Original Article

The effects on the endocrine system under hepatotoxicity induction by phenobarbital and di(2-ethylhexyl)phthalate in intact juvenile male rats

Takafumi Yamaguchi^{1,2}, Minoru Maeda¹, Keiko Ogata¹, Jun Abe¹, Toru Utsumi¹
and Koji Kimura²

¹Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.,
3-1-98 Kasugade-Naka, Konohana-ku, Osaka 554-8558, Japan

²Laboratory of Animal Reproductive Physiology, Graduate School of Environmental and Life Science, Okayama
University, 1-1-1, Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan

(Received November 28, 2018; Accepted April 12, 2019)

ABSTRACT — Phenobarbital (PB) and Di (2-ethylhexyl) phthalate (DEHP), an anti-epileptic drug and a plasticizer used in flexible polyvinylchloride formulations, respectively, are well-known typical hepatotoxicants. This study investigated the effects of PB (100 mg/kg/day) or DEHP (500 mg/kg/day) on the endocrine system in intact juvenile/peripubertal male rats exposed for 31 days beginning on postnatal day 23. Slight hormone level changes, histopathological changes in thyroid gland or induction of UDP-glucuronosyltransferase in liver were observed in both the PB and DEHP groups. One of the assumed mechanisms inducing thyroid effects is predictable to be secondary changes based on the enhancement in thyroid hormone metabolism via the induction of hepatic microsomal enzymes. No reproductive system-related changes in organ weights, histopathology, and sexual maturation were observed in both groups. Lower testosterone level was observed in the PB group. CYP2B and CYP3A, which are involved in testosterone metabolism, were induced in liver of the PB group. There was no change of 17 β -hydroxysteroid dehydrogenase activity in testis of both groups. Lower testosterone level in the PB-treated male rats was attributed to an indirect, hepatotoxicity-associated effect on the reproductive system and not to direct effects on testis such as the antiandrogenic activity and the inhibition of steroidogenesis. These results did not indicate that PB or DEHP exposure affects the endocrine system directly.

Key words: Phenobarbital, Di(2-ethylhexyl)phthalate, Hepatotoxicant, Testosterone, CYP, Rat

INTRODUCTION

The World Health Organization (WHO), through the International Program on Chemical Safety (IPCS), defines endocrine disrupting chemicals (EDCs) as “exogenous substances or their mixture that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO/IPCS, 2002). On the other hand, there is as yet no global consensus on a standardized method of EDCs identification. Many chemicals indirectly affect the endocrine system via lead (representative) toxicities such as hepatotoxicity, renal toxicity, and body weight loss (Kortenkamp *et al.*, 2012). Toxicity studies in compliance with the US Environmental Pro-

tection Agency (EPA) or Office of Economic Cooperation and Development (OECD) test guidelines provide scientific information regarding direct or indirect effects on the endocrine system (U.S. EPA, 2011; Day *et al.*, 2018). Indirect effects that are non-specific secondary consequences of lead toxicity should not be considered when evaluating EDCs (Stump *et al.*, 2014). Under the circumstances, it is recommended to conduct the toxicity studies in compliance with EPA or OECD test guidelines such as the stably transfected human androgen receptor transcriptional activation assay and Hershberger assay to verify direct effects, but there is no recommendation regarding tests verifying indirect effects, which still have not been developed. Hepatotoxicity is one of the most common adverse effects of chemical exposure. Phenobarbital

Correspondence: Takafumi Yamaguchi (E-mail: yamaguchit3@sc.sumitomo-chem.co.jp)

(PB) and Di(2-ethylhexyl)phthalate (DEHP) are typical hepatotoxicants used frequently in toxicological research. While data on the liver effects by PB or DEHP exposure have been abundantly reported, the relationship between hepatotoxicity and the effect on endocrine system remains unclear.

PB has been widely utilized as an anti-epileptic drug to control seizures and is well known an inducer of numerous cytochrome P450s and UDP-glucuronosyl transferase (UGT) isoforms related to metabolism of steroid hormones and thyroid hormones in liver (Waxman and Azaroff, 1992). Thyroid gland tumors, induced in rats by PB exposure, increased levels of pituitary thyroid-stimulating hormone (TSH) secretion as a compensatory response to increased thyroid hormone glucuronidation and biliary excretion (IARC, 2001). However, there is not enough information on the relationship between the induction of hepatic microsomal enzymes by PB exposure and the metabolism of steroid hormones.

