Low Dose Exposure to Di-2-Ethylhexylphthalate in Juvenile Rats Alters the Expression of Genes Related with Thyroid Hormone Regulation

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Abstract

Phthalates widely used in the manufacture of plastics have deeply penetrated into our everyday lives. Recently, a concern over the toxicity of phthalates on thyroid, has been raised but in most of cases, the doses employed were unrealistically high. To investigate the effects of phthalates on thyroid, we investigated the effects of the repeated oral exposure to low to high doses (0.3, 3, 30 and 150 mg/kg) di-2-ethylhexylphthalate (DEHP) from weaning to maturity for 90 days in juvenile rats on the thyroid. The histological examination revealed that DEHP significantly induced hyperplasia in the thyroid from the doses of 30 mg/kg, which was confirmed with Ki67 staining. In line with this finding, increased mRNA expression of thyrotropin releasing hormone (*Trh*) was observed in the thyroid of female at 0.3 mg/kg and 150 mg/kg as determined by RNAseq analysis. Moreover, significantly increased expression of parathyroid hormone (*Pth*) in the female at 0.3 mg/kg, and thyroglobulin (*Tg*) and thyroid hormone responsive (*Thrsp*) in the male at 0.3 mg/kg were noted in the blood, of which changes were substantially attenuated at 150 m/kg, alluding the meaningful effects of low dose DEHP on the thyroid hormone regulation. Urinary excretion of mono-2-ethylhexyl-phthalate (MEHP), a major metabolite of DEHP was determined to be 4.10 and 12.26 ppb in male, 6.65 and 324 ppb in female at 0.3 and 30 mg/kg DEHP, respectively, which fell within reported human urine levels. Collectively, these results suggest a potential adverse effects of low dose phthalates on the thyroid.

Key Words: Phthalate, di-2-ethylhexylphthalate (DEHP), Thyroid, Juvenile toxicity, Thyroid hormone, Proliferation

INTRODUCTION

Phthalates, a family of phthalic acid esters are widely used as plasticizers in the manufacture of polymer-products offering flexibility, durability and elasticity to coating materials, cable, film, tubes, wire, flooring materials, cleanser, automobile, soap, shampoo, hair-sprayer, color-cosmetics and even personal care products (Takatori *et al*., 2004; Calafat and McKee, 2006). Phthalates such as di-2-ethylhexylphthalate (DEHP), diisodecylphthalate (DIDP), and diisononylphthalate (DINP) have been used worldwide and their global production has reached nearly 10 million tons per year. Unfortunately, phthalates are associated with undesirable human health effects that include abnormal reproductive organ developments (Liu
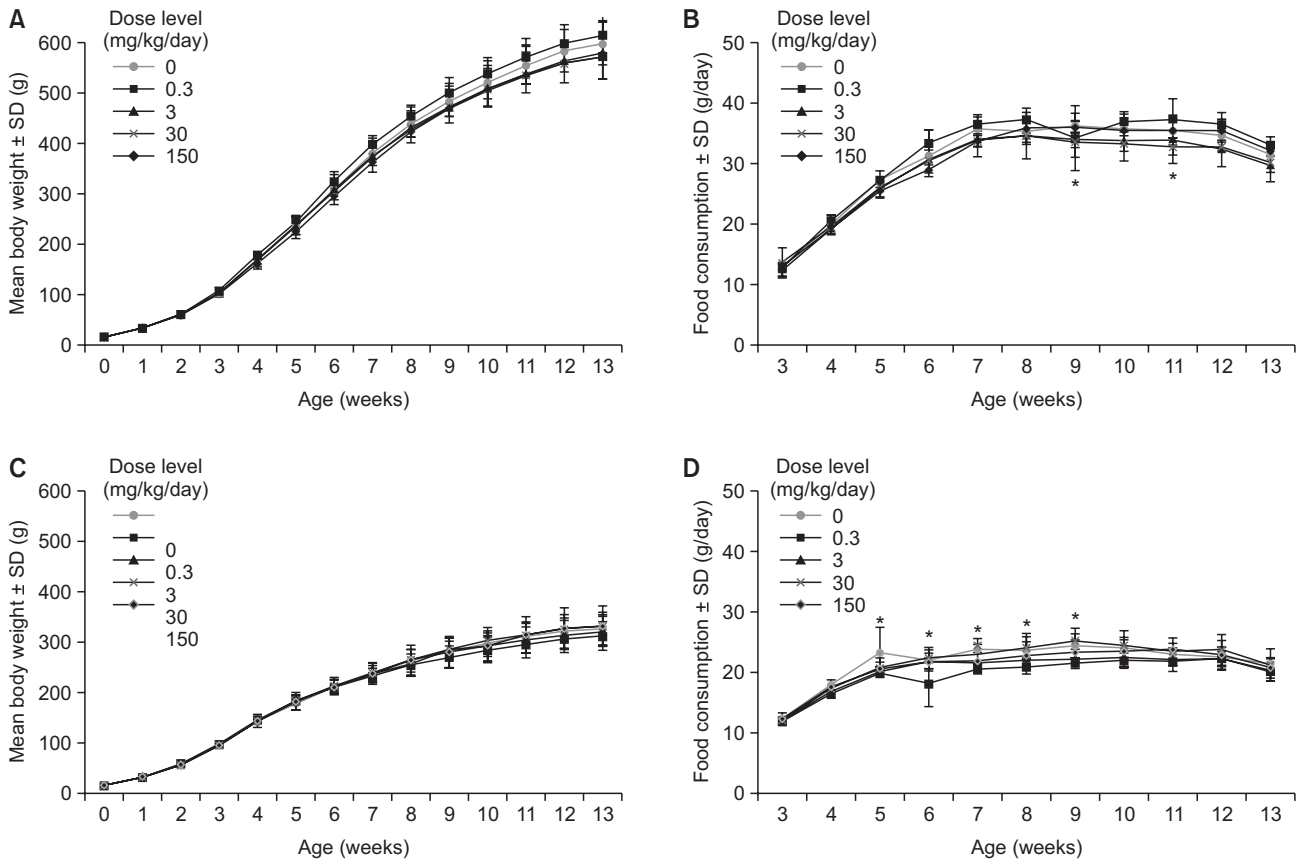
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Fig. 1. Body weight and food consumption of juvenile rats exposed to DEHP for 90 days. Body weights of male (A) and female (C) rats orally exposed to DEHP at 0, 0.3, 3, 30 and 150 mg/kg/day. Food consumptions of male (B) and female (D) rats. Data are mean ± SD of 9-10 animals. *$p < 0.05$ (One-way ANOVA) followed by Bonferroni *post hoc* to examine the difference from 0 mg/kg/day.

