Food and Chemical Toxicology 124 (2019) 265–272

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Di-2-ethylhexylphthalate promotes thyroid cell proliferation and DNA damage through activating thyrotropin-receptor-mediated pathways *in vitro* and *in vivo*

Seoyoung Kim[a], Ga-Young Park[a], Young Jo Yoo[a], Ji Seong Jeong[b], Ki Taek Nam[c], Sun-Ha Jee[d], Kyung-Min Lim[a,*], Yun-Sil Lee[a,**]

[a] Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, 03760, Republic of Korea
[b] Developmental and Reproductive Toxicology Research Group, Korea Institute of Toxicology, Daejeon, 34114, Republic of Korea
[c] Severance Biomedical Science Institute, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, 03722, Republic of Korea
[d] Department of Epidemiology and Health Promotion and Institute for Health Promotion, Graduate School of Public Health, Yonsei University, Seoul, 03722, Republic of Korea

ARTICLE INFO

Keywords:
Phthalates
Di-(2-ethylhexyl) phthalate (DEHP)
Thyroid
DNA damage
Proliferation
Thyrotropin receptor (TSH)

ABSTRACT

Phthalates are being suggested to be associated with altered thyroid function and proliferative changes, but detailed mechanisms remain unclear. Here, we examined the effects of di-(2-ethylhexyl) phthalate (DEHP) on DNA damage and proliferation in thyroid using thyroid carcinoma cell line, 8505C, *in vitro* and the rats orally treated with DEHP at 0, 0.3, 3, 30 and 150 mg/kg for 90 days from post-natal day 9 *in vivo*. Exposure to DHEP (1–50 μM) induced cellular proliferation, as evidenced by increased cell viability and DNA synthesis. Activation of γH2AX, a sensitive biomarker for DNA damage was observed following the exposure to DHEP (from 5 to 50 μM) with increased comet tail moment (5–100 μM) in comet assay, reflecting that DNA damage also occurred. When upstream signaling was examined, both thyrotropin receptor (TSHR)-ERK1/2 axis and TSHR-AKT axis were activated with upregulation of Pax8, a master transcriptional factor for thyroid differentiation and proliferation. Thyroid tissue from juvenile rats orally exposed to DEHP also confirmed DNA damage responses and the activation of TSHR signaling, which was evident from 0.3 to 3 mg/kg respectively. Notably, deletion of TSHR through siRNA attenuated these DEHP-induced events *in vitro*. Collectively these results suggest that DEHP induces DNA damage and cellular proliferation in thyroid, which appears to be from TSHR activation, providing an important insight into endocrine disrupting activities of phthalates on thyroid.

1. Introduction

Phthalates are widely used as plasticizers to confer the polymer products with flexibility as in the manufacture of cables, tubings, coatings, flooring materials, toys, and even in cosmetics and personal care products (Calafat and McKee, 2006; Takatori et al., 2004). Accordingly, phthalates exist ubiquitously in human community and surrounding environment and inevitably, humans are exposed to high level of phthalates through oral, dermal and inhalation routes (Calafat and McKee, 2006; Deutschle et al., 2008; Miao et al., 2017). Average daily exposure to phthalates was reported to be 0.003–0.03 mg/kg/day (7.7–77 μmol/kg/day) (ATSDR, 2002) and children is considered more susceptible to phthalates with the high daily exposure levels hovering over 233–352 μg/kg bw/day in extreme conditions as the intensive care unit (Weuve et al., 2006). Unfortunately, phthalates are associated with diverse adverse human health effects that include abnormal reproductive organ developments (Liu et al., 2018), tumorigenesis (Miao et al., 2017; Takeshita et al., 2006), and reproductive toxicity (Latini et al., 2003; Martino-Andrade and Chahoud, 2010), and respiratory toxicity (Lopez-Carrillo et al., 2010; Ventrice et al., 2013).

Special attention is directed on the human exposure and toxicity of di-(2-ethylhexyl) phthalate (DEHP) which has been profusely used in the manufacture of polyvinylchloride (PVC) that is widely applied in various consumer products, such as building materials, clothing, cosmetics and personal care, cleaning materials and medical products (Deutschle et al., 2008; Latini, 2005). DEHP is the most widely used plasticizer and known to be produced 100-500 million tons per annum in US alone (Nazaroff et al., 2012). Harmful effects of DEHP on human

* Corresponding author.
** Corresponding author.
E-mail addresses: kmlim@ewha.ac.kr (K.-M. Lim), yslee0425@ewha.ac.kr (Y.-S. Lee).

https://doi.org/10.1016/j.fct.2018.12.010
Received 1 September 2018; Received in revised form 16 November 2018; Accepted 7 December 2018
Available online 10 December 2018
0278-6915/

