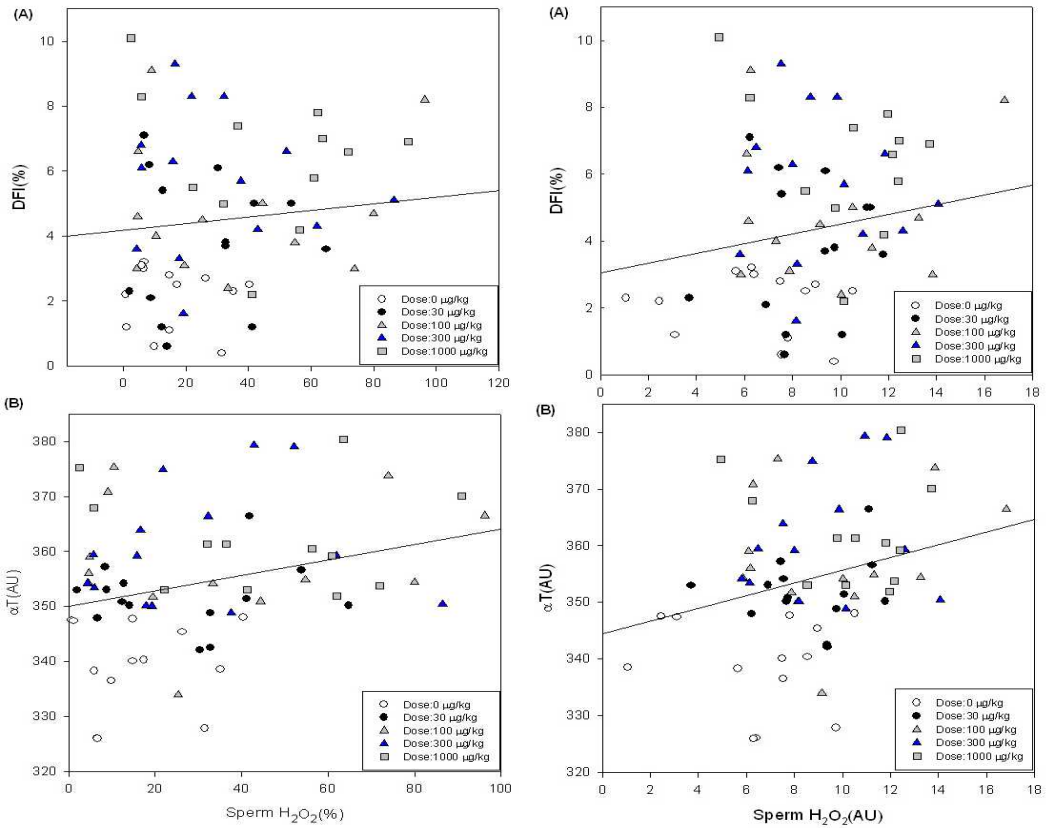

Figure 1. Relationships among sperm chromatin structure assay (SCSA) and hydrogen peroxide (H_2O_2) generation by general linear model. (Left A, B) Increased DFI(%) and αT in relation to percentage of sperm with excessive H_2O_2 generation [DFI (%) = 4.01 + 0.02 × sperm H_2O_2, $p = 0.15$ for Left A; αT = 350.21 + 0.14 × sperm H_2O_2(%), $p = 0.025$ for Left B]. (Right A, B) Increased DFI(%) and αT in relation to intensity of sperm with H_2O_2 generation. [DFI (%) = 3.04 + 0.17 × sperm H_2O_2 (AU), $p = 0.09$ for Right A; αT(AU) = 342.79 + 1.31 × sperm H_2O_2(AU), $p = 0.01$for Right B].

Discussion:
It has been known for some time that the spermatozoa of subfertile patients contain particularly high levels of 8-hydroxy-2'-deoxyguanosine. It was the major oxidized base adduct formed when DNA is subjected to attack by ROS[7]. It seems reasonable to propose that oxidative stress is one of the major contributors to DNA damage in the male germ line[8]. Furthermore, oxidative stress and DNA damage might be induced by xenobiotics that activate ROS generation by spermatozoa. Animal studies have concluded that high phthalate exposure might lead to significant ROS production in the testicular environment bringing about depletion in the testicular antioxidant system as well as the hormone levels in the serum[9]. In an epidemiological study found that the mean DFI % of 5.3–23.8[10] indicated that the spermatozoa would be able to fertilize an oocyte and give concern to healthy offspring, considering that the threshold value for DFI % in vivo fertility potential respectively[11, 12]. Our study has found that the LOEL for sperm toxicity was found to be oral 30 μg DEHP/kg/day in sperm chromatin

DNA integrity and 1000 μg DEHP/kg/day in sperm H_2O_2 generation and liver weight on postnatal Days 56–105 in the male rats. This study represents the first indication of the adverse effect of DEHP on sperm function.

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