et al., 2018), tumorigenesis (Takeshita *et al*., 2006; Miao *et al*., 2017), and reproductive toxicity with respect to spermatogenesis and fetal development (Latini *et al*., 2003; Martino-Andrade and Chahoud, 2010), and respiratory toxicity (Lopez-Carrillo *et al*., 2010; Ventrice *et al*., 2013).

Especially concern over the toxicity of DEHP is increasing due to the heavy use of polyvinylchloride (PVC) for food packaging materials and resultant ubiquitous presence of DEHP in community and environment (Latini, 2005). Indeed, human is being exposed to DEHP for life time from *in utero*. The average daily exposure of human to DEHP is estimated to be 0.003-0.03 mg/kg/day (7.7-77 μmole/kg/day) (Agency for Toxic Substances and Disease Registry, 2002) and exposure of children is considered more serious because DEHP penetrates into placenta, excreted into breast milk and is heavily used in the packaged food materials and toys (Main *et al*., 2006; Schettler, 2006). Reflecting this, Cutanda *et al*. (2015) demonstrated that urine level of phthalates is higher in children than mothers. In the worst case scenario examining the infants admitted to Neonatal Intensive Care Unit, measurement of urine level of phthalate metabolites suggested the daily exposure level to reach up to 233-352 μg/kg bw/day (Weuve *et al*., 2006).

Biological effects of phthalates are largely considered from their endocrine disruptor activity (Ohtani *et al*., 2000; Feige *et al*., 2007). Reproductive organs (testis and uterus) (Mylchrest *et al*., 1999), adipose tissues (Campioli *et al*., 2011) and

thyroids (Liu *et al*., 2015; Dong *et al*., 2017) have been suggested as the major target organs of phthalate-mediated toxicity. Of these, recent interests are being directed to the effects of phthalates on the thyroid tissue and thyroid hormone regulation. Indeed, correlation between serum thyroid hormone levels and DEHP metabolites has been revealed in US, Danish and Taiwanese population (Boas *et al*., 2006; Meeker *et al*., 2007; Meeker and Ferguson, 2011; Wu *et al*., 2013). This issue has drawn further attention in relation with increased incidences of thyroid cancer worldwide (Davies and Welch, 2006). To elucidate the effects of phthalates on the thyroid, *in vivo* rodent studies have been conducted which revealed the alteration of thyroid following the exposure to DEHP (Liu *et al*., 2015; Dong *et al*., 2017), where significant effects on thyroid hormone and metabolism were noted. However, these studies employed extremely high doses of DEHP ranging 150 mg/kg to 600 mg/kg, which are rather unrealistic in terms of human exposure levels of 0.003-0.03 mg/kg/day (7.7-77 μmole/kg/day).