DEHP (when utilized in products such as medical devices/supplies) is detected at higher concentration in pregnant women and infants (Lottrup *et al.*, 2006). Many scientists suggest that phthalates including DEHP are EDCs with antiandrogenic, estrogenic, or thyroid-disrupting effects (Ema and Miyawaki, 2001; Dong *et al.*, 2017; Ghisari and Bonefeld-Jorgensen, 2009; Cha *et al.*, 2018). DEHP is a hepatotoxicant and one of several peroxisome proliferators (PPs) that activate the expression of peroxisome proliferator-activated receptor α (PPAR α) in liver of rodents and induce multiple cytochrome P450s (Rusyn *et al.*, 2006; IARC, 2013).

One example of the indirect effects of microsomal enzyme inducers on endocrine system is to enhance testosterone metabolic clearance, resulting in a decrease in circulating hormone levels (Coulson *et al.*, 2003; Rasoulpour *et al.*, 2015; Masubuchi *et al.*, 1997; Wilson and LeBlanc, 1998). There is a possibility that the increased clearance of androgen affects puberty and fertility in vertebrates. It is essential to elucidate the mode of action (MoA) of each effect on endocrine system when evaluating EDCs. Therefore, the purpose of this study was to investigate indirect adverse effects on components of the endocrine system including male reproductive organs and thyroid gland by PB or DEHP (known hepatotoxicants that induce hepatic microsomal enzymes). Ahead of hepatic microsomal enzyme induction, PB and DEHP activate CAR (Constitutive androstane receptor) and/or PPAR α (peroxisome proliferator-activated receptor α). They are different type of enzyme inducers as agonists of single or multiple nuclear receptors (Eveillard *et al.*, 2009; Ren *et al.*, 2010). Liv-

er effects such as hepatocellular hypertrophy are observed following enzyme induction. However, the effects on hormone levels have not always been reported in all hepatotoxicants indicating hepatocellular hypertrophy and there is not plenty of case studies to investigate the comparison of induced cytochrome P450s and hormone fluctuation. This study inspected the hormonal effects by PB or DEHP exposure on endocrine system including reproductive organs and thyroid gland in intact juvenile/peripubertal male rats. We especially examined whether there are any relationship between changes in hormone levels and the induction pattern of hepatic microsomal enzymes, and it is expected to proceed the additional investigation to apply as one of reference research when assessing EDCs.

MATERIALS AND METHODS

Test chemicals

The test chemicals, phenobarbital sodium (PB) and di-2-ethylhexyl phthalate (DEHP), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan; Lot No. EPG6760 with 98.0% purity and Lot No. DCP6102 with 97.0% purity, respectively). The test solutions were prepared by dissolving PB in corn oil at a concentration of 20 mg/mL and DEHP at a concentration of 100 mg/mL.

Animals

All animal experiments were performed in accordance with The Guide for Animal Care and Use of Laboratory Animals in the Environmental Health Science Laboratory of Sumitomo Chemical Co., Ltd. (Osaka, Japan). CrI:CD (SD) male rats at postnatal day (PND) 14 were purchased with foster dams from Charles River Laboratories Japan, Inc. (Hino Breeding Center, Shiga, Japan). Each dam had 8 male pups. All pups were weaned from each dam at PND 21. The first administration of test solution was on PND 23 in pups with body weights ranging from 63.5 to 86.4 g. During the course of the study, the environmental conditions in the animal room were maintained within a temperature range of 22–26°C and a relative humidity range of 40–70%, with frequent ventilation (more than 10 times per hour), and a 14 hr light (6:00–20:00) / 10 hr dark (20:00–6:00) illumination cycle. One dam and 8 pups were housed before weaning in each suspended aluminum cage with stainless steel wire-mesh front and floor, and then two pups were housed in each cage after weaning. The animals were allowed free access to unsterilized pellet diet CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water. The diet was further analyzed for contents of genistein and daidzein. The con-

tents of genistein and daidzein, which are phytoestrogens, were within 2.2 mg/100 g and 1.6 mg/100 g, respectively. On the day of the group allocation (PND 22), all pups were weighed, and assigned by the stratified randomization method using a computer program. Prior to dosing, it was confirmed that mean body weight was not significantly different among the groups and that each individual body weight was within $\pm 20\%$ of the mean body weight of all animals. The animals were not fasted overnight prior to sacrifice.

