Effects of Di(2-ethylhexyl) Phthalate on Regulation of Steroidogenesis or Spermatogenesis in Testes of Sprague-Dawley Rats

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Di(2-ethylhexyl) phthalate (DEHP) used as a common plasticizer additive in the manufacture of plastics, such as polyvinyl chloride (PVC). This study examined the effect of DEHP on steroidogenesis or spermatogenesis in the testes of Sprague-Dawley male rats treated orally with 250, 500, 750 mg/kg over a 30-day period. The expression levels of the steroidogenic- or spermatogenic-related genes were analyzed in the testis using a reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. High doses of DEHP (500 and 750 mg/kg) significantly decreased the testicular sperm counts and daily sperm production (DSP). In addition, serum testosterone levels were significantly lower in the DEHP treatment groups than in the control. The mRNA levels of SR-B1, StAR, PBR and CYP17 increased in a dose-dependent manner. These increases were significant at 500 and 750 mg/kg. In the other hand, the mRNA levels of CYP19 decreased significantly in testes of rats exposed to DEHP 500 and 750 mg/kg. Dose-dependent decreases in Spag4 and LDHA mRNA in the testis were observed after DEHP exposure, while there was a significant decrease in thyroid hormone receptor (TR) α 1 protein levels. High doses of DEHP significantly increased the expression of peroxisome proliferator-activated receptor (PPAR)-r and retinoid X receptor (RXR)- α protein but markedly decreased the expression of RXR-r. These results suggest that DEHP exposure can alter the expression of the spermatogenic- or steroidogenic-related genes resulting in a decrease in sperm production in the testis. This study is expected to be helpful in research examining the mechanisms for how DEHP reduces the expression pattern of the genes involved steroid hormone synthesis after chronic exposure to DEHP.

Key words —— di(2-ethylhexyl) phthalate, spermatogenesis, steroidogenesis, daily sperm production, sperm count, gene expression

INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is most commonly utilized as an additive in the manufacture of plastics. DEHP is a well known reproductive and hepatic toxicant but DEHP is rapidly absorbed and converted to mono(2-ethylhexyl) phthalate (MEHP), which also causes male reproductive organ damages in animals, particularly in the testis when DEHP is administered orally to rats.^{1–3)} The testicular toxicity of DEHP in animals is characterized by the marked degeneration of seminiferous tubules resulting in testicular atrophy.^{4, 5)} It has been suggested that Sertoli cell is the primary targets of phthalates in testicular toxicity, which ultimately decreases sperm production and sperm count.^{6, 7)} In addition, DEHP-induced testicular damage has been reported to be closely associated with changes in the testosterone levels as well as testicular zinc depletion by interfering with steroid biosynthesis.^{8, 9)} However, the precise molecular mechanisms for how DEHP affects the regulation of the steroidogenic- or spermatogenicrelated genes are unclear. Previous our studies

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showed that chronic oral exposure to DEHP and di(n-butyl) phthalate (DBP) caused significant damage to Sertoli cells as well as germ cell degeneration in the testis of Sprague-Dawley rats.^{5,10)} In addition, Sjöberg *et al.*¹¹⁾ suggested that the age-related difference in the testicular response after the oral administration of DEHP might be due to pharmacokinetic differences rather than to tissue sensitivity differences when DEHP is administered to male rats at various ages. Based on these results, immature rats were used in this study because they are more sensitive to DEHP-induced testicular damage than mature rats when DEHP is administrated orally.

There are a few reports on the gene expression related spermatogenesis or steroidogenesis in the testis after DEHP exposure.^{12, 13)} Recent studies focused on the mediation of the peroxisome proliferator-activated receptor (PPAR) subtypes as potential mediators of phthalate-induced testicular toxicity.⁵⁾ Although the mechanism of steroid biosynthesis pathway in the testis is known, recent studies indicate a close link between PPAR and other nuclear receptors in the testes.^{14, 15)} Lovekamp-Swan et al.¹⁶) suggested that PPAR ligands suppressed aromatase mRNA levels in the rat ovarian granulose cells but had no effect on P450scc mRNA. It is unclear if this effect was mediated by estrogen receptor (ER). ER β was assumed to mediate the DBP-induced suppression of CYP19 because ER α was not detected in the testes, and ER mediated the DBP-induced inhibition of testosterone production.¹⁷⁾