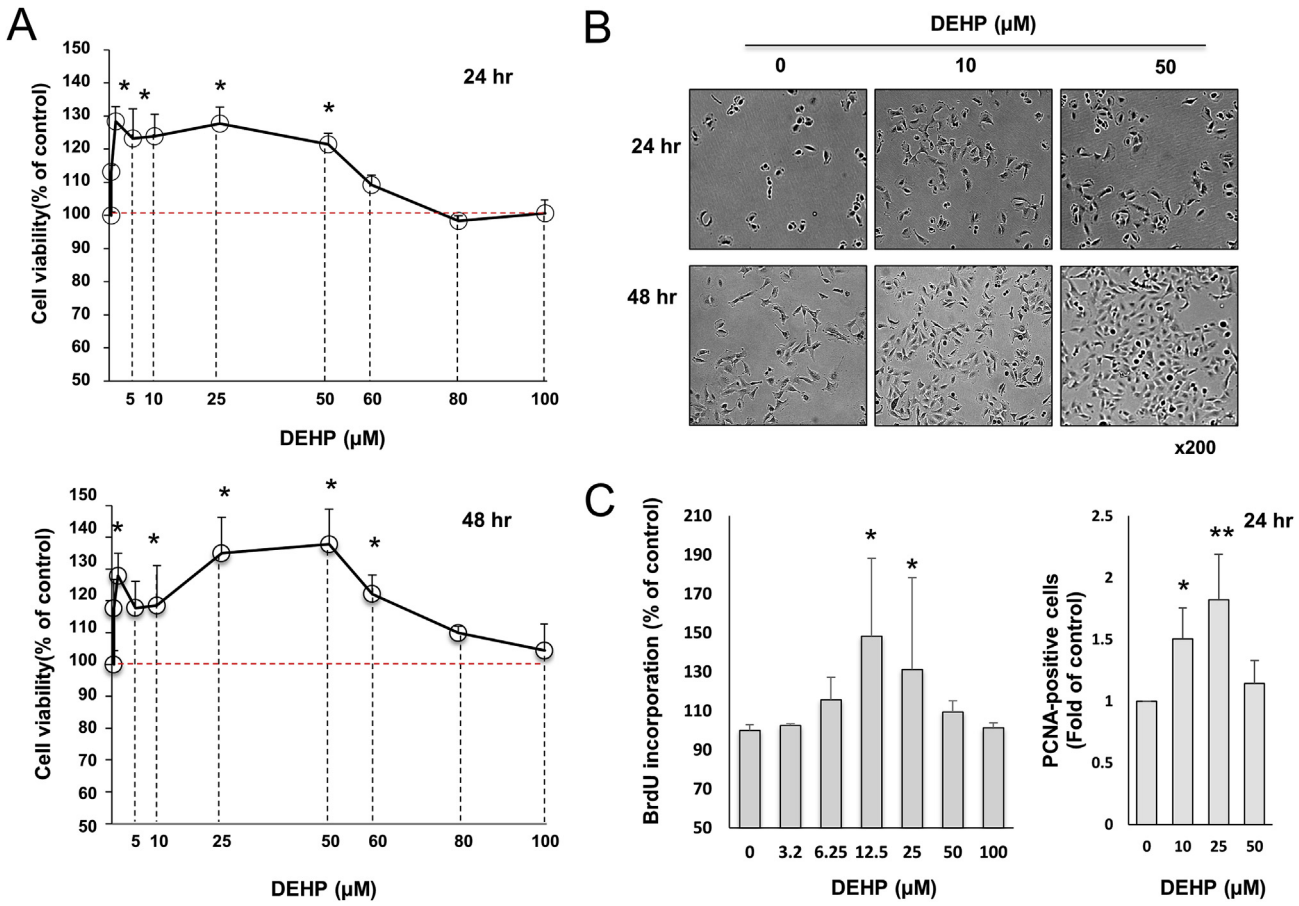

Fig. 1. Effects of DEHP on cellular proliferation in thyroid cells. (A) Cell viability of 8505C cells was assessed after exposure to range of DEHP concentrations (0.1–100 μM) for 24 or 48 h using MTT assay. (B) 8505C cells were exposed to DEHP (1–50 μM) for 48 h, and cell morphology was observed and photographed by phase contrast microscopy (magnification, × 200) (C) At 24 h after treatment with indicated doses of DEHP, BrdU incorporation and flow cytometry for 8505C cells stained with PCNA and propidium iodide was evaluated. Data are expressed as mean ± SD from at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ vs untreated control.

health are largely considered to arise from its endocrine disrupting activity (Feige et al., 2007; Ohtani et al., 2000). Endocrine tissues like reproductive organs (Liu et al., 2018; Mylchreest et al., 1999), adipose tissues (Campioli et al., 2011) and thyroids (Dong et al., 2017; Liu et al., 2015) are examined as the target organs for DEHP with respect to abnormal organ development, altered cell differentiation and hormone dysregulation.

Especially, effects of DEHP on the thyroid tissue and thyroid hormone are drawing recent attention. Reduced serum thyroid hormone levels have been well-documented in the human populations with higher urine phthalate metabolites in various regions around the globe (Boas et al., 2006; Gao et al., 2017; Meeker et al., 2007; Meeker and Ferguson, 2011; Wu et al., 2013). Rodent studies have also confirmed the effects of DEHP on the thyroid (Dong et al., 2017; Liu et al., 2015), where significant influences on thyroid hormone and metabolism were observed. Especially, proliferative changes were noted in the thyroid *in vivo*, alluding a concern of potential thyroid carcinogenicity of DEHP. However, detailed mechanisms underlying this proliferative changes and effects of DEHP exposure on DNA integrity have not been addressed to our best knowledge.

Here we investigated whether DEHP can induce proliferative changes and DNA damages in thyroid carcinoma cell line, 8505C, *in vitro* and in the thyroid tissue of rats orally treated with DEHP for 90 days from the juvenile period to full maturation *in vivo*. Mechanism underlying was also explored with respect to thyrotropin signaling and related down-stream pathways to shed light on the potential risk of thyroid carcinogenesis from phthalates.