Chemical treatment

The rats were divided into 3 treatment groups (PB, DEHP and vehicle control), with 10 animals per group. The dosing volume was 5 mL/kg and the dose was 100 mg/kg/day for PB and 500 mg/kg/day for DEHP administered once daily (8:00-10:00) on 31 consecutive days beginning on PND 23. Corn oil (Nacalai Tesque, Inc., Kyoto, Japan) was used as the vehicle. The doses for each chemical, which were expected to induce hepatotoxic effects according to the toxicological literature such as IARC Monographs (IARC, 2001; Jo *et al.*, 2011; IARC, 2013), were selected. Test solutions were administered orally by gavage using a flexible catheter joined to a plastic disposable syringe. Clinical signs (twice daily), body weight (once daily), and food consumption (weekly) were monitored throughout the study. All animals were observed for preputial separation (PPS) beginning on PND 30 every morning (8:00-12:00). The age on the day of PPS completion was recorded for all animals.

Blood collection

On PND 54, each animal was moved to a holding room 1 hr prior to blood sampling. Blood samples from all animals were randomly collected by decapitation without anesthesia at 2 to 3 hr after final administration. Serum samples were separated by centrifugation from blood samples, stored at -80°C , and then used for serum chemistry and hormone analysis.

Serum chemistry and hormone analysis

A Clinical Biochemistry Analyzer JCA-BM1250 (JEOL Ltd., Tokyo, Japan) was used to measure serum levels of total protein, albumin, total cholesterol, triglycerides, phospholipids, urea nitrogen, creatinine in all animals.

Serum testosterone level was measured using a Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a Thermo TSQ™ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Bedford, MA, USA) with an atmospheric pressure chemi-

cal ionization (APCI) source. Serum samples were spiked with the stable deuterium-labelled internal standard testosterone 16, 16, 17-d3. The UFLC conditions were as follows: detection wavelength, 254 nm; analytical column, COSMOSIL 3C18-EB Packed Column (Nacalai Tesque, Inc.); mobile phase, gradient elution with 0.1% formic acid in water and 0.1% formic acid in methanol. Operating conditions of APCI positive mass spectrometry were as follows: vaporizer temperature, 400°C ; sheath gas flow rate, 10 arb, aux gas flow rate, 5 arb; discharge current, 4.0 μA ; capillary temperature, 200°C . The detected analyte ions included precursor ion 289, which was converted to product ions 97 and 109 for testosterone, and precursor ion 292, which was converted to product ions 97 and 109 for testosterone-d3.

Thyroid stimulating hormone (TSH) level was measured in all serum samples by radioimmunoassay using the rat thyroid stimulating hormone (rTSH) [^{125}I] assay system (Code RK-554 SCETI K.K., Tokyo, Japan) and Accu-FLX γ 7010 (Hitachi Aloka Medical, Ltd., Tokyo, Japan) gamma counter for quantitative measurement. Total triiodothyronine (T3) and thyroxine (T4) levels were measured by chemiluminescent enzyme immunoassay with the Access2 Immunoassay System (Beckman Coulter Inc., Brea, CA, USA).

Preparation of liver and testis homogenates

Six animals from each group were selected for measurement of enzyme activities in liver and left testis. The liver was homogenized in 50 mM Tris/HCl buffer (pH 7.4, containing 154 mM KCl). The homogenate was centrifuged at $9,000 \times g$ for 20 min at 4°C to separate the S9 fraction. The testis was homogenized in 50 mM Tris/HCl buffer (pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, and 0.1 mg/mL PMSF). The protein level in the liver S9 and testis homogenate fractions was determined with bovine serum albumin as the protein standard.