The overall aim of this investigation was to determine the molecular mechanism of DEHP toxicity on the regulation of testicular gene expression. To accomplish this, the present study examined the expression of nuclear receptors, such as PPAR and retinoid X receptor (RXR), which regulate the transcription of their specific responsive genes. The expression of these genes was further correlated with sperm production and testosterone levels. In addition, the expression of two genes, Spag4 and lactate dehydrogenase A (LDHA), was analyzed. The results show that oral administration of DEHP to male rats caused more pronounced testicular damage by altering the expression of the spermatogenicor steroidogenic-related genes.

MATERIALS AND METHODS

Animals and Treatments ----- Sprague-Dawley

male rats were obtained from Charles River Laboratories (Tokyo, Japan) and housed under a controlled temperature $(22 \pm 2^{\circ}C)$ and lighting (12 hr light and dark cycles). The animals were given access to an animal diet (PMI, Brentwood, MO, U.S.A.) and tap water *ad libitum*. DEHP (250, 500 or 750 mg/kg) was administered to the prepubertal rats (4 weeks of age, weighing approximately 80–100 g) by oral intubations for 30 days. The control group was given corn oil in the same manner. The doses used in this study were based on previous reports showing the adverse effects of DEHP on male reproductive development in adult male rats.⁵⁾ The institutional animal care committee of Pusan National University approved the experimental protocol.

Body and Reproductive Organ Weight — The body weights were recorded daily before dosing. After administering the test compound for daily 30 days (approximately 24 hr after the final dose), animals were anesthetized with diethyl ether. The testes were excised, weighed, frozen in liquid nitrogen, and stored at -80° C until use. Throughout the study period, each animal was observed at least once daily for any clinical signs of toxicity related to the DEHP treatment. During the experimental periods, all the cages were checked in the morning and afternoon for the presence of dead or moribund animals.

Sperm Count and Daily Sperm Production -For sperm count, cauda epididymis were removed and three to four deep cuts were made along the proximal and distal cauda of each epididymis, placed in 5 ml M199 (Sigma Chemical Co., St. Louis, MO, U.S.A.) medium supplemented with 0.5% bovine serum albumin (BSA, Sigma) at room temperature for 10 min. Tissues were removed, and the supernatant diluted (1:100) with M199 media and the total sperm number was determined with a hemocytometer. Daily sperm production in the testis of the rats was measured, as described previously by Robb et al.¹⁸⁾ The right testis was decapsulated and homogenized for 1 min in 10 ml of a 0.9% NaCl solution containing 0.5% Triton X-100 with a tissue mizer, followed by sonication for 30 sec. After diluting the sample 10fold, it was stained with 2% eosine and transferred to the chambers of a hemocytometer. The daily sperm production was calculated by dividing the total number of spermatids or sperm per gram of testicular parenchyma (testis weight minus the weight of the capsule) by 6.1 days, which is the duration of the step 19 spermatids in the seminiferous epithelial

cycle.19)

Hormonal Analyses — The serum testosterone levels were measured using Radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories, Inc., Webster, TX, U.S.A.) according to the manufacturer's protocols. All serum samples were analyzed in duplicate.

RNA Isolation and Reverse Transcription (RT)-PCR—— The total RNA (about 2 µg) extracted from testes was used to synthesize the cDNA using a SuperScriptTM kit (Invitrogen). The first cDNA strand was synthesized from the total RNA using the specific primers. Table 1 indicates the primers and PCR conditions for SR-B1, PBR, StAR, P450scc, CYP17, CYP19, Spag4, LDHA and β -actin. The RT reaction was carried out at 50°C for 1 hr, and quenched by heating at 94°C for 2 min. The RT-PCR reactions were performed using a RT-PCR system (Applied Biosystems) according the manufacturer's instructions. Approximately 2 µl cDNA per sample was used for amplification. The cDNA fragments were generated under the following conditions: initial denaturation: 94°C for 5 min. The PCR products were separated by electrophoresis on a 2% agarose gel and detected under UV light.