Here we investigated whether the subchronic exposure to low dose DEHP in juvenile animals from their weaning to maturity may affect the thyroid with a special attention to the proliferative changes in the thyroid. To identify the possible effects on the thyroid, expression of genes related with thyroid hormone homeostasis was examined with RNAseq technique in the thyroid tissue and blood of the exposed animals.
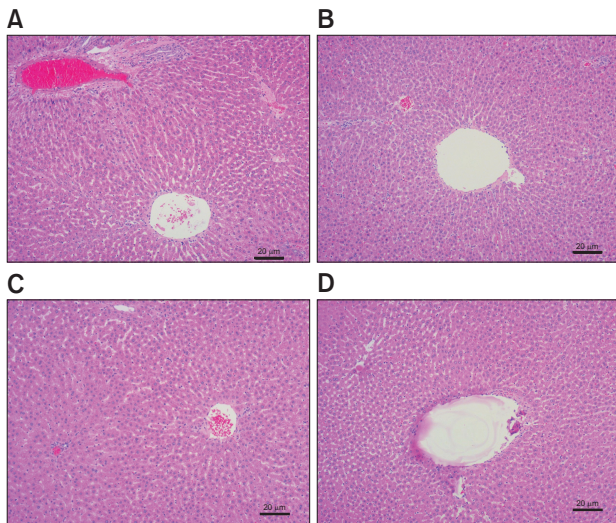
Fig. 2. Histology of livers of DEHP-treated juvenile rats, H&E. (A) DEHP 0 mg/kg male, (B) DEHP 0 mg/kg female, (C) DEHP 150 mg/kg male (D) DEHP 150 mg/kg male. (Bar=20 μm). Representative slides of 9-10 animals per a group.

Fig. 3. Histology of thyroids of DEHP-treated juvenile rats, H&E. (A) DEHP 0 mg/kg male, (B) DEHP 0 mg/kg female, (C) DEHP 150 mg/kg male (D) DEHP 150 mg/kg male. (Bar=20 μm). Representative slides of 9-10 animals per a group.
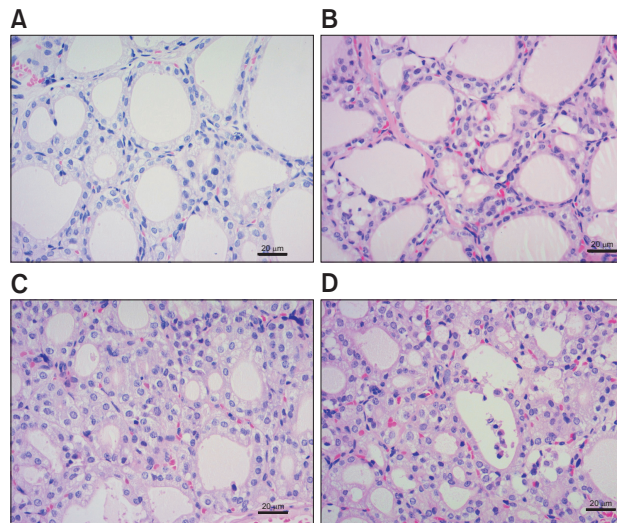
We employed 0.3, 3, 30 and 150 mg/kg to cover the highest human exposure level reported and the lowest level investigated *in vivo* previously. We also measured urinary excretion of mono-2-ethylhexyl-phthalate (MEHP), a major metabolite of DEHP to estimate the systemic exposure to DEHP (Koo and Lee, 2007) in an effort to seek the relevance of our findings to human exposure scenario.

MATERIALS AND METHODS

Materials

Di-2-ethylhexylphthalate (DEHP, synonym Dioctyl phthalate, ≥99.5%) was purchased from Sigma-Aldrich (St. Louis, CA, USA). Dose formulations were prepared on the day of dosing with corn oil for oral gavage administration as appropriate. LC-MS grade water (Tedia, NJ, USA), acetonitrile (Burdick & Jackson, MI, USA), and ammonium acetate (Sigma-Aldrich) were used in the mobile phase of LC system.

Animals and husbandry

F0 generation male and female Sprague-Dawley (Crl:CD) rats were obtained from Orient Bio Inc (Gyeonggi, Korea). For obtaining pregnant animals, two females were placed in the cage with one male overnight. F1 generation male and female pups were selected on postnatal day (PND) 4. All procedures were in compliance with Animal Welfare Act and Guide for the Care and Use of Laboratory Animals (by ILAR publication) and assessed by the Institutional Animal Care and Use Committee (IACUC) of KIT (The approval number is 1704-0150). The animal room was maintained in a controlled environment (temperature: 23 ± 3°C, humidity: 30-70%, light cycle: 12 h light/12 h dark cycle, light intensity: 150-300 Lux, air changes: 10 to 20 times/h). A standard rodent pellet diet (Lab Diet® #5053 PMI Nutrition International, Brentwood, MO, USA; irradiated by gamma-ray) and filtered, ultraviolet light-irradiated municipal tap water were provided to the animals *ad libitum*.