2. Materials and methods

2.1. Cell cultures and reagents

8505C (Thyroid gland carcinoma) cells were cultured in Dulbecco's minimal essential medium (DMEM) (GIBCO, Gaithersburg, MD USA), supplemented with heat-inactivated 10% fetal bovine serum (FBS, GIBCO), 0.1 mM non-essential amino acids, glutamine, HEPES, and antibiotics at 37 °C in a 5% CO_2 humidified incubator. DEHP (Bis(2-ethylhexyl) phthalate)) was purchased from Sigma-Aldrich (San Diego, CA, USA).

2.2. Cell transfection

TSHR expression was suppressed using specific si-RNAs. si-TSHR or si-control (used as negative control) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), TSHR si-RNA plasmid (sc-36754) and sh-control plasmid (sc-36869) were transfected with Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA). For transfection, cells were seeded in culture dishes and transfection was performed after 24 h using Opti-MEM media (Invitrogen, Carlsbad, CA, USA).

2.3. Western blotting

Cells were washed twice in phosphate-buffered saline (PBS) and then lysed with lysis buffer containing protease inhibitors for 15 min on ice. Whole cell lysates were centrifuged at 13,000 rpm for 20 min to

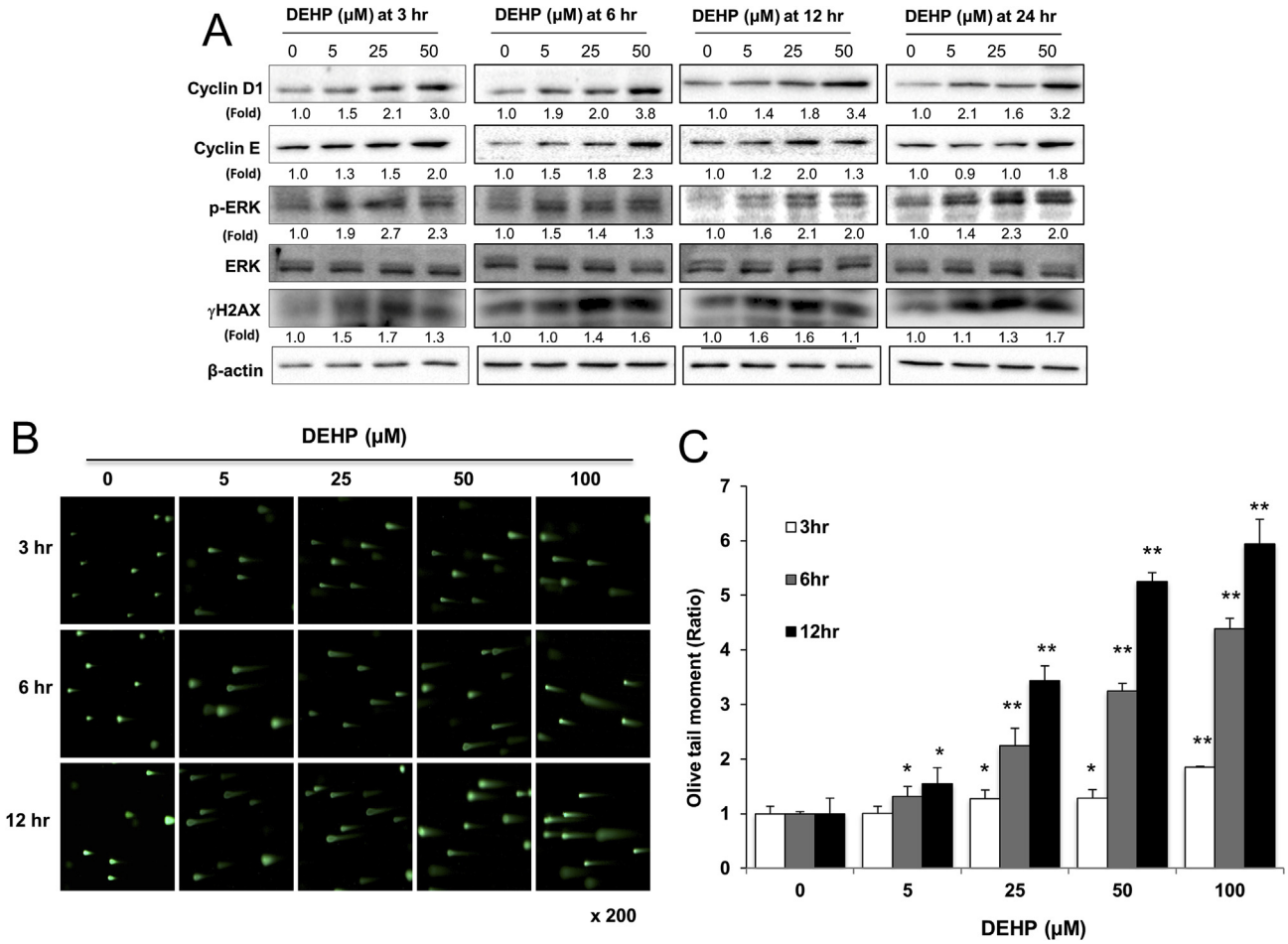

Fig. 2. Effects of DHEP on DNA damage responses in thyroid cells. (A) 8505C cells were treated with indicated doses and time of DEHP. Western blotting was performed at indicated time and concentrations. (B) 8505C cells were treated with DEHP for 6, 12 and 24 h. Quantification of the percentage of comet tails, which indicate DSBs. Tail length, indicating the extent of DNA fragmentation, measured for each cell showing a comet. The comet tail moments represent the averages of 250 cells in three independent experiments. *$p < 0.05$ and **$P < 0.01$ vs untreated control (ANOVA).