Western Blot Analysis ----- The frozen testes were thawed and homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 100 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. After incubation for 60 min at 4°C, the homogenate was centrifuged at 16000 q for 20 min at 4°C, and the supernatant was collected to determine the protein concentration. The protein concentration of the cell extract was determined in triplicate using a BioRad (Hercules, CA, U.S.A.) protein assay kit. Equal amounts of the total protein were resolved on a 10% sodium dodecyl sulfate (SDS)polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dried milk in PBS containing 0.1% Tween-20 for 30 min, and incubated for 1 hr at room temperature with the primary antibodies such as anti-TR α 1 (1:500), PPAR- α (1:500), PPAR-r (1:200), RXR- α (1:200), and

Table 1.	Rat Specific	Primer Sets fo	r RT-PCR	Analysis
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Gene	Primers	PCR Conditions	Product Size
LDHA	F: ACAGTCCACACTGCAAGCTG	94°C 5 min, 23 cycles (94°C	304
	R: TTCCACTGCTCCTTGTCTGC	$40 \text{sec}, 60^{\circ}\text{C}$ $40 \text{sec}, 72^{\circ}\text{C}$	
		40 sec), 72°C 5 min, 4°C	
PBR	F: CCATGCTCAACTACTATGTATGGC	94°C 5min, 25 cycles (94°C	233
	R: GTACAACTGTCCCCGCATG	$40 \sec, 60^{\circ}C 40 \sec, 72^{\circ}C$	
		45 sec), 72°C 5 min, 4°C	
Spag4	F: GCACCGAAGGAGTTCTCTGT	94°C 5 min, 35 cycles (94°C	221
	R: GGGATGCCATGGACTCTAAA	$40 \sec, 60^{\circ}C 40 \sec, 72^{\circ}C$	
		45 sec), 72°C 5 min, 4°C	
SR-B1	F: CCATTCATGACACCCGAATCCT	94°C 5 min, 28 cycles (94°C	100
	R: TCGAACACCCTTGATTCCTGGT	$15 \text{ sec}, 60^{\circ}\text{C} 30 \text{ sec}, 72^{\circ}\text{C}$	
		45 sec), 72°C 5 min, 4°C	
StAR	F: CATCCAGCAAGGAGAGGAAG	94°C 5 min, 24 cycles (94°C	496
	R: CGTGAGTTTGGTCTTTGAGG	$30 \sec, 55^{\circ}C = 30 \sec, 72^{\circ}C$	
		45 sec), 72°C 5 min, 4°C	
P450scc	F: CGCTCAGTGCTGGTCAAAA	94°C 5 min, 25 cycles (94°C	688
	R: TCTGGTAGACGGCGTCGAT	$30 \sec, 55^{\circ}C = 30 \sec, 72^{\circ}C$	
		45 sec), 72°C 5 min, 4°C	
CYP17	F: GACCAAGGGAAAGGCGT	95°C 12 min, 24 cycles (94°C	302
	R: GCATCCACGATACCCTC	30 sec , 54°C 30 sec , 72°C	
		45 sec), 72°C 5 min, 4°C	
CYP19	F: ATAATGTCACCATCATGGTCCCGG	95°C 12min, 30 cycles (94°C	579
	R: GCATGATGTGTCTCATGAGGGTCA	$30 \sec, 60^{\circ}C = 30 \sec, 72^{\circ}C$	
		45 sec), 72°C 5 min, 4°C	
β -actin	F: TGGAGAAGATTTGGCACCAC	95°C 12 min, 20 cycles (94°C	450
	R: AGTCTAGGGCAACATAGCAC	30 sec, 55°C 30 sec, 72°C	
		45 sec), 72°C 5 min, 4°C	

RXR-r (1:200). The blots were washed three times for 15 min with PBST containing 0.1% Tween-20 and incubated for 1 hr with horse rabbit peroxidaseconjugated anti-rabbit or anti-mouse immunoglobin G (1:1000). The membranes were washed again 4 times. The blots were developed using an Enhanced Chemiluminescence System (ECL, Amersham Corp., Cardiff, U.K.). An image analyzer was used to determine the relative band intensities.