Animal experiments

PND 4 rats were considered to be equivalent to preterm human babies (Bowman *et al*., 2011) and the PND 7 are believed to be similar to humans at birth (Beck *et al*., 2006). On PND 4, each pups are cross-fostered between litters such that each litter is comprised of pups originating from at least 6 different litters, with no litter-mates of the same sex, and containing no pups from the original litter. Pups were assigned to toxicology and toxicokinetic groups. Male and female pups were administered by oral gavage once daily at dose level of 0, 0.3, 3, 30 and 150 mg/kg/day from PND 6 to 96 for 13-week. Parameters including mortality, clinical sign, body weight, food consumption, sexual maturation, organ weight, clinical pathology, macro- and microscopic findings were examined. Urine was collected for 16 h from dosing the day 91 for the analysis of MEHP, a major metabolite of DEHP. Urine was stored in –80°C prior to analysis. Thyroid and liver were fixed in formalin, embedded in paraffin, and undergone section to stain with H&E.

Ki-67 immunohistochemistry and quantitative analysis

For examination of thyroid cell proliferation, we processed immunohistochemical staining for Ki-67 as described previously (Kim *et al*., 2016; Jeong *et al*., 2017). To perform immunohistochemistry, samples were fixed in 4% paraformaldehyde and then embedded in paraffin. The sections were de-paraffinized and rehydrated sequentially. Antigen retrieval was carried out using pH6.0 Target Retrieval solution (DAKO, Carpinteria, CA, USA) in a pressure cooker for 15 min. After cooling for more than 1 h on ice, the sections were incubated in 3% H_2O_2 for 30 min to block endogeneous peroxidase activity. The sections were washed twice with PBS and incubated with Protein block serum-free (DAKO) for 2 h at 25°C to reduce non-specific signal. Sections were incubated overnight at 4°C with primary antibody specific for Ki-67 (abcam, Cambridge, UK) at a 1:200 dilution. After washing three times with PBS, the sections were incubated for 15 min with HRP-
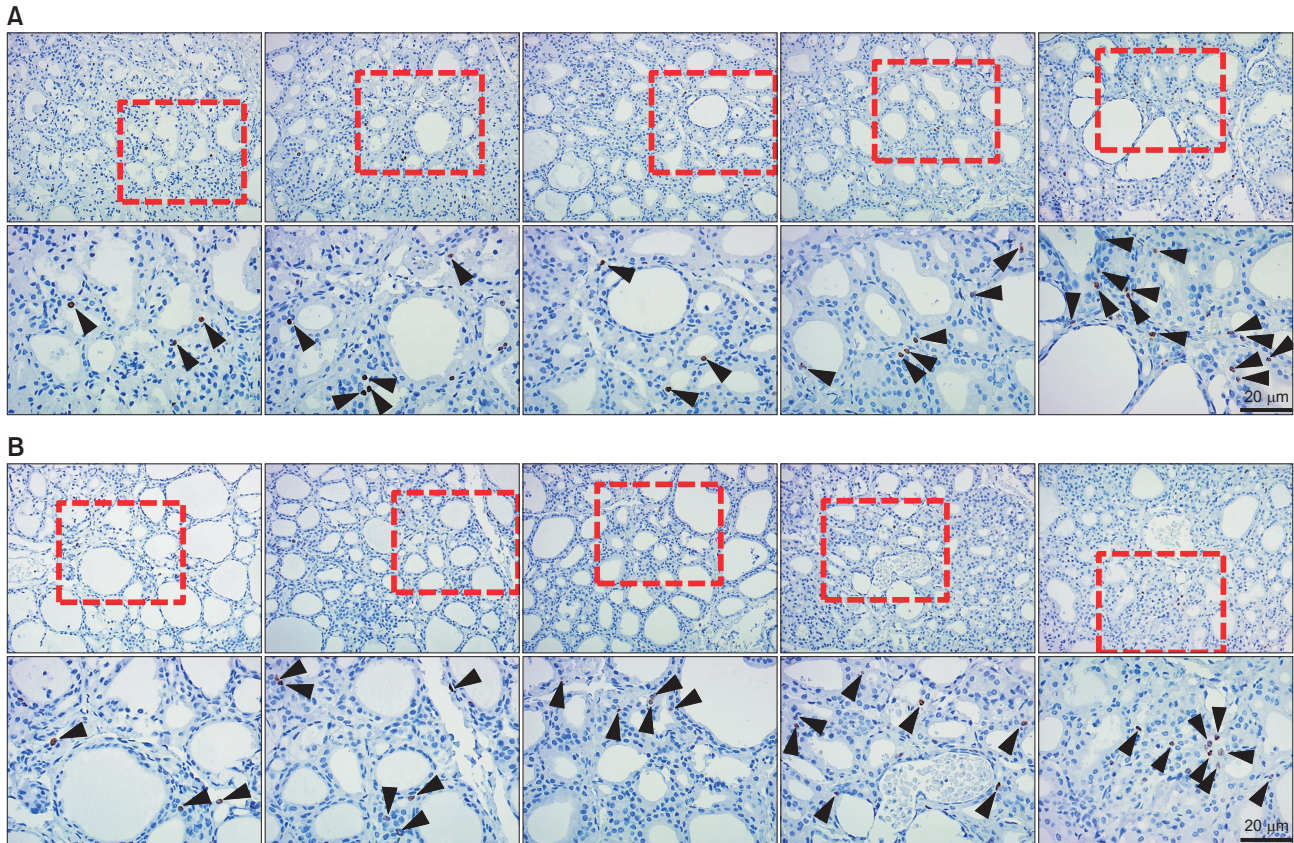
Fig. 4. Cell proliferation in the thyroids of DEHP-treated juvenile rats. Photomicrograph of the immunohistochemistry for Ki-67 to evaluate thyroid cell proliferation, (A) DEHP 0, 0.3, 3, 30 and 150 mg/kg male, (B) DEHP DEHP 0, 0.3, 3, 30 and 150 mg/kg female. Representative slides and arrow heads indicating Ki-67^{+} cells.

