

The Effects of Subacute Inhalation of Di (2-ethylhexyl) Phthalate (DEHP) on the Testes of Prepubertal Wistar Rats

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Abstract: The Effects of Subacute Inhalation of Di (2-ethylhexyl) Phthalate (DEHP) on the Testes of Prepubertal Wistar Rats: Norie KURAHASHI, *et al.* Department of Public Health, Hokkaido University Graduate School of Medicine—In animal studies using oral dosing for short periods, di (2-ethylhexyl) phthalate (DEHP) is well known for its reproductive toxicity, especially for its testicular toxicity. However, extending the period of DEHP exposure in prepubertal rats resulted in significant increases in testosterone. This suggests that the reproductive effect of DEHP might be associated with the timing and the term of exposure. Moreover, the route of exposure may induce differences in its effect because tissue levels of metabolites of DEHP after inhalation are thought to be different from those after oral administration. We researched the effects of inhalation of DEHP on testes of prepubertal rats. Our results showed that inhalation of DEHP by 4-wk-old male Wistar rats at doses of 5 or 25 mg/m³, 6 h per day, for 4 and 8 wk significantly increased the concentration of plasma testosterone and weight of seminal vesicles. However, the concentration of luteinizing hormone (LH), follicular stimulating hormone (FSH) and the expression of mRNAs of androgen biosynthesis enzyme, cytochrome P450 cholesterol side-chain-cleavage enzyme (P450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17 α -hydroxylase/17, 20 lyase (CYP17) and aromatase (CYP19) did not change. Rats with precocious testes did not increase in any of the DEHP groups. We also found that the estimated effective dose in this study was less than those reported in previous studies which used oral dosing. Our study showed that inhaled DEHP increased plasma

testosterone concentrations in prepubertal rats and suggested that their effects were more sensitive to inhalation of DEHP than oral dosing.

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Key words: Di (2-ethylhexyl) phthalate (DEHP), Inhalation, Rats, Testosterone, Testis, Expression of mRNA of androgen biosynthesis enzyme

Di (2-ethylhexyl) phthalate (DEHP) is a manufactured chemical that is commonly added to plastics to make them flexible. DEHP is present in plastic products such as wall coverings, floor tiles, some toys, automobile upholstery and tops, packaging film and sheets, medical tubing and blood storage bags¹, and it is widely found in food and the environment. A recent investigation by Blount *et al.*² demonstrated that the urinary levels of phthalates in humans are much higher than generally expected. Although DEHP has a relatively low vapor pressure, it appears to be a common air contaminant and is globally present³. The release of DEHP directly into the atmosphere is the most important mode of entry into the environment³. Absorption of DEHP from the atmosphere is most likely to occur by inhalation since the dermal absorption of DEHP in rats is low⁴.

The concentration of DEHP in Canada for the general population has been documented to be 3 $\mu\text{g}/\text{m}^3$ ⁵. Uhde *et al.*⁶ reported that the maximum DEHP concentration in a room with wallcoverings coated by polyvinyl chloride was 0.94 $\mu\text{g}/\text{m}^3$. In another study, the median concentration of DEHP in 27 houses was reported as 0.11 $\mu\text{g}/\text{m}^3$ ⁷. In the air inside cars, DEHP has been found at levels ranging from 1 to 34 $\mu\text{g}/\text{m}^3$ ¹. Thus, the entire general population might have inhalation exposure to DEHP. Children and adolescents are generally recognized as being particularly at risk for greater exposure to environmental contaminants because children's air intakes relative to body weight are high compared to adults⁸.

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DEHP is well known for its testicular toxicity in laboratory animals⁹⁻¹⁵ and mono ethylhexyl phthalate (MEHP) which is a metabolite of DEHP is thought to be responsible for the testicular toxicity attributed to DEHP exposure¹⁶⁻¹⁸.

In oral DEHP exposure, DEHP is hydrolyzed in the small intestines and absorbed as MEHP and 2-ethylhexanol¹⁹. On the other hand, DEHP can be introduced directly into the circulatory system by inhalation as evidenced by identification of DEHP derivatives in the blood of infants exposed to DEHP during respiration therapy²⁰. Eighty percent of the oral dose was converted to MEHP while only 1% of the intra-arterial or intraperitoneum dose was converted²¹. Thus, after inhalation, tissue levels of DEHP are thought to be higher and the levels of MEHP might be lower than those after oral administration. Disparities in the amounts of metabolites may induce the differences in the toxic effects of DEHP seen between oral administration and inhalation.