Data Analysis — Data are expressed as mean \pm S.D. (n = 6 animals). The data for the mean initial or necropsy body weights, organ weights, and hormone levels were analyzed for any homogeneity of the variance using a Bartlett's test. Nonparametric analysis of variance was applied when the samples were found to be homogeneous. Statistical analysis of the RT-PCR data was also carried out using a Dunnett test, which compared the relative expression ratios from each treatment group with the control.

RESULTS

Clinical Signs, Body and Testis Weight

DEHP was administered orally to pubertal male rats for 30 days in order to determine its effects on male testicular development and growth. During the study period, the clinical signs were similar in both the control and DEHP-treated groups (data not shown). In addition, the body weight gains of the DEHP-treated groups were similar to the control (Fig. 1). In contrast, the testes and epi-





didymides weights in the high dose groups (500 and 750 mg/kg) were significantly lower than control, respectively (Fig. 2).

Daily Sperm Production and Testosterone Levels

Sperm counts and daily sperm production as a result of DEHP exposure were significantly lower in the 500 and 750 mg/kg groups than the control (Fig. 3A). Serum testosterone concentrations were also examined because it plays a role in cholesterol transport and may influence the steroidogenic pathway. As expected, the RIA data indicated that the serum testosterone levels were significantly lower in the high doses (500 and 750 mg/kg) groups (Fig. 3B).

Steroidogenic- and Spermatogenic-related Gene Expression

According to previous data,⁵⁾ there was a significant change in the genes involved in cholesterol transport or lipid metabolism in the testes following



Fig. 2. Effects of DEHP on Testes and Epididymis Weights Four-week old rats were treated with DEHP (250, 500 or 750 mg/kg) for 30 days. The data is reported as the mean \pm S.E. for 6 animals/group. The asterisk indicates a significant difference from the control (p < 0.05).



Fig. 3. Effects of DEHP on the Sperm Count, Daily Sperm Production (DSP), and Serum Testosterone Levels

Four-week old rats were treated with DEHP (250, 500 or 750 mg/kg) for 30 days. (A) Sperm count and DSP were measured as described in Materials and Methods. (B) Serum testosterone levels were measured using RIA. The data is reported as the mean \pm S.E. for 6 animals/group. The asterisk indicates a significant difference from the control (p < 0.05).

high dose of DBP (750 mg/kg) exposure. Therefore, the expression levels of the steroidogenicrelated genes, such as SR-B1, PBR, StAR, P450scc, CYP17, and CYP19 were also examined. Similarly, high doses (500 and 750 mg/kg) of DEHP produced a significant increase in the mRNA levels of StAR, PBR, SR-B1, P450scc and CYP17. However, CYP19 mRNA was not expressed absolutely in the DEHP treatment groups (Fig. 4A).

The expression levels of the spermatogenicrelated genes, such as Spag4 and LDHA, were also investigated. There was a dose-dependent decrease in the Spag4 and LDHA mRNA levels after DEHP exposure. High doses of DEHP produced a significant decrease in the levels of Spag4 and LDHA mRNA (Fig. 4B).

Thyroid Hormone Receptor (TR), PPARs and RXRs Expression

RT-PCR analysis was carried out to determine the expression pattern of TR α 1 and TR β mRNA in the testes. The DEHP treatment produced a dose-dependent increase in TR α 1 mRNA levels. A high dose of DEHP (750 mg/kg) significantly upregulated TR α 1 mRNA expression. In contrast, the TR β mRNA levels were significantly lower in all groups exposed to DEHP (Fig. 5A). The total protein extracts were examined by Western blot analysis to compare the testicular changes in the PPAR and RXR expression levels. As shown in Fig. 5B, the levels of PPAR- γ and RXR- α protein expression were significantly higher in the high doses DEHP (500 and 750 mg/kg) group. However, high doses (500 and 750 mg/kg) of DEHP significantly reduced the PPAR- α and RXR- γ mRNA levels in the testis.