Enzyme activities

Enzyme activities of CYP2B (Yamada *et al.*, 2014), CYP4A (Pinot *et al.*, 1998), CYP3A (Saito *et al.*, 2006), and UGT (Barter and Klaassen, 1992) in liver and of 17 β -HSD (Hu *et al.*, 2009; Fukuta *et al.*, 1999) in testis were measured as previously described with minor modifications. The assay conditions are summarized as Supplemental data separately. For each enzyme, liver S9 or testis homogenate was incubated with substrate and other ingredients, and the reaction was stopped by addition of organic solvent. After the centrifugation, cleavage products in each supernatant were analyzed and quantitated.

Pathology

The wet weights of the dissected tissues and organs were examined macroscopically and weighed. Relative organ weights (organ weight to body weight ratios) were calculated on the basis of the final body weight on the day of euthanasia. The dissected tissues and organs included testes, epididymides, ventral prostate, seminal vesicles plus coagulating glands (SVCG), levator ani plus bulbocavernosus muscle complex (LABC), thyroid gland, liver, and kidneys. The organs and tissues except testes and epididymis were preserved in the fixative 10% neutral-buffered formalin after measurement of organ weights and gross pathology. Right testis and epididymis were primarily fixed in formalin-sucrose-acetic acid solution and then preserved in 10% neutral-buffered formalin. Liver, kidney, thyroid gland, right testis, and epididymis were processed, embedded, sectioned, and stained with hematoxylin and eosin. Stained histologic sections were examined by light microscopy.

Statistical analysis

For the incidence of clinical signs, a one-tailed (upper) Fisher exact probability test was used, while a two-tailed test was performed for all other analyses. In each analysis, the significant differences were evaluated at probab-

ity levels of 1 and 5%. Comparing the treatment effect to the vehicle effect on body weight, body weight gain, food consumption, age at preputial separation, body weight at preputial separation, biochemistry, enzyme activities, and organ weights, the data were analyzed using an F-test. When the difference between the variances was not significant, the Student t-test was performed. When the difference between the variances was significant, the Welch test was performed. Macroscopic findings and non-graded histopathological findings were analyzed by the Fisher exact probability test to determine significant differences between the treatment and vehicle control groups. Graded histopathological findings were analyzed by Mann-Whitney U test to determine significant between-group differences.

RESULTS

Clinical signs, food consumption, PPS, body and organ weights

A summary of age on the day of PPS completion, body weight, and organ weight is presented in Table 1. No treatment-related clinical signs were observed in both the PB and DEHP groups throughout the treatment period. No statistically significant and consistent changes

Table 1. Summary of age on the day of PPS completion, body and organ weights.

Measurements	Vehicle control	PB	DEHP
Age on the day of PPS completion (days)	42.2 ± 1.32	44.4 ± 3.28	43.8 ± 2.15
BW on the day of PPS completion (g)	247.1 ± 25.18	259.0 ± 31.60	269.9 ± 24.83
BW at PND54	362.2 ± 37.08	339.3 ± 13.82	369.1 ± 26.17
Liver (g)	16.85 ± 2.287	20.02 ± 1.439**	25.65 ± 2.970**
Liver (g%, weight to BW ratio)	4.64 ± 0.294	5.90 ± 0.317**	6.93 ± 0.402**
Kidneys (g)	2.71 ± 0.341	2.72 ± 0.253	3.06 ± 0.285*
Kidneys (g%, weight to BW ratio)	0.75 ± 0.056	0.80 ± 0.054	0.83 ± 0.048**
Testes (mg)	2945 ± 285.8	3035 ± 495.8	2996 ± 397.4
Testes (mg%, weight to BW ratio)	815.1 ± 51.93	895.3 ± 150.58	812.1 ± 95.19
Epididymides (mg)	476.8 ± 57.27	443.9 ± 34.77	461.3 ± 62.23
Epididymides (mg%, weight to BW ratio)	131.8 ± 10.81	131.0 ± 11.57	124.5 ± 13.70
Thyroid gland (mg)	21.23 ± 5.290	20.71 ± 3.772	20.34 ± 3.082
Thyroid gland (mg%, weight to BW ratio)	5.90 ± 1.457	6.11 ± 1.129	5.47 ± 0.657
Ventral prostate (mg)	244.3 ± 71.17	219.9 ± 41.18	258.2 ± 22.88
Ventral prostate (mg%, weight to BW ratio)	67.3 ± 17.01	64.7 ± 11.08	70.2 ± 7.80
LABC (mg)	816.5 ± 151.84	640.1 ± 52.01**	728.6 ± 72.77
LABC (mg%, weight to BW ratio)	225.3 ± 32.86	188.7 ± 14.09**	198.1 ± 22.39*
SVCG (mg)	649.1 ± 191.56	601.8 ± 122.50	634.0 ± 136.69
SVCG (mg%, weight to BW ratio)	178.9 ± 48.89	177.0 ± 34.15	171.9 ± 35.02