remove cell debris. The protein concentration of lysates was determined by Bio-Rad Protein Assay. Cell lysates were separated on SDS polyacrylamide electrophoresis gels. The separated proteins were transferred to nitrocellulose membranes. Membranes were incubated with primary antibody and then with horseradish peroxidase–conjugated secondary antibody in 5% skim mlk. Immunoblotting was performed with primary antibodies against the following proteins: Cyclin E (sc-481), Cyclin D (sc-753), γH2AX (Millipore 05-636), p-ERK (sc-7383), ERK (CST-9102S), Cleaved PARP (CST-9541S) p-AKT (CST-4051S), AKT (CST-4692S), Pax8 (ab53490), TSHR (sc-53542). The immunoblots were visualized with the ChemiDoc™ Imaging System from Bio-Rad. β-actin (sc-47778) staining represents the loading controls.

2.4. RNA extraction, RT-PCR and quantitative RT-PCR analysis

The 8505C cells were seeded into 100 cm dishes at a density of 2×10^5 cells/well in 7 mL medium. Total RNA was isolated using TRIzol reagent (Qiazen, Valencia, CA, USA), and 1 μg of RNA was used to synthesize cDNA using the ReverTra Ace qPCR RT reverse transcription (RT) master mix with genomic DNA (gDNA) remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions and a previous study (Kim et al., 2018a). The obtained cDNA was used to determine the amount of Pax8 and Actin mRNA using PCR with Taq DNA polymerase. Actin was used as an internal control. The primers that were used for amplification of the Pax8 transcripts were as follows: PAX8 forward, 5′-GGCTCCACCTACTCCATCAA-3′ and reverse, 5′-GGA AGGGGTGGAGCTAGAAC-3'; and Actin forward, 5′-GCACCACACCTTC TACAA TG-3′ and reverse, 5′-TGCTTGCTGATCCACATCTG-3′ and relative gene expression levels were calculated using the image analyzer (Image J, NIH, Bethesda, MD, USA).

2.5. Neutral comet assay

8505C cells were treated with DEHP for 6, 12 or 24 h and then subjected to a comet assay to detect double strand DNA damage, using a commercially available assay system (Trevigen, Helgerman, MD, USA). Briefly, after DEHP treatment, cells were harvested and mixed with low-melting-temperature agarose (LMA). After lysis, electrophoresis was performed at 100V for 15 min using Bio-rad (Sub-cell GT) electrophoresis system. Slides were stained with SYBR Green dye for 10 min. 250 cells per sample were captured under a Zeiss fluorescent microscope (Cal Zeiss, Oberkohen, Germany), and digital fluorescent images were obtained using AxioVision software. The relative length and intensity of SYBR Green (Sigma-Aldrich, CA, USA)–stained DNA tails to heads is proportional to the amount of DNA damage present in the individual nuclei and is measured by Olive tail moment using TriTek CometScore software (TriTek, Valencia, CA, USA).

2.6. Cell proliferation assays

Cell proliferation was evaluated by MTT assay and BrdU cell proliferation assay. For the MTT assay, 5×10^3–$1x10^4$ cells were seeded