conjugated rabbit secondary antibody (DAKO) at 25°C. For immunohistochemistry, DAB (DAKO) was used for antibody development and Mayer's Hematoxylin (DAKO) was used for counter staining.

For quantitative analysis, all thyroid area in each group samples was captured using Olympus BX43 microscope 40x lens (Olympups Optical Co., Shinjuku, Japan). Using the ImageJ (National Institutes of Health, Bethesda, MD, USA), we counted total cell of each group thyroid and calculated the percentages of Ki-67 positive cells, respectively.

Construction of RNA-seq libraries

Total RNA was extracted from ~30 mg of each tissue sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA library was prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The mRNA was fragmented, followed by first and second strand cDNA synthesis. The cDNA was subject to adenylation of 3' ends and adapter ligation. cDNA sample was purified using AMPure XP beads (Beckman Coulter, CA, USA) and then used in 15 cycles of PCR amplification (Eastwin, Beijing, China). The cDNA library quality and size distribution was checked with a Bioanalyzer and DNA 1000 chip (Agilent Technologies). Library fragment sizes of all the samples were

ranged between 250 and 500 bp, with a peak at ~310 bp. All libraries were quantified with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by NextSeq 500 sequencing system (Illumina).

RNA-seq data analysis

FastQC was used to check the sequence quality of the reads. The cutadapt was then used to clean the reads by removing adapter sequences. The cleaned reads were aligned to the Ensembl rn5 rat transcriptome to obtain gene-level expression count data with subread R package (Liao *et al*., 2013). For each gender and tissue, differential expression was tested for 150 mg/kg and 0.3 mg/kg versus vehicle control group, in which the vehicle control group was used as the control. The differential expression analysis was performed with DESeq2 R package (Love *et al*., 2014).

Urine analysis for mono-ethylhexyl phthalate, MEHP

Analysis for the determination of urinary excretion of MEHP was conducted as previously reported (Kato *et al*., 2005; Teitelbaum *et al*., 2008). Urine 0.5 ml of method blank, MEHP standards (1, 2, 5, 10, 20, 50, 100 and 200 ppb, Accustandard. Inc., New Haven, CT, USA), quality control samples, (QL 5 ppb, QM 50 ppb, QH 200 ppb) and samples were mixed with 2 M ammonium acetate buffer 1 ml and β-glucuronidase (Sigma-Aldrich) 20 μl and incubated for 15 h in 37°C incubator. After hydrolysis, the samples were mixed with 2 N HCl 100

Table 1. Counts of Ki67 positive cells in the thyroid tissue of rats orally administered with DEHP for 90 days

DEHP (mg/kg)	Male			Female		
	Ki67	Non-Ki67	Total cell	Ki67	Non-Ki67	Total cell
0	74	18268	18342	47	14437	14484
0.3	119	22798	22917	95	23933	24028
3	85	19844	19929	57	16275	16332
30	136	25830	25966	111*	18786	18897
150	285*	22620	22905	115*	19256	19371

*$p < 0.05$ by χ^2 test.

μl, 200 ppb internal standard (MEHP-$^{13}C_2$, Cambridge Isotope Laboratories. Inc. Tewksbury, MA) 50 μl and ethyl acetate 4 ml for extraction. After agitation in vortex for 5 min, samples were centrifuged at 4000 rpm for 10 min and then, supernatant was separated into 3 ml glass tube. After vaporization in N_2 concentrator, the residue was re-dissolved into 60% acetonitrile 100 μl. 10 μl aliquot was injected into LC-MS/MS instrument (Agilent 1200 series (LC) / Agilent 6460 QQQ (MS), Agilent, Santa Clara, CA, USA).