PPS, Preputial separation; BW, body weight; PND, Post-natal day; LABC, Levator ani plus bulbocavernosus muscle complex; SVCG, Seminal vesicles plus coagulating glands

Values expressed as mean ± S.D. (n = 10).

* p < 0.05, ** p < 0.01: Significantly different from the vehicle control group.

in food consumption and body weight were observed in both the PB and DEHP groups compared with the vehicle control group throughout the treatment period. There was no significant delay in PPS completion in both PB and DEHP groups compared with the vehicle control group, and the between-group difference in body weight on the day of PPS completion was also not statistically significant. Absolute and relative liver weights increased significantly in both the PB and DEHP groups relative to the vehicle control group as already reported in many articles (IARC, 2001; IARC, 2013). Absolute and relative kidney weights increased in the DEHP group compared with the vehicle control group. Absolute LABC weight in the PB group and relative LABC weight in both the PB and DEHP groups decreased compared with the vehicle control group.

Serum chemistry

A summary of serum hormone levels is presented in Table 2. Testosterone level was lower in the PB group than the vehicle control group, but was unchanged in the DEHP group. TSH level was higher in both the PB and DEHP groups than the vehicle control group. Although the difference in the DEHP group was not statistically significant, TSH level was higher in the DEHP group than the PB group. T3 level was slightly though significantly lower in both the PB and DEHP groups than the vehicle control group, and T4 level was significantly lower in the DEHP group. Higher Alb level was observed in the DEHP group compared with the vehicle control group. There were no statistically significant changes in any other parameters.

Enzyme activities

Enzyme activities of CYP2B, CYP3A, CYP4A, UGT, and 17 β -HSD are shown in Fig. 1. 7-pentoxoresorufin O-depentylase, lauric acid ω -hydroxylase, and testosterone 6 β -hydroxylase activities are regarded as the indicator of CYP2B, CYP4A and CYP3A induction, respectively. Hepatic CYP2B, CYP3A, CYP4A and UGT activities were increased in the PB group. Among them, the most

highly induced enzyme was CYP2B (27.2-fold control level). In the DEHP group, hepatic CYP4A activity (6.5-fold control level) was greatly increased, while CYP2B and CYP3A activities were not affected. Furthermore, UGT activity (1.3-fold control level) was slightly increased, but not statistically significant ($p=0.097$). 17 β -HSD in testis was not induced in both the PB and DEHP groups.

Pathology

Liver enlargement was observed macroscopically in both the PB and DEHP groups. Histopathological examination showed greater centrilobular hepatocellular hypertrophy in the PB group in contrast to the vehicle control group (Fig. 2B) and greater diffuse hepatocellular hypertrophy and eosinophilic granular change in the DEHP group in contrast to the vehicle control group (Fig. 2C). The histopathological effects on liver replicated findings already reported in many articles (Haines *et al.*, 2019; Rusyn *et al.*, 2006).