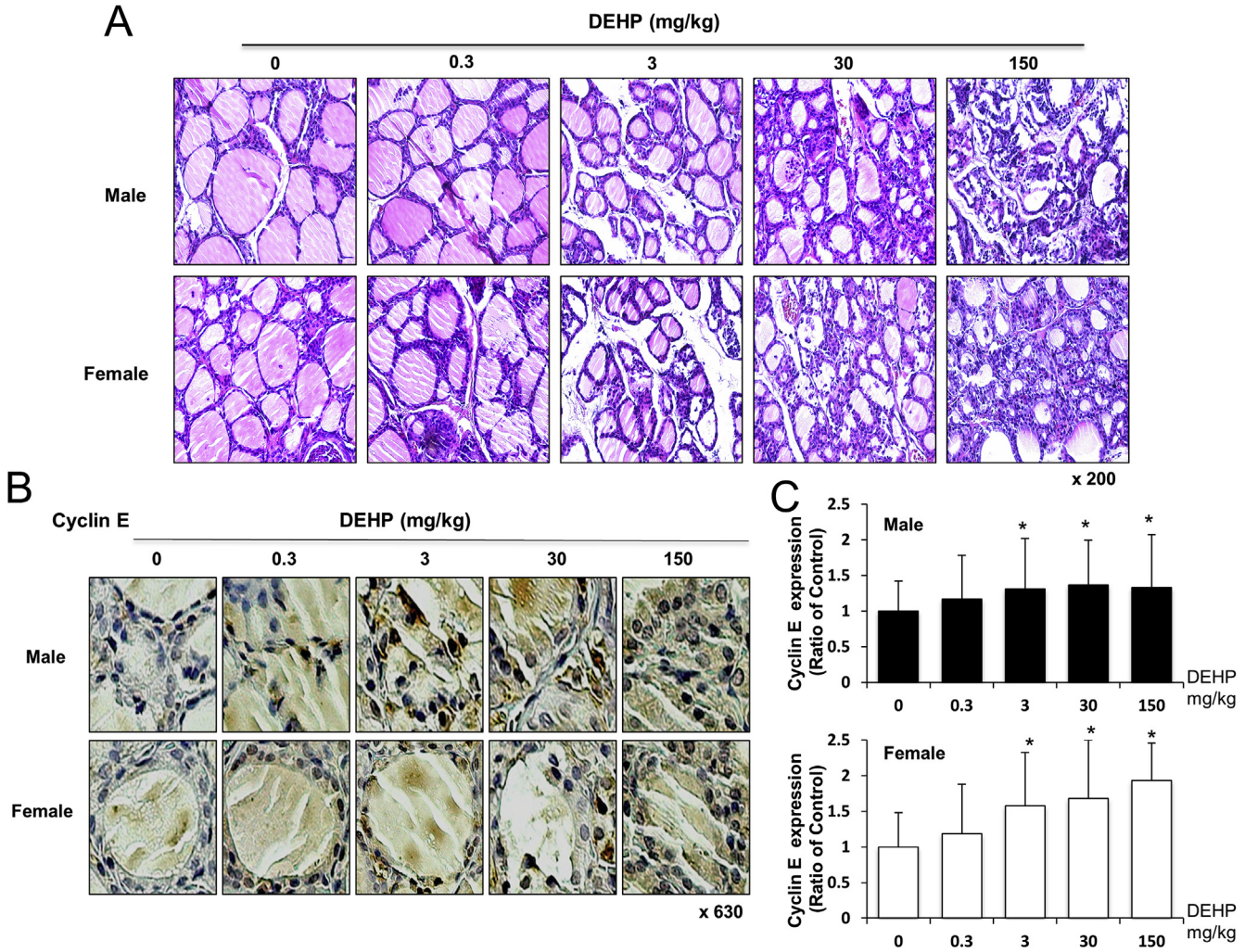

Fig. 3. Effects of DHEP on morphology and proliferation markers in rat thyroid. (A) Representative H&E staining of thyroid tissue from different treatment groups (magnification, ×200). (B) Immunohistochemistry analysis of cyclin E in rat thyroid tissues after oral treatment with BPA (0.5 mg/kg or 150 mg/kg) for 90 days. (C) Quantification of cyclin E positive areas in each slide were analyzed using GraphPad Prism software 5.0. Graphs represent mean ± SD of three mice per group, $*p < 0.05$ and $**P < 0.01$ (ANOVA) vs untreated control.

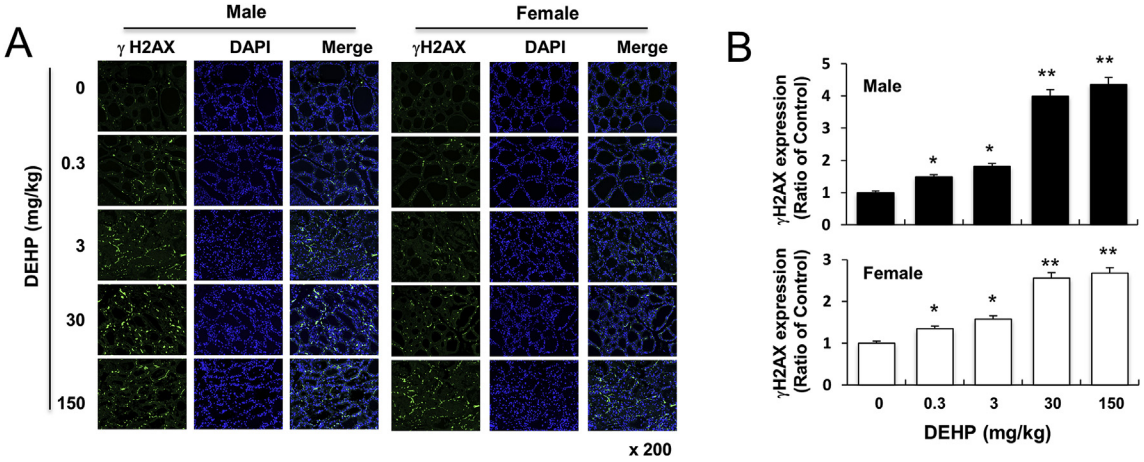

Fig. 4. Effects of DHEP on DNA damage responses in thyroid tissues. (A) Expression of p-H2AX was evaluated using immunofluorescence in rat thyroid tissues after oral treatment with BPA (0.5 mg/kg or 150 mg/kg) for 90 days. (B) Data are expressed as mean ± SD of three mice per group. $*P < 0.05$ and $**P < 0.01$ vs untreated control.

into 96-well plates. At the end of the exposure, cells were incubated with 100 μL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT; Sigma) for 2 h, and the absorbance determined colorimetrically at 540 nm using an ELISA plate reader. The 5-bromo-2-deoxyuridine (BrdU) assay was performed with the BrdU Cell Proliferation Assay kit according to the manufacturer's instructions