DISCUSSION

These results showed that a high dose of DEHP administered daily to prepubertal rats for 30 days significantly affects the testicular gene expression profiles involved in steroidogenesis and spermatogenesis. Previous studies reported that neonatal exposure to DEHP significantly affected testicular growth and development as a result of the reduced testosterone production.^{14, 20, 21} However, the molecular mechanisms of DEHP-induced testic-



Fig. 4. Transcript Levels for StAR, SR-B1, PBR, P450scc, CYP17 and CYP19 mRNA Levels in the Testis

Four-week old rats were treated with DEHP (250, 500, or 750 mg/kg) for 30 days. The RNA was also isolated from the testis of Sprague-Dawley rats. (A) The RT-PCR products representing StAR, PBR, SR-B1, P450scc, CYP17 and CYP19 transcripts were separated on agarose gels. (B) Expression levels of mRNA were normalized to β -actin expression, and relative mRNA values were depicted as mean \pm S.D. (C) Transcript levels for Spag4 and LDHA mRNA levels in the testis. (D) Expression levels of mRNA were normalized to β -actin expression levels of mRNA were normalized to β -actin expression levels of mRNA values were depicted as mean \pm S.D. Photo is representative of three separate blots. The asterisk indicates a significant difference from the control (*p < 0.05; **p < 0.01).



Fig. 5. Effect of DEHP on the Regulation of Nuclear Receptors in the Testes of Sprague-Dawley Rats

Four-week old rats were treated with DEHP (250, 500, or 750 mg/kg) for 30 days. (A) The RNA was also isolated from the testis of Sprague-Dawley rats. The RT-PCR products representing TR α 1 and TR β transcripts were separated on agarose gels. Photo is representative of three separate blots. (B) Expression levels of mRNA were normalized to β -actin expression, and relative mRNA values were depicted as mean \pm S.D. The asterisk indicates a significant difference from the control (*p < 0.05; **p < 0.01). (C) Western blotting analysis of the PPAR and RXR proteins in the testis of Sprague-Dawley rats. The PPAR- α , PPAR-r, RXR- α and RXR-r proteins were detected by Western blot analysis using their respective antibodies. A representative blot from three separate experiments is shown. (D) The ratios of PPAR- α , PPAR-r, RXR- α and RXR-r proteins to β -actin protein expression were determined from five separate experiments by comparing the relative intensities of protein bands. Data are presented as the mean \pm S.D. The asterisk indicates a significant difference from the control (*p < 0.05; **p < 0.01).

ular genes associated with steroidogenesis and spermatogenesis expression in rodents are still unclear. This study examined the differentially expressed genes involved in the testicular toxicity caused by long-term exposure to DEHP.

In general, a decrease in the estrogen levels in the testes is closely related to the sperm motility. Accordingly, it is reasonable to speculate that there may be disturbances in endogenous estrogen biosynthesis during the early period of reproductive tissue differentiation. Furthermore, the targeted disruption of the aromatase gene (CYP19) is a disturbance in acrosome formation.^{22, 23)} Therefore, these reports support the data showing that exposure to DEHP might result in a low sperm number and sperm motility. RT-PCR analysis, which was used to examine the molecular mechanism of DEHP on sperm motility, showed that DEHP exposure decreased the levels of LDHA mRNA expression in a dose-dependent manner. Lactate is produced from pyruvate subsequent to LDHA action on Sertoli cells and is transported across the plasma membrane to the germ cells.²⁴⁾ A significant decrease in lactate production was observed in cultured Sertoli cells obtained from rat testes exposed to flutamide in utero, and a decrease in the levels of LDHA mRNA was observed in both the whole testis and isolated Sertoli cells.²⁵⁾ Indeed, it was reported that spermatids produce factors that increase the level of lactate production in Sertoli cells and stimulate lactate production and LDHA expression in Sertoli cells.^{26,27)} The levels of Spag4 expression were also measured to determine the factors involved in the enhanced testicular toxicity of DEHP. Taken together, it was reported that the Spag4 mRNA levels were significantly lower in the testes chronically exposed to DBP than in the controls.²⁸⁾ Based on these data, the Spag4 gene may play an important role in rodent spermatogenesis.