In the testis and epididymis, only one animal of the DEHP group exhibited unilateral soft testis, small seminal vesicle, and epididymal defect. Histopathological examination revealed diffuse atrophy of the seminiferous tubules characterized by multinucleated giant cell formation, and decreased numbers of mature sperm. Only one animal in the PB group unilaterally exhibited enlarged testis. Histopathological examination revealed diffuse dilatation of the seminiferous tubules with decreased numbers of mature sperm, unilateral decrease in the amount of sperm, and desquamated spermatogenic cells and cell debris in the epididymis. It is unclear whether the findings in the testis were present bilaterally because the histopathological examination was conducted unilaterally and gross findings in the testis and epididymis were unilaterally observed. The above-mentioned testicular changes were observed in only one animal in the DEHP and PB groups. Similar or suspected histopathological changes in reproductive tissues were not observed in any other animals of each group. In addition, the animals showing the testicular changes and the animals showing lower testo-

Table 2. Summary of hormone levels in serum chemistry.

Measurements	Vehicle control	PB	DEHP
Testosterone (ng/mL)	2.6 $\pm$ 1.66	0.5 $\pm$ 0.32**	1.7 $\pm$ 1.12
TSH (ng/mL)	10.5 $\pm$ 2.16	12.8 $\pm$ 2.51*	13.5 $\pm$ 6.32
T3 (ng/mL)	0.7 $\pm$ 0.10	0.6 $\pm$ 0.10*	0.6 $\pm$ 0.09*
T4 (μ g/dL)	6.04 $\pm$ 1.041	6.07 $\pm$ 1.020	4.27 $\pm$ 0.736**

Values expressed as mean $\pm$ S.D. (n = 10).

* $p < 0.05$, ** $p < 0.01$: Significantly different from the vehicle control group.

Fig. 1. Enzyme activities of CYP2B (A), CYP3A (B), CYP4A (C), and UGT (D) in liver and 17 β -HSD (E) in testis after treatment of PB and DEHP. Data are expressed as mean $\pm$ S.D. (n = 6) * p < 0.05, ** p < 0.01, indicates a significant difference from the vehicle control value.

sterone levels in each group did not coincide. The above findings in the testis and epididymis are considered to be spontaneous findings that can commonly be seen in rats of this strain, and the incidences of these findings were not statistically significantly different among the PB, DEHP, and vehicle control groups. Therefore, they were not considered to be exposure-related pathological changes.

In thyroid gland, the areas of reduced colloid and

increased follicular cell height were increased in the PB and DEHP groups in contrast to the vehicle control group (Fig. 2E). One animal of the PB group also showed follicular cell hyperplasia (Fig. 2F).

DISCUSSION

Phenobarbital (PB) and Di(2-ethylhexyl)phthalate

Effects on endocrine system by Phenobarbital and DEHP with male rats

Fig. 2. Histopathological lesions induced by exposure of PB and DEHP. H&E staining. (A) Control group; normal liver. Bar = 50 µm. (B) PB group; centrilobular hepatocellular hypertrophy. Bar = 50 µm. (C) DEHP group; diffuse hepatocellular hypertrophy. Bar = 50 µm. (D) Control group; normal thyroid gland. Bar = 20 µm. (E) PB group; reduced colloid area and increased height of follicular cell in the thyroid gland. Bar = 20 µm. (F) PB group; hyperplasia of follicular cell in the thyroid gland. Bar = 20 µm.

(DEHP) are well-known hepatotoxicants, with abundant toxicological information available (Haines *et al.*, 2019; Rusyn *et al.*, 2006). Many articles have suggested that phthalates including DEHP, in particular, are endocrine disrupting chemicals (EDCs) (Martinez-Arguelles *et al.*, 2013; Akingbemi *et al.*, 2004). PB and DEHP are recognized hepatic microsomal enzyme inducers and some of these induced enzymes are involved in the metabolism of steroid hormones such as testosterone and estradiol. However, very little is known about the relationship between the change in hormone level and the degree of enzyme activity induction. This study investigated the hormonal effects by PB or DEHP exposure on endocrine system including reproductive organs and thyroid gland in intact

juvenile/peripubertal male rats.