Recently, Parks *et al.*²² reported that the testicular atrophy of rats exposed to DEHP during gestation was induced by decreasing fetal testosterone levels. Kim *et al.*¹⁵ reported that significant decreases of both the testis weight and testosterone concentration were observed in 4-wk-old rats treated with DEHP for 5 d. Similarly, Akingbemi *et al.*¹⁴ reported that in prepubertal rats exposed to DEHP for 14 days by gavage, Leydig cell testosterone production was reduced. In contrast, extending the period of DEHP exposure to 28 d resulted in significant increases in Leydig cell testosterone production^{14,23}. These reports suggest that the reproductive effect of DEHP is associated with the timing and/or term of exposure.

Although there are many studies about oral exposure to DEHP, there is little information on inhalation toxicity. Kilmish *et al.*²⁴ exposed 9-wk-old Wistar rats to DEHP aerosols in a head-nose inhalation system for 28 d and found no testicular toxicity, but the effects in prepubertal rats exposed to DEHP by inhalation are unknown. It is important to research the effects of inhalation during the prepubertal and pubertal periods, because this phase is a time of dramatic endocrine changes that are required for sexual maturation and might be the periods during which rats are most susceptible to any latent reproductive and developmental toxic effects of DEHP²⁵.

The object of the present study was to investigate the effects of DEHP inhalation on the testes of prepubertal rats and to test the hypothesis that the effect on the testis differs according to the route of exposure.

Materials and Methods

Animals and tissue collection

All procedures were performed according to a protocol approved by the Animal Care and Use Committee of the Laboratory of Animal Experimentation in Hokkaido University. Thirty-six 21-d-old male Wistar rats were

obtained from the Institute for Animal Experimentation in Hokkaido University. Rats were housed under controlled temperature and lighting conditions with a 12 h day: night cycle. Commercial food pellets (Oriental Yeast CO., Ltd.) and tap water were available *ad libitum* except during experimental exposure to DEHP.

Rats at 28 d of age were divided into 3 groups (control, low-dose and high-dose) with 12 animals per group. Allocation of animals to dose groups was done by body weight randomization to ensure equal weight distribution among the groups. Rats, except for those in the control group, were exposed by inhalation for 6 h per day, 5 d per week to low-dose (5 mg/m³) or high-dose (25 mg/m³) concentrations of DEHP. To research the effects of DEHP on male prepubertal rats with regard to testis development, we analyzed half the rats after 4-wk of inhalation, and the remaining animals were exposed to DEHP for another 4 wk.

Animals were exposed whole body in stainless steel inhalation chambers (70 × 70 × 52 cm, with a pyramidal top) under dynamic airflow conditions (approximately 15 air changes/h). The flow through each chamber was maintained at 25 l/m³. Di (2-ethylhexyl) phthalate (CAS No. 117-82-7), 99.9% pure, was purchased from Amersham Pharmacia Biotech UK, Ltd. DEHP was continuously supplied to a special exposure machine (Shibata Scientific Technology, Ltd.). DEHP was contained in a flask and vaporized with bubbling by glass-filtered air. The base of the flask was immersed in a temperature-controlled oil bath maintained at 90°C for low-dose DEHP and 130°C for high-dose DEHP. The DEHP vapor concentration was assayed with a gas chromatograph (GC-8APF, Shimadzu Corporation, Ltd.). The analytical column was a packed glass column (Silicone OV-1.5%, 2.0 m × 3.2 mm I.D.), carrier gas flow was 60 ml/min N₂, and the column temperature was 220°C. We measured the DEHP concentration once a day. The mean concentration of DEHP in the gas chamber was 5.1 ± 1.3 mg/m³ for low-dose DEHP and 24.6 ± 5.2 mg/m³ for high-dose DEHP exposure. Control rats were exposed to air under identical conditions.