Microarray analysis indicated that a number of genes are involved in the xenobiotic metabolism, apoptosis, steroid hormone biosynthesis, and cholesterol metabolism were significantly altered after a DEHP treatment.^{29, 30)} It was reported that exposure to DEHP produced a significant dosedependent decrease in the level of StAR, P450scc, and P450c17 mRNA.³¹⁾ In contrast, subchronic exposure to DEHP in prepubertal rats enhanced androgen biosynthesis in Leydig cells and increased the serum testosterone levels, which is probably due to a compensatory mechanism.³²⁾ In addition, they also reported increased Leydig cells steroidegenesis in rats after a DEHP treatment.³³⁾ These results are similar to those obtained in this study that prepubertal exposure to DEHP for 30 days enhanced the expression of SR-B1, StAR, PBR, P450scc and CYP17 involved in the steroidogenic pathway. In this study, the mechanisms responsible for these observations are unclear. Moreover, it is not known why these genes were upregulated after chronic DEHP exposure.

In general, cholesterol uptake by the cells is mediated by SR-B1, and intracellular cholesterol is also transported from the outer to inner mitochondria membrane via StAR.^{34, 35)} StAR functions in concert with PBR to regulate cholesterol transport across the mitochondrial membrane.³⁶⁾ Several studies reported that PBR is an 18 kDa protein that is expressed ubiquitously, and is implicated in the regulation of various cell functions, including cholesterol transport, steroidogenesis, immune function, and apoptosis.^{37,38)} It was reported that PBR is over-expressed in steroid-producing tissues. such as the testis and adrenal glands.³⁹⁾ In this study, the levels of StAR, PBR and SR-B1 mRNA were significantly higher in the rats exposed to DEHP at 500 and 750 mg/kg.

This study examined the expression of TR because crosstalk of the steroid hormone receptor is important for regulating testicular growth and development. The testicular level of the TR mRNA expression was measured using RT-PCR assays. Both TR α 1 and TR β expression were identified in the testes of the control rats. However, the levels of TR α 1 mRNA increased dose-dependently after DEHP exposure, but TR β was not detected in the DEHP treatment testis. Previous study reported that TR has a direct effect on the development of the prepuberal testes, which may lead to further modulation of the effect of gonadotropins on the testicular function.⁴⁰⁾ It was reported that the TR α 1 mRNA and protein are abundant in Sertoli cells in developing testis.⁴¹⁾ These results showed that TR, mainly TR α 1, is expressed at low levels in the testes of normal rats. The levels of TR α 1 mRNA were expressed at significantly higher levels in the testis of the DEHP exposed rats than in unexposed rats. This

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suggests that the DEHP-induced changes in TR α 1 expression may play a critical role in initiating testicular cell growth and differentiation. These results are similar to those reported previously in that TR α 1 expression is low and confined to periductal mesenchymal cells surrounding the proximal ducts in normal developing testes.⁴¹⁾ Therefore, chronic exposure to DEHP differentially alters the testicular expression of the TR α 1, which is significantly up regulated. This suggests that TR α 1 is an essential factor that mediates the adverse effects of DEHP, which contributes to the DEHP-induced dysgenesis of testicular growth and delayed development.

The molecular responses of the testis were further examined to gain better insights regarding the mechanism for the toxic action of DEHP on the testis. In perticular, the expressions of the nuclear receptors, such as PPAR and RXR, which regulate the transcription of their specific responsive genes, were examined to assess potential changes in gene expression in the testis after exposure to DEHP. These results indicate that PPAR- γ and RXR- α affect the production of steroid hormones in the testes after exposure to DEHP.

In conclusion, prepubertal exposure to DEHP alters male testicular growth and morphogenesis in a dose-dependent manner. DEHP increased the expression of several steroidogenic genes (SR-B1, StAR, PBR and P450scc). However, the changes in CYP19 expression leads to low serum estrogen levels, which is likely to have adverse reproductive effects via a hypothalamus-pituitary-gonadal (HPG) axis negative feedback mechanism. It is conceivable that the nuclear receptors, PPAR- γ and RXR- α , are involved in down-regulating the aromatase and testosterone levels, which underlie the disturbed development of the male reproductive system as a result of exposure to DEHP. These results suggest that DEHP is broadly reactive through multiple pathways involved in maintaining steroid and lipid homeostasis. However, further research will be needed to better understand how the genes associated with the biosynthesis of steroid hormones are regulated in the testis after chronic DEHP exposure.

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