Analytes were separated on CAPCELL PAK C18 MG II 3.0 mm×150 mm 3 μm (Shiseido, Tokyo, Japan) with a gradient condition with mobile phase A (0.1% acetic acid in D.W) and B (0.1% acetic acid in acetonitrile) varying mobile phase A from 90% to 0% for 20 min then back to 90% by 30 min at the column temperature of 35°C with the flow rate of 0.3 ml/min. MS detector condition was as follows; capillary voltage −4000 V, EMV 700, nebulizer 50 psi, gas temperature 350°C, gas flow 10 ml/min. MEHP was detected as 277.2 → 134.1 while IS was 281.2 → 137.1. Limit of quantitation (LOQ) for MEHP is 5 ng/ml.

Statistics

Statistical analyses for comparisons of the various dose groups with the vehicle control group were conducted using Pristima System (Version 6.4. Xybion Medical Systems Corporation, Morris Plains, NJ, USA). Difference from control (0 mg/kg) group was examined using Student *t*-test. When multiple comparison tests for different dose groups were conducted, variance of homogeneity was examined using the Bartlett's Test. Homogeneous data was analyzed using the Analysis of Variance (ANOVA) and the significance of inter-group differences were analyzed using Dunnett's Test or Bonferroni *post hoc* test. Heterogeneous data was analyzed using Kruskal-Wallis Test and the significance of inter-group differences between the control and treated groups were assessed using Dunn's Rank Sum Test. RNAseq data were tested with likelihood ratio test in DESeq2 R package.

RESULTS

Effects of DEHP on body weight change, food consumption, hematology and blood chemistry in juvenile rats

To examine the effects of DEHP exposure during young ages on the thyroid, juvenile rats (at the time of weaning, postnatal day 6) were orally administered with DEHP dissolved in corn oil for 90 days. There were no meaningful clinical signs related with the treatment. The body weight was not affected by the exposure to DEHP at any dose levels in both sexes (Fig. 1A, 1C) but reduced food consumption were noted sporadically in 30 mg/kg male group and all the dosed female groups (Fig. 1B, 1D). Especially in 0.3 mg/kg female group, a significant reduction of food consumption as high as 18% was noted from day 30 to day 65.

Hematology as well as blood coagulation assay revealed that no significant differences in both male and female rats exposed to DEHP were noted (Supplementary Table 1). Blood chemistry showed that γ-glutamyl transpeptidase (GGT) decreased at the dose of 30 mg/kg and 150 mg/kg in female (0.27 ± 0.21 and 0.18 ± 0.23 versus 0.60 ± 0.34, $p < 0.05$, student *t*-test, Supplementary Table 2). Other statistically significant changes noted in dosing groups compared with the control were not considered treatment-related, as these values were within the normal ranges of biological variation.

Effects of oral treatment of DEHP on the sexual maturation and organ weights

To examine the effects of DEHP on the sexual maturation of male and female juvenile rats, preputial separation and vaginal opening time were examined respectively. Exposure to DEHP did not affect the sexual maturation of juvenile rats of both sexes (data not shown).

However, measurement of absolute (Supplementary Table 3) and relative organ weights (Supplementary Table 4) revealed that kidney and liver of male and female rats dosed with 150 mg/kg DEHP showed increased weights. In addition, mammary glands of male dosed at 30 mg/kg and left ovaries of female dosed at 3 and 30 mg/kg decreased in absolute or relative weights.

Histopathological examination of the liver and thyroid of the juvenile rats exposed to DEHP