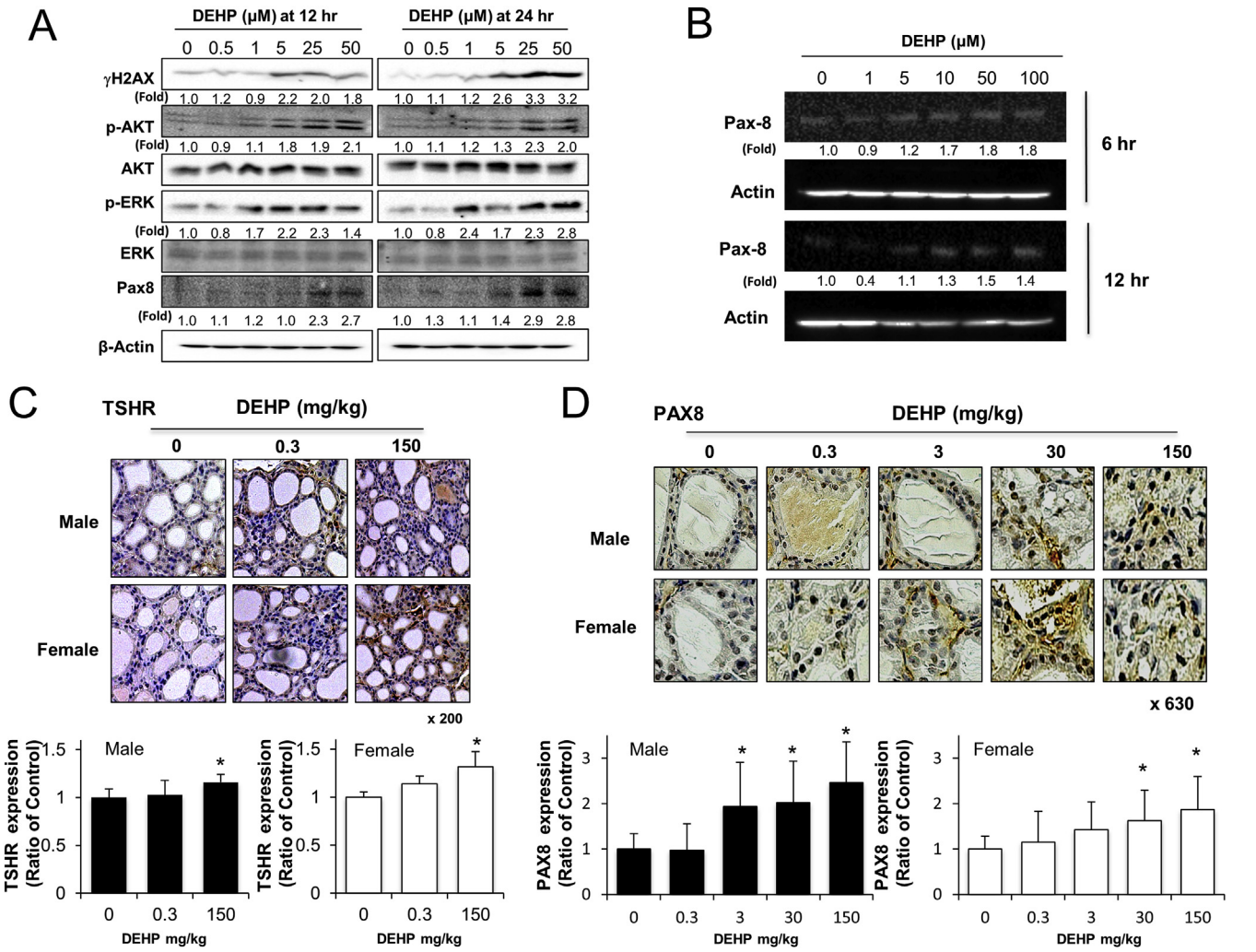

Fig. 5. Activation of TSHR pathways by DEHP. (A) 8505C cells were treated with indicated doses of DEHP for indicated time points and Western blotting was performed. Protein levels were quantified using Image J software, and data are expressed as the fold change relative to the negative control. Data are representative of three independent experiments with similar results. (B) 8505C cells were treated with indicated doses of DEHP for 6 and 12 h Pax8 mRNA level was measured by RT-PCR. The levels of mRNA or protein were quantified using Image J software, and data are expressed as the fold change relative to the negative control. Immunohistochemistry analysis for TSHR(C) and Pax8 (D) in rat thyroid tissues after oral treatment with DHEP (0.5 mg/kg or 150 mg/kg) for 90 days. Data are representative of three mice per group with similar results. Photographs of four representative stained tissues are presented. Quantification of TSHR positive and Pax8 positive areas in each slide were analyzed using GraphPad Prism software 5.0. Graphs represent mean ± SD, $*p < 0.05$ and $**P < 0.01$ vs untreated control (ANOVA).

(Cell Signaling Technology, Inc., Danvers, MA, USA). Briefly, cells were seeded into three wells of a 96-well plate at 0.5×10^4, 1×10^4 and 2×10^4 cells/well and incubated at room temperature for 24 h. BrdU (10 μL) was added to each well and incubated at room temperature for an additional 4 h. Following removal of the medium, 100 μl/well fixing/denaturing solution was added and incubated at room temperature for 30 min, followed by antibody detection solution for 1 h. The plate was washed three times and then anti-mouse IgG HRP-conjugated secondary antibody was added and incubated for 30 min, followed by 100 μL TMB Substrate for 30 min. The reaction was terminated with STOP solution and absorbance was measured at 450 nm on a microplate reader. Cell proliferation was calculated as the ratio of treated samples to control samples.