The rats were dissected under ether anesthesia on the day which exposure was finished. Blood was obtained by heart puncture. Plasma was collected by centrifugation at 1,500 rpm for 15 min and stored at -80°C for measurement of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Testes were removed, and weighed on a scale. We also weighed the epididymis, seminal vesicles and ventral prostate. One testis was used for histopathological examination. The other testis was immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Hormone determinations

The plasma concentration of testosterone was measured

Table 1. Primer Sets for Real-Time Quantitative RT-PCR Analyses

Gene	Forward primer	Reverse primer
Cyclophilin	CACTGGTGGCAAGTCCATCTA	CATTTGTGTTTGGTCCAGCATT
P450 _{scc}	TCCAGAGGACCCAGGTCAA	CATGAGGTGTCAAATCTTGGACTT
3 β -HSD	CTAGTCCACAAGCTTTCTTCCTAAT	ACTCAGCTCTAAGTGACAGGAAATTG
CYP17	ACTATCCGAGAAGTGCTGCGTAT	TGGAGTCAACGTTAGCCTTGTG
CYP19	TGGAATCCATCAAGCAGCATT	GCGTGTTAGAAGTGTCCAGCAT

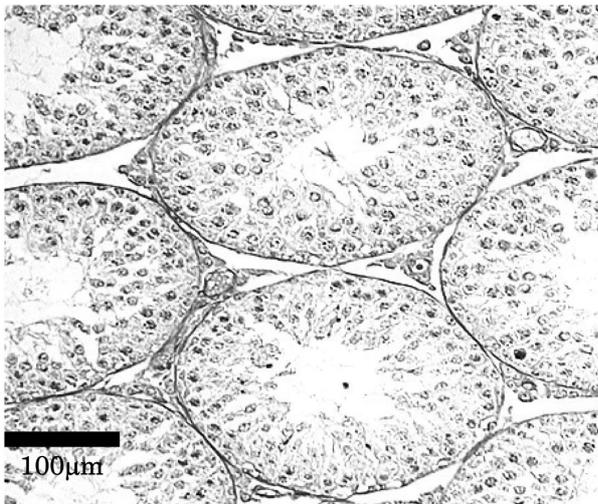


Fig. 1A. Immature seminiferous tubules in the testis of a control rat after 4 wk exposure to DEHP by inhalation (8 wk of age at the examination). Although these tubules are post-meiotic tubules, only a few elongated spermatids are observed in the seminiferous epithelia.

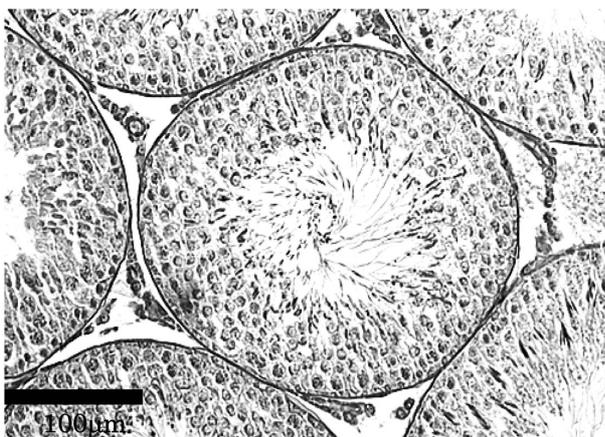


Fig. 1B. Mature seminiferous tubule in the testis of a rat treated by inhalation of low-dose DEHP for 4 wk (8 wk of age at the examination). This tubule is at stage VII and many elongated spermatids are observed at the inner edge of the seminiferous epithelium.

by radio immunoassay (RIA). We measured plasma concentrations of LH and FSH by enzyme immunoassay (EIA). Testosterone was measured at Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. The test kits for LH and FSH used were the Rat Luteinizing Hormone (rLH) Biotrak EIA system and Rat Follicle Stimulating Hormone EIA system (Amersham Pharmacia Biotech UK, Ltd.), respectively. LH and FSH were assayed according to the manufacturer's suggested protocol.