Previously, DEHP administration was known to affect liver and thyroid at the doses above 250 mg/kg (Liu *et al*., 2015; Dong *et al*., 2017). While there were no treatment related histological findings in liver (Fig. 2), hyperplasia and hypertrophy of thyroid glands were evident in the rats administered with DEHP 150 mg/kg for 90 days (Fig. 3). To further confirm this, thyroid tissues were immunohistochemically stained for Ki67 to quantitate the proliferative changes (Fig. 4). Significant increases in proliferating cells in the male rats dosed at 150 mg/kg and female at 30 and 150 mg/kg were demonstrated (Table 1), reflecting that DEHP exposure may induce proliferative alteration of thyroid tissue.
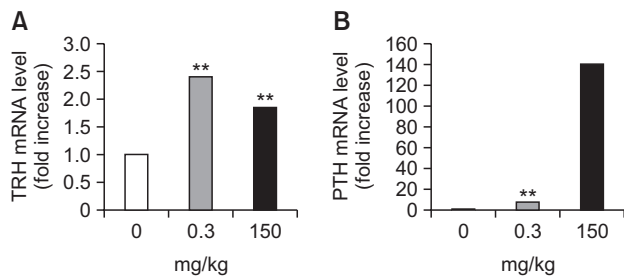
Fig. 5. Gene expression changes in the thyroid and blood of female rats exposed to DEHP at 0.3 mg/kg and 150 mg/kg. Of the genes examined by RNAseq, statistically, significantly changed genes with more than 2 fold in the thyroid and blood were shown, (A) *Trh* mRNA levels normalized by the mean of vehicle control in the thyroid of female rats (B) *Pth* mRNA levels in the blood of female, (N=3), **significant at *p*<0.01 by likelihood ratio test in DESeq2 R package.
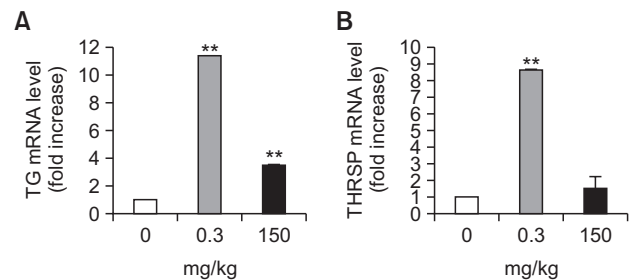

Fig. 6. Gene expression changes in the blood of male rats exposed to DEHP at 0.3 mg/kg and 150 mg/kg. Of the genes examined by RNAseq, statistically, significantly changed genes with more than 2 fold in the thyroid and blood were shown, (A) *Tg* mRNA levels in the blood of male, (B) *Thrsp* mRNA levels in the blood of male. (N=3), **significant at *p*<0.01 by likelihood ratio test in DESeq2 R package.

Expression of genes related with thyroid physiology examined with RNAseq technique in the thyroid and blood of the rats exposed to 0.3 mg/kg and 150 mg/kg DEHP

Previous studies have shown that high doses of DEHP can upregulate the genes like thyroid peroxidase, thyroid deiodinase 1, thyroid stimulating hormone receptor, sodium/iodide symporter (NIS), and thyroglobulin but it was not examined whether lower doses also can alter the genes related to thyroid physiology. To identify the gene expression changes induced by DEHP regarding thyroid hormone homeostasis, expression of related genes in the thyroid and blood of the rats treated with 0.3 mg/kg were quantitated with RNAseq technique. As a result, in the thyroid, thyrotropin-releasing hormone (*Trh*) gene was found to be up-regulated significantly in the female rats dosed with 0.3 mg/kg DEHP (Fig. 5A). Interestingly, significantly increased mRNA expression of parathyroid hormone (*Pth*) in the female 0.3 mg/kg (Fig. 5B), and thyroglobulin (*Tg*) (Fig. 6A) and thyroid hormone responsive (*Thrsp*) (Fig. 6B) in the male 0.3 mg/kg group were also found in the blood reflecting that DEHP may affect the thyroid hormone regulation, which is in line with the histopathological examination. Interestingly, these changes were substantially attenuated at 150 mg/kg, reflecting that distinct effects of low dose DEHP may exist.

Urine analysis for mono-ethylhexyl phthalate, MEHP

To estimate the systemic exposure of DEHP in this study, urine was collected for 16 h on the final dosing day and measured for the concentration of MEHP, a major bio-metabolite of DEHP using LC/MSMS. To assess the total MEHP (free and conjugated forms), samples were treated with β-glucuronidase before analysis. As shown in Table 2, urinary excretion of MEHP increased dose-dependently by DEHP (from 4.10 to 12.26 ppb in male, from 6.65 to 324 ppb in female at 0.3 to 30 mg/kg) where the female exhibited higher systemic exposure over male.

DISCUSSION

Here we demonstrated that the exposure to DEHP during young ages may induce proliferative changes in the thyroid and alter gene expression related to thyroid hormone regulation. Ratio of proliferative cells was significantly increased in female rats dosed at 30 and 150 mg/kg and in male at 150 mg/kg. Although evident proliferative changes were not observed in 0.3 mg/kg, significant changes in the mRNA levels of the genes such as *Trh*, *Pth*, *Tg* and *Thrsp* were induced, reflecting that low dose phthalates may also influence thyroid hormone regulation. Urinary excretion of MEHP increased dose-dependently but a considerable gender difference was noted where the female rats exhibited higher systemic exposure amounting to 1.6 to 146 fold over male as the dose increases. Importantly, highest urinary metabolites of phthalates in human were reported at the ranges of 47.1 ppb to 331 ppb which was coincided with the levels shown in our study (from 4.10 to 12.26 ppb in male, from 6.65 to 324 ppb in female at 0.3 to 30 mg/kg) suggesting the potential adverse effects of the low dose phthalates on the thyroid in human.