2.7. Flow cytometry

Flow cytometry was performed using the BD FACS Callibur (Becton Dickinson). The cells were trypsinized and centrifuged at 1300 rpm for 5 min and resuspended in PBS at a concentration of about 1×10^6 cells/1 mL. The washed cells equally diluted and resuspended in 100 μl staining buffer (PBS, 1% FBS, 0.09%, NaN_3), for further addition of FITC-PCNA (BD #556030) antibody, in the dark, for 20 min at room temperature. The cells were washed three times in staining buffer and resuspended in 1 ml staining buffer. And add 10 μl of 0.5 μg/ml PI (Sigma P4170) staining solution. Data were analyzed with BD CellQuest Pro software.

2.8. Immunofluorescence and immunohistochemistry

Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, washed three times with PBS, and then incubated with anti-γH2AX (ab11174) antibodies diluted 1:100 in PBS/5% FBS for 1 h in a humidified chamber at room temperature. Excess antibody was removed by washing the coverslips three times with PBS before incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Dako, Glostrup, Denmark) at a 1:200 dilution in PBS/5% FBS for 4 h. After three washes with PBS, coverslips were mounted on microscope slides using ProLong anti-fade mounting reagent (Molecular Probes, Eugene, OR USA). Cells were imaged and analyzed using a confocal laser-scanning microscope (Leica

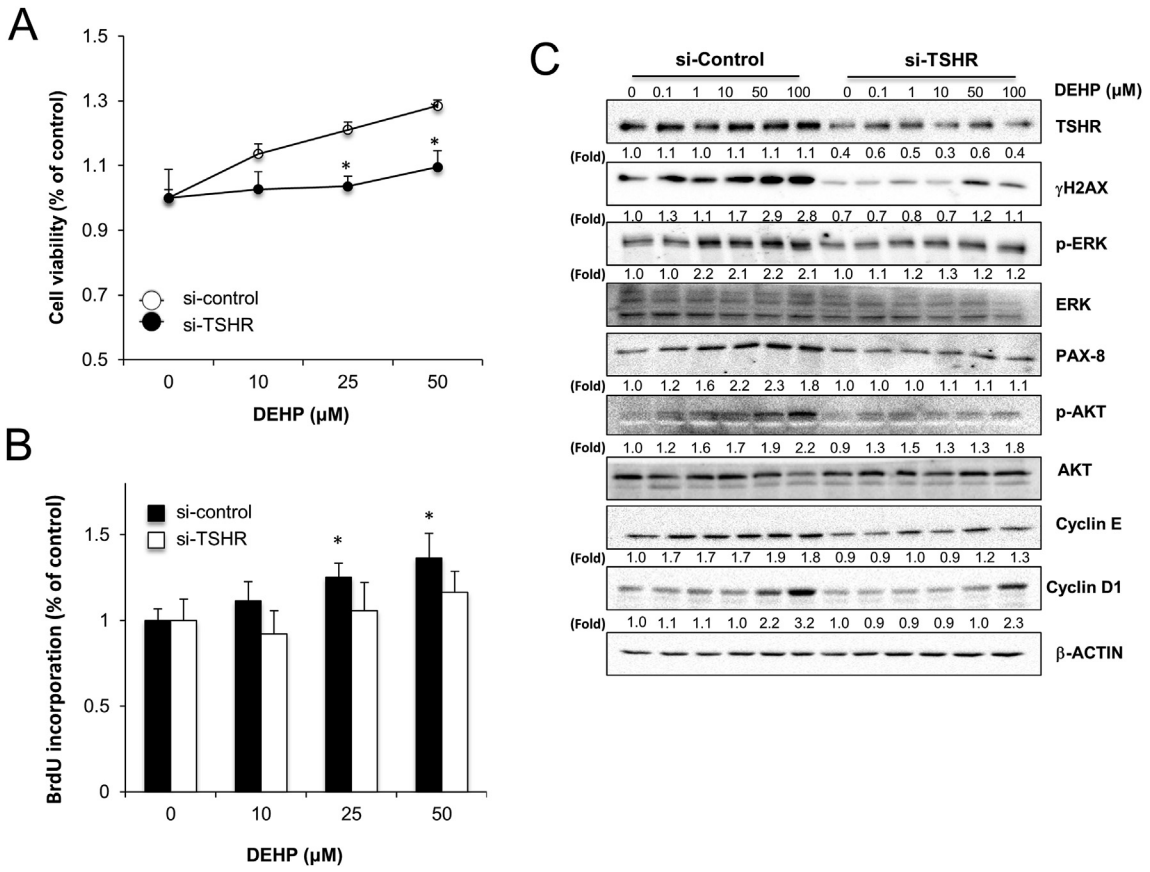

Fig. 6. Dependency of TSHR in DHEP-mediated cellular proliferation and DNA damage responses in thyroid cells. MTT assay (A) or BrdU incorporation assay (B) in si-Control or si-TSHR transfected 8505C cells was evaluated at 12 h after DHEP treatment. Data are expressed as mean ± SD from at least three independent experiments. *$P < 0.05$ vs untreated control. (C) si-Control or si-TSHR transfected 8505C cells were treated with indicated doses of DEHP for 24 h and Western blotting was performed. Protein levels were quantified using Image J software, and data are expressed as the fold change relative to the negative control. Data are representative of three independent experiments with similar results.