Reverse transcription and real-time polymerase chain reaction

Total RNA was isolated from testis using ISOGEN (Nippon Gene Co., Ltd.) and the phenol-chloroform extraction method. Synthesis of cDNA was performed with 5 μ g of total RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences UK, Ltd.) according to the manufacturer's suggested protocol. Semiquantitative PCR was performed using SYBR Green PCR Master Mix reagent kits according to the manufacturer's instructions for quantification of gene expression (Applied Biosystems) and the results were analyzed on a Gene Amp 5700 Sequence Detection System (Applied Biosystems). The reverse transcription-polymerase chain reaction (RT-PCR) was used for the relative quantitation of mRNAs of cytochrome P450 cholesterol side-chain-cleavage enzyme (P450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and cytochrome P450 17 α -hydroxylase/17, 20 lyase (CYP17) and aromatase (CYP19) aromatized from testosterone to estrogen. Cyclophilin was used as an internal calibrator for all RT-PCR reactions. Primers were chosen with the assistance of a computer program Primer Express (Applied Biosystems). The primer sequences of cyclophilin, P450_{scc}, 3 β -HSD, CYP17 and CYP19 are listed in Table 1.

Histology of the testis

Tissue was fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μ m, and stained with PAS and hematoxylin. All cross-sections of the seminiferous tubule in 1 transverse section of the testis were examined. In addition to the histopathologic changes, the progression of spermatogenesis was also evaluated. When the

Table 2. Effects of prepubertal (from 28 d of age) administration by inhalation of low-dose (5 mg/m³) or high-dose (25 mg/m³) DEHP on organ weights of male rats

	Control	Low-dose DEHP	High-dose DEHP
Body weight (g)			
4 wk exposure	176.6 ± 18.7	175.5 ± 9.5	169.7 ± 13.2
8 wk exposure	272.0 ± 12.3	283.7 ± 20.4	273.3 ± 24.2
Testis weight (g)			
4 wk exposure	0.807 ± 0.225	0.948 ± 0.097	0.936 ± 0.166
8 wk exposure	1.332 ± 0.035	1.369 ± 0.049	1.344 ± 0.100
Epididymis weight (g)			
4 wk exposure	0.144 ± 0.029	0.165 ± 0.031	0.157 ± 0.038
8 wk exposure	0.416 ± 0.076	0.374 ± 0.039	0.388 ± 0.055
Seminal vesicle weight (g/100 g bw)			
4 wk exposure	0.104 ± 0.051	0.174 ± 0.055	0.165 ± 0.044
8 wk exposure	0.327 ± 0.034	0.426 ± 0.035*	0.428 ± 0.017*
Ventral prostate weight (g/100 g bw)			
4 wk exposure	0.054 ± 0.021	0.076 ± 0.015	0.066 ± 0.011
8 wk exposure	0.111 ± 0.021	0.118 ± 0.013	0.126 ± 0.017

Results are expressed as Mean ± SD (n=6). * $p < 0.01$ (significantly different using Scheffe's test after one-way ANOVA)

progression of spermatogenesis was not sufficient quantitatively (Fig. 1A), the seminiferous tubule was classified as an immature tubule, and when the progression was quantitatively sufficient (Fig. 1B), the tubule was classified as a mature tubule. Almost all immature tubules were post-meiotic tubules in this study. After the examination, the proportions of tubules with histopathologic changes and immature tubules were evaluated based on a semiquantitative grading system²⁶. Histologic examinations were carried out blind to the treatment.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS (version 10.0). When statistically significant effects ($p < 0.05$) were detected in the overall ANOVA model, Scheffe's test was used for comparisons among the three groups.

Results

Body and reproductive organ weights

After 4 or 8 wk of exposure to DEHP, there were no significant differences in terminal body weight. The testis and epididymis weights in rats treated with DEHP for 4 wk were slightly heavier than those of the control rats. After 4 and 8 wk of exposure, the weights of the seminal vesicles of both the low- and high-dose groups were heavier than that of the control group. The difference was marginally significant ($p = 0.06$) after 4 wk and significant ($p < 0.01$) after 8 wk. There was no significant change in the weight of the ventral prostate at any of the doses tested (Table 2).