Along with other endocrine disruptors like bisphenol A (Chung *et al*., 2017; Jeong *et al*., 2017; Kim *et al*., 2018), carcinogenic effects of phthalates have drawn attention due to their ubiquitous presence in community and environment. The association of phthalate exposure with the risk of cancer in human has been illuminated by the seminal study of López-Carrillo L *et al*., who showed that exposure to DEHP may be associated with increased risk of breast cancer (Lopez-Carrillo *et al*., 2010). Potential risk of pancreatic cancer has been also suggested (Selenskas *et al*., 1995; Ventrice *et al*., 2013). The carcinogenic effects of phthalates have been explained by the activation of peroxisome proliferator-activated receptors and aryl hydrocarbon receptor mediated transcription pathways (Hsieh *et al*., 2012). Mechanism behind the proliferative effects of DEHP on the thyroid can also be speculated into its endocrine disrupting (antagonistic) effects of thyroid hormone homeostasis and hypothalamus-pituitary-thyroid axis (Zhai *et al*., 2014). Liu *et al*. (2015) showed that *in vivo* exposure to high dose of DEHP for 30 days in rats reduced circulating T3, T4 thyroid hormone and thyrotropin releasing hormone (TRH) which reached statistical significance at 500-750 mg/kg. In line with this, Liu *et al*. (2015) found that suppression of biosynthesis, and transport of thyroid hormone was noted as determined by reduced sodium iodide symporter and thyroid peroxidase levels, which they attributed to the elevation of hepatic enzymes involved in the thyroid hormone elimination and

Table 2. Urine levels of MEHP (µg/L or ppb) of rats orally administered with DEHP for 90 days

DEHP (mg/kg)	Male (N=4) Mean ± SD (Min, Max)	Female (N=4) Mean ± SD (Min, Max)
0	4.31 ± 0.18 (4.07, 4.36)	4.82 ± 0.79 (4.26, 5.95)
0.3	4.10 ± 0.09 (4.00, 4.23)	6.65 ± 1.47 (5.18, 8.45)
3	4.91 ± 0.50 (4.43, 5.62)	21.64 ± 5.38 (15.31, 28.37)
30	12.26 ± 5.34 (6.47, 19.38)	324 ± 207.80 (106.28, 604.95)
150	75.55 ± 44.02 (29.19, 134.48)	11,037.95 ± 15,900.81 (11,037.95, 34,867.47)

resultant acceleration of thyroid hormone metabolism. Dong *et al.* (2017) also confirmed the decreased thyroid hormone levels in the rats exposed to DEHP at 150, 300, and 600 mg/kg for 3 and 6 months.

While evidence on the disruption of thyroid hormone homeostasis by DEHP is strong, previous studies have not addressed the possible implication on the proliferative changes on thyroid induced by DEHP. Dong *et al.* demonstrated that exposure to the high dose of DEHP induced pronounced fat degeneration, fat drops, empty lipocytes, and apoptosis cell in thyroid tissues (Dong *et al.*, 2017) but it remains unclear whether DEHP can induce proliferation of thyroid cells, which is a vital line of evidence linking DEHP exposure and thyroid cancer. Liu *et al.* (2015) demonstrated that extremely high doses of DEHP (500 and 750 mg/kg) induced hyperplasia but it is hard to find a relevance of this dose level to realistic human exposure. To fill this gap, we examined lower dose ranges that cover the reported human exposure level, and the lowest dose explored for the effects on thyroid in other studies. Human urine levels of phthalate metabolites were reported to be from 47.1 ng/ml (mono-n-butyl phthalate (MnBP), median) in school children (Wang *et al.*, 2015) to 331 ng/ml (total phthalate metabolites, median) in China (Guo *et al.*, 2011), that were in proximate ranges with the urinary excretion levels we measured. Indeed, quantitation of MEHP in the pooled urine samples revealed that female 30 mg/kg group, which showed significantly increased Ki67 expressing proliferating cells in the thyroid, showed 106.28-604.95 ng/ml. Moreover, gene expression changes in the thyroid and blood could be observed at 0.3 mg/kg doses which showed urinary MEHP levels of 4.00 to 4.23 ng/ml in males and 5.18 to 8.45 ng/ml in females, which further supports the relevance of our results to the real human exposure.

In conclusion, we demonstrated that exposure to low-dose DEHP in juvenile rats from young ages to maturity, may induce proliferative changes in thyroid tissues without changes in liver histology. Even without clear histological changes, significant changes in the mRNA levels of the genes related with thyroid hormone regulation such as *Trh*, *Pth*, *Tg* and *Thrsp* were induced at 0.3 mg/kg DEHP. The urinary excretion of MEHP was comparable to the reported human urinary phthalate metabolite levels, supporting the relevance of our findings to real human exposure. Further studies on the links of low dose phthalate exposure with human thyroid cancer are warranted to fully elucidate the potential risk of thyroid toxicity of phthalates.

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