In the PB group, there were no consistent changes in food consumption, significant changes in body weight, and severe renal toxicity throughout the treatment period. A dose level (100 mg/kg/day) was considered to be adequate without causing excessive stress or severe toxicity to each treated animal. Liver effects such as increased liver weight and hepatocellular hypertrophy are adaptive changes related to hepatic microsomal enzyme induction and their observation was expected based on the existing toxicological literature (IARC, 2001). Testosterone level was significantly decreased, although its level in the DEHP group was unchanged. It is reported that PB exposure during the late prenatal development period of

rats decreased plasma and brain testosterone levels during the late fetal, early postnatal, pubertal, and adult periods (Gupta *et al.*, 1982). However, there is insufficient information about the effect of PB exposure on testicular function or sexual maturation. The weight of levator ani plus bulbocavernosus muscle complex (LABC) which is a skeletal muscle whose mass is regulated by androgens (e.g., testosterone), was significantly decreased. Since LABC weight is highly sensitive to serum androgen level, there is a possibility that the weight change correlates with lower testosterone level. Slight effects on thyroid gland, including higher TSH (thyroid stimulating hormone) level, lower T3 (triiodothyronine) level, reduced colloid area, and increased follicular cell height were observed.

The anticipated reason for lower testosterone level in the PB group is considered to be as follows. PB induces liver effects such as increased liver weight and hepatocellular hypertrophy, which are adaptive changes related to hepatic microsomal enzyme induction. In addition, analysis results of hepatic enzyme activities showed that hepatic cytochrome P450s (CYPs) such as 7-pentoxoresorufin O-depentyldase (CYP2B), testosterone 6 β -hydroxylase (CYP3A), and lauric acid ω -hydroxylase (CYP4A) were induced by PB exposure. It is generally known that testosterone is metabolized by CYPs such as CYP2B and CYP3A in liver (Turan *et al.*, 2001; Kandel *et al.*, 2017). In this study, while PB lowered testosterone level, DEHP, a well-known CYP4A inducer, had no effect on testosterone level, CYP2B, and CYP3A activities. Therefore, it seems likely that lower testosterone level was caused by induction of certain CYPs responsible for testosterone metabolism such as CYP2B and CYP3A. Furthermore, 17 β -hydroxysteroid dehydrogenase activity did not change in testis in both the PB and DEHP groups. The results of this study support the elucidation of the MOA for the lowering of testosterone level induced by PB exposure. There were no changes in the ventral prostate and seminal vesicles plus coagulating glands (SVCG), which have a close relationship with serum androgen level, and no clear delays in age at preputial separation (PPS). It was predicted that the level of luteinizing hormone (LH) secreted from the pituitary would be within normal range, because no weight changes and no clear histopathological findings were observed in male reproductive organs. Testicular weight and Leydig cell hyperplasia should be increased when testis continually receives LH stimulation. Therefore, the reduction of testosterone level detected in the present study is not likely to be a significant change affecting the ability of negative-feedback system between testis and pituitary to maintain homeostasis.

The feedback-effective range of plasma testosterone level is very restricted and differs in individual rats (Damassa *et al.*, 1976). It would be assumed that lower testosterone level without histopathological change and effect on sexual maturation is neither an adverse effect nor a physiologically meaningful phenomenon.

The plausible MOAs of the changes detected in the thyroid gland in the PB group are as follows. PB activates CAR (Constitutive androstane receptor) and then is well-known to induce various enzymes including CYP2B and UGT (UDP-glucuronosyltransferase) (Qatanani *et al.*, 2005). UGT enhances metabolism of circulating thyroid hormones such as T3 and T4 by glucuronic acid conjugation and biliary excretion of the conjugated hormone (Meek *et al.*, 2003; Papineni *et al.*, 2015). Therefore, the induction of UGT results in a decrease in T3 and T4 half-life. The increase in TSH level stimulated by PB exposure might be attributed to feedback on the HPT (hypothalamic-pituitary-thyroid) axis triggered by a decrease in circulating levels of thyroid hormones. Elevation of the circulating TSH level stimulates the thyroid gland to secrete T3 and T4 continuously (which occurs in conjunction with increased follicular cell height), and to deplete the pool of thyroid hormone (which occurs in conjunction with reduced colloid area).