Plasma concentrations of testosterone, LH and FSH

After 4 wk exposure, plasma testosterone had significantly increased in rats in the low-dose DEHP group compared to the control group ($p < 0.05$), and it was also higher in the high-dose DEHP group than in the control group, but the difference was not significant. After 8 wk exposure, the concentration of plasma testosterone had increased significantly in both the low- and high-dose groups ($p < 0.05$) (Fig. 2). Plasma LH and FSH levels did not change at any exposure level (Figs. 3, 4).

The expression of mRNA of steroidogenic enzymes

Figures 5-A and B show the expression of the mRNAs of four enzymes involved in testosterone biosynthesis, P450scc, 3 β -HSD, CYP17 and CYP19. The expression of the mRNAs of the four enzymes did not significantly change.

Histology of the testis

In the testis of the rats with 4 wk exposure to DEHP (8 wk of age at the examination), regardless of the treatment, there were few histopathologic changes except for germ cell degeneration. The proportion of tubules with germ cell degeneration was relatively high at stage VII and exceeded 5% in some rats. However, this germ cell degeneration was not regarded as pathologic because increased germ cell degeneration is associated with immaturity of the seminiferous tubule²⁶. The proportion of immature tubules varied widely among the rats exposed for 4 wk. Table 3 shows the results based on a semiquantitative grading system²⁶. The number of rats with grade 1 (proportion of immature tubules less than

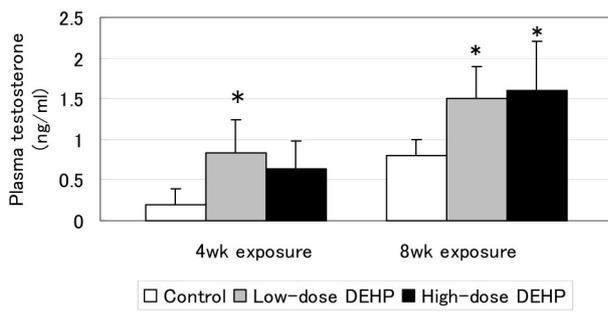


Fig. 2. Mean plasma testosterone of male rats exposed to DEHP by inhalation for 4 or 8 wk. Results are expressed as Mean \pm SD (n=6). * p <0.05 (significantly different using Scheffe's test after one-way ANOVA)

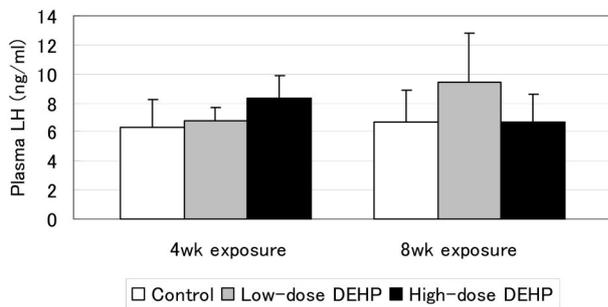


Fig. 3. Mean plasma LH of male rats exposed to DEHP by inhalation for 4 or 8 weeks. Results are expressed as Mean \pm SD (n=6).

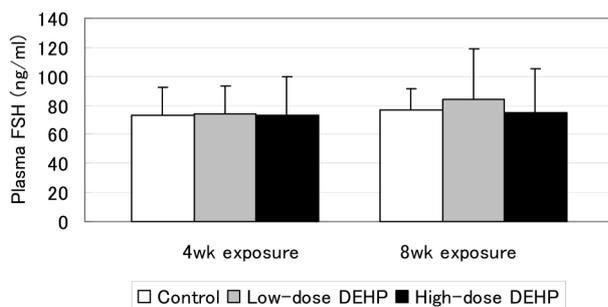


Fig. 4. Mean plasma FSH of male rats exposed to DEHP by inhalation for 4 or 8 wk. Results are expressed as Mean \pm SD (n=6).

5%) slightly increased in the low-dose DEHP group, but there was no difference in the immaturity of the seminiferous tubules between the high-dose DEHP group and the control group.

In the testes of the rats exposed for 8 wk (12 wk of age at the examination), histopathologic changes, including germ cell degeneration, were few. In all the rats, all seminiferous tubules were classified as mature tubules

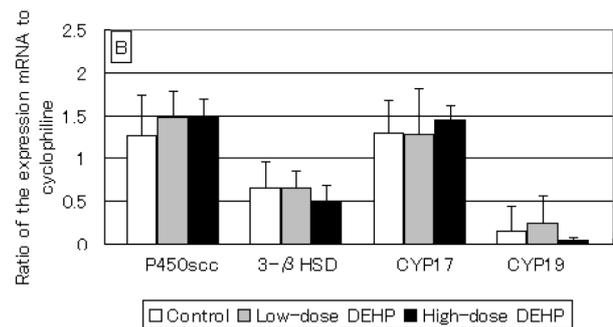
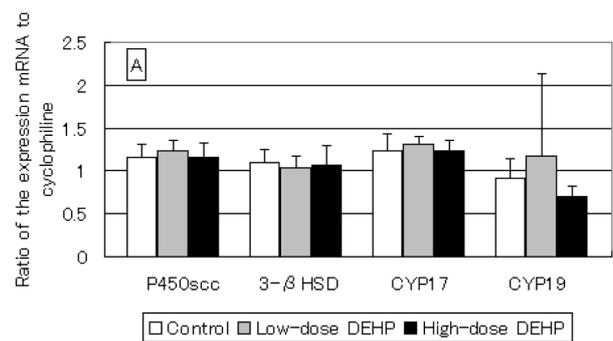


Fig. 5A, B. Changes of the expression of mRNA in the testis (A) after exposure by inhalation to DEHP for 4 wk and (B) for 8 wk. Values are Mean \pm SD (n=6). Results are expressed as Mean \pm SD (n=6).

and the progression of spermatogenesis was quantitatively sufficient.

Discussion

In this study, 4- and 8-wk inhalation of DEHP from 4 weeks of age increased the plasma testosterone concentration and no apparent histopathological damage was observed in the testes of the treated rats. Increased testosterone caused the relative weight of the seminal vesicle to significantly increase. These results support those of Akingbemi *et al.*²³⁾ who reported that testosterone was increased in rats with oral intake of DEHP from 21 to 90 d of age. Moreover, Akingbemi *et al.*¹⁴⁾ reported that oral intake of DEHP for 14 d by gavage in prepubertal rats reduced Leydig cell testosterone production, but that extending the period of DEHP exposure to 28 d resulted in significant increases in Leydig cell testosterone production. Considering their and our results together, testosterone may increase in prepubertal rats subacutely exposed to DEHP, regardless of the route of exposure. It is possible that the endocrine-system of rats subacutely exposed to DEHP during the prepubertal period might be disrupted, because normal sexual development depends on the delicate balance of hormones^{27, 28)}.

Table 3. The proportion of immature seminiferous tubules in the testes of the rats after 4 weeks exposure to DEHP by inhalation

Grade	Proportion of immature tubules	Control	Low-dose	High-dose
1 (minimal)	< 5%	3 (50%)	5 (83%)	3 (50%)
2 (slight)	5–25%	2 (33%)	0 (0%)	2 (22%)
3 (moderate)	25–50%	0 (0%)	1 (17%)	1 (17%)
4 (marked)	50–75%	1 (17%)	0 (0%)	0 (0%)
5 (severe)	> 75%	0 (0%)	0 (0%)	0 (0%)

There are four possible mechanisms that might have increased testosterone production in this study. First, animals in the group exposed to DEHP might have grown more rapidly than those in the control group. Body development usually has a strong association with reproductive development. However, our study showed that the final body weight of exposed rats was the same as for control rats, and “overgrowth” did not seem to be the cause of high plasma testosterone in the exposed group. Second, a high concentration of LH might increase testosterone production because LH stimulates the process of differentiation in developing Leydig cells and maintains steroidogenic enzyme gene expression and cell volume in mature cells²⁹. Testosterone is produced almost exclusively by Leydig cells in the testis. LH secreted by the pituitary is the primary regulator of Leydig cell function³⁰. However, in our study, plasma LH levels did not increase significantly. Third, steroidogenic enzymes involved in androgen synthesis might be directly stimulated. Androgen synthesis is the process of conversion of cholesterol into testosterone and this process is catalyzed by the following enzymes: P450_{scc}, 3 β -HSD, and CYP17. Moreover, testosterone undergoes aromatization to estradiol by aromatase (CYP19). Our results showed that plasma testosterone significantly increased without changing the expression of the mRNAs of the steroidogenic enzymes. Fourth, DEHP may reduce the metabolic clearance rate of testosterone. It has been suggested that there is a decrease in the metabolic clearance rate of testosterone during the developmental period of the male rat, and this may be partially responsible for the increasing concentrations of steroids observed in developing immature male rats³¹. In this study, it was not clear the why testosterone was increased by DEHP inhalation. To elucidate the mechanism, we need to further study to investigate estrogen and hormone receptors because the androgen-estrogen balance is important for regulating the reproductive function in males and there is the possibility of multiple crosstalks between androgen, estrogen and steroid hormone receptors^{23, 32}.

Although earlier papers have reported that plasma testosterone in adult rats was decreased after exposure to

DEHP^{14, 15, 21}, it was increased in prepubertal rats after exposure to DEHP, in Akingbemi’s report^{14, 23} and this report. What induces the difference of effect on testosterone related to exposure timing? In urine samples of workers who were occupationally exposed to DEHP, not only MEHP but also mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono (5-carboxy-2-ethylpentyl) phthalate were found as the major metabolites³³. In urine sample from the general population, concentrations of MEHHP and MEOHP were approximately 10-fold higher than the concentration of MEHP^{34, 35}. Moreover, Koch *et al.*³⁶ reported that concentrations of MEHHP and MEOHP in urine were significantly higher for children than for adults. The difference of the concentrations of metabolites between children and adults might be responsible for the difference of the effect on testosterone between our results, together with those of Akingbemi *et al.*^{14, 23} (testosterone increased by subacute exposure in prepubertal rats), and previously reported results (testosterone decreased by short exposure in adult or fetal rats). Although there is little information about the biologic activities of MEHHP and MEOHP, they also may play important roles in increasing testosterone in rats exposed to DEHP during the prepubertal period.

The results of our study suggested that the effect of DEHP inhalation on the endocrine system might be stronger than that of oral administration. Exposure for 6 hr per day to target concentrations of 5 and 25 mg/m³ DEHP in our study gave estimated doses of 1.0 and 5.1 mg/kg/day for the male, on the assumption of 100% deposition and absorption³⁷. In our study, the plasma testosterone concentration and relative weight of the seminal vesicle increased in the low-dose DEHP group and therefore the low-observed-effect level (LOEL) was 1.0 mg/kg, but Akingbemi *et al.*¹⁴ reported the LOEL of DEHP affecting steroidogenesis to be 10 mg/kg and the no-observed-effect level (NOEL) 1 mg/kg. Although there is no report about metabolites produced after inhalation of DEHP, higher concentrations of MEHHP and MEOHP were found in plasma following intravenous infusions of DEHP in rats³⁸ and the level of MEHP was lower after intravenous exposure than after oral

administration²¹). After inhalation of DEHP, the plasma concentrations of MEHHP and MEOHP may be higher than those after oral administration. Thus, the different concentrations of both MEHHP and MEOHP in tissue may also be responsible for this difference of the LOEL between inhalation and oral administration.

In our study, inhalation of DEHP for 4 wk increased testosterone non-linearly. There was no significant difference between the high-dose group and the low-dose group, because the slope of the dose-response curve was slight with regard to the dosage used in our study.

Many countries have set a threshold exposure level of DEHP in the workplace of 5 mg/m³ 1.39). In occupational exposure, Dirven *et al.*³³) reported that the highest DEHP concentration, 1.26 mg/m³, was found in a PVC boot factory and a cable factory. Other workplace levels of DEHP ranging from 0.02 to 4.1 mg/m³ were reported at facilities using or manufacturing the compound⁴⁰). Although the effect of DEHP on adults is thought to be less than those on children, we need to investigate the effects on workers occupationally exposed to it. Gann *et al.*⁴¹) reported that high levels of circulating testosterone are associated with an increased risk of prostate cancer. Moreover a previous study demonstrated that in males with high testosterone levels, the risk of precocious puberty might increase⁴²). Therefore, we also need to further investigate children environmentally exposed to DEHP.

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