Microsystems, Wetzlar, Germany). Immunohistochemistry was performed with the following antibodies: TSHR (sc-53542) or Cyclin E (sc-481) and Pax8 (ab53490). For antigen retrieval, slides were placed in citric acid buffer (pH 6.0) and heated at 100 °C for 10 min. Slides were incubated overnight at 4 °C with antibodies. Quantification of images was measured with image analyzer (Image J, NIH, Bethesda, MD, USA). All statistical analyses of images were performed using GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA, USA).

2.9. Thyroid tissues of rats exposed to DEHP from juvenile to maturation

Thyroid tissues of rats (Sprague-Dawley (Crl:CD), Orient Bio Inc. Gyeonggi-do, Republic of Korea) exposed to DEHP from juvenile (Post Natal Day 9) to maturation (for 90 days) were obtained from another study which is currently in press. Briefly, two females were placed in the cage with one male overnight. F1 generation male and female pups were selected on postnatal day (PND) 4. Animal welfare was provided according to Korea Institute of Toxicology (KIT, Daejun, Korea) protocols. All procedures were in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals of KIT. On PND 4, all pups were cross-fostered between litters such that each litter was 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. Male and female pups were administered DEHP by oral gavage daily at dose levels of 0, 0.5, and 150 mg/kg/day in 0.3% carboxymethylcellulose suspension from PND 6 to 96 (13-weeks). Thyroid tissues were fixed in formalin, embedded in paraffin, and then sectioned prior to staining with H&E or immunocytochemistry.

2.10. Statistical analysis

Statistical significance was determined by ANOVA or Student's t-test. Differences were considered significant if the p value was less than 0.05.

3. Results

3.1. DHEP induced cellular proliferation on the thyroid carcinoma cell line, 8505C

To investigate the effects of DHEP on thyroid, we first evaluated its effect on the proliferation of 8505C cells, thyroid gland carcinoma cells after exposure to various concentrations of DEHP for 24 and 48 h. As a result, DHEP induced more than 30% increase in the cell viability (Fig. 1A and B). The first peak increase occurred at relatively low concentrations of 2.5 μM at both 24 and 48 h and second one was at 25 μM at 24 h and 50 μM at 48 h. Moreover, DHEP increased cell viability consistently at the concentrations below 80 μM at 24 h and below 100 μM at 48 h but these effects were significantly attenuated at higher concentrations above 50 μM of DHEP while no cytotoxicity could be observed. To investigate whether the increase in cell viability following DHEP treatment was due to cellular proliferation, BrdU incorporation (an index of DNA synthesis) was measured following DHEP exposure. As shown in Fig. 1C, DHEP increased BrdU incorporation with a peak effect at 12.5 μM in 8505 cells at 24 h, which was further confirmed with PCNA analysis showing a peak effect between 10 and 25 μM. Consistent with the viability assay, the proliferative effects of DHEP

were attenuated at the higher concentrations from 50 μM.

3.2. DHEP increased the expression of cell cycle-related proteins in accompany with increased γH2AX expression

To further confirm DHEP–induced proliferative effects, cell cycle related proteins such as cyclins D1, and E were examined, which revealed the increases of these proteins by DHEP in a dose dependent manner (Fig. 2A). Peak effect was seen at 6 h of DHEP treatment. Meanwhile, at the tested concentrations of DHEP (100 μM or lower), a cell death marker like cleaved PARP1 (C-PARP) could not be detected (data not shown), suggesting that cytotoxicity was not involved. In line with this, another proliferative markers, pERK1/2 also was increased by DHEP dose dependently. Since cellular proliferation and DNA damage can occur simultaneously (Kim et al., 2018c), we examined the phosphorylation of histone H2AX (γH2AX), a sensitive marker of a DNA damage after DEHP treatment. Of note, γH2AX phosphorylation was also increased which corresponded well with cyclin D1 and E expression. Comet assay which identifies DNA damage, also confirmed DNA damages by DEHP as measured by significantly increased comet tail moments from the low concentrations of 5 μM (Fig. 2B).

To examine whether DHEP effects shown *in vitro* may occur *in vivo*, the thyroid tissues from the rats exposed to DHEP of 0.3, 3, 30, and 150 mg/kg/day for 13-weeks were examined. Consistent with the previous report (B&T citation), hyperplasia and hypertrophy of thyroid glands were evident in the rats exposed to DEHP (Fig. 3A). This could be further supported by increased cyclin E expression as determined by immunohistochemistry, which achieved statistical significance from 3 mg/kg (Fig. 3B and C). In addition, both female and male rats showed increased γH2AX expression in dose-dependent manners. Of note, this effect was observed even at the lowest dose of 0.3 mg/kg (Fig. 4A and B).

3.3. DHEP activated thyroid stimulating hormone receptor (TSHR) pathways

To elucidate the mechanism underlying DHEP-induced cell proliferation, we evaluated the TSHR pathway because TSHR plays a key role in the proliferation and differentiation of thyroid cells (De Felice et al., 2004). DHEP increased TSHR expression which was corresponded with the activation of p-AKT and p-ERK, downstream pathways of TSHR activation (Fig. 5A). Of note, these changes were accompanied with increases of Pax 8