In the DEHP group as in the PB group, a dose level (500 mg/kg/day) was considered to be adequate for liver effects in each treated animal without causing excessive stress or severe toxicity throughout treatment period, and the results were consistent with existing toxicological information (IARC, 2013). Slight thyroid gland effects, including lower T4 (thyroxine) and T3 (triiodothyronine) levels, reduced colloid area and increased follicular cell height, were observed. DEHP has been reported to cause the weight change and histopathological alteration of testes, disturb the hypothalamic-pituitary-thyroid axis, and then suppress the testosterone production (Ha *et al.*, 2016; Lin *et al.*, 2009; Akingbemi *et al.*, 2001; Barakat *et al.*, 2017). However, even though the dose level was sufficiently higher with compared to those of previous reports, testosterone level remained unchanged in the DEHP group in the present study. In addition, sexual maturation and reproductive organs were unaffected in this study, although DEHP is generally regarded as an EDC with antiandrogenic and estrogenic effects (Ema and Miyawaki, 2001; Ghisari and Bonefeld-Jorgensen, 2009; Cha *et al.*, 2018). Under the situation indicating clearly liver effect, it is unclear why no reproductive function-related effects were detected in the present study. DEHP may have higher sensitivity of liver effect rather than the effect on reproductive organs at lower dose level. There

might be a threshold for the DEHP-treated effects or a difference in treatment sensitivity among *in utero*, lactational, and juvenile exposures. The difference of species, its strains or treatment period/route can be also envisaged as other reasons.

The putative MOAs of the changes detected in the thyroid gland in the DEHP group are as follows. DEHP activates not only PPAR α (peroxisome proliferator-activated receptor α) but also CAR (Eveillard *et al.*, 2009; Ren *et al.*, 2010). As above-mentioned in thyroid effects by PB exposure, UGT induced via CAR activation enhances metabolism of circulating thyroid hormones such as T3 and T4. Therefore, the results of this study are considered to support that the thyroid gland response to DEHP exposure arises from CAR activation. Furthermore, thyroid effects via PPAR α activation have been also reported as synergistic acceleration of thyroid hormone degradation related to PPAR α -dependent CAR induction (Wieneke *et al.*, 2009). However, rodents such as rat and mouse express PPAR α mRNA at high levels in liver, whereas human PPAR α mRNA is expressed at low levels in liver (Palmer *et al.*, 1998; IARC, 2013). In contrast to rodents, attributable risk of effects related to PPAR α activation is considered to be low in human. Additionally, it is reported that DEHP has a potential to cause the fluctuation of mRNA expressions of Type 1, 2 and 3 iodothyronine deiodinase (D1, D2 and D3) in liver, and the reduction of gene/protein levels of transthyretin (TTR) and thyroperoxidase (TPO), sodium iodide symporter (NIS) in serum (Liu *et al.*, 2015; Dong *et al.*, 2019). Therefore, thyroid effects by DEHP exposure may originate in the combined effects on metabolism, biosynthesis, biotransformation and biotransport of thyroid hormones. Since the ratio of free thyroid hormone level over total one in rodents is much lower than that in human, rodents is generally sensitive to the thyroid effects and it is unknown whether human has same risk or not (Döhler *et al.*, 1979; Jahnke *et al.*, 2004).

In conclusion, the effects on testosterone level and thyroid gland by PB exposure were considered to be secondary changes resulting from increased hormone metabolism by hepatic microsomal enzyme induction. The effects on thyroid gland by DEHP exposure may be induced by multiple MOAs such as increased metabolism, inhibitory biosynthesis, biotransformation and biotransport of thyroid hormones. In particular, no plausible evidence has yet appeared to suggest that PB adversely affects male reproductive organs despite its use in medical therapy for epileptic patients over many years. Therefore, the change in hormone levels via hepatic microsomal enzyme induction may not have significant toxicological meaning. Further-

more, since the sensitivity of enzyme induction in liver by chemical exposure differs between species, the functional abnormality may not be observed in the reproductive organs of humans. It is assumed that a robust hormonal feed-back system (which maintains the normal physiological state) protects the living organism from the indirect effects caused by increased hormone metabolism, in contrast to the direct effects on reproductive organs such as the inhibition of steroidogenesis in testis. Further research to determine the toxicological significance of small changes in hormone level is consequently needed.

Conflict of interest---- The authors declare that there is no conflict of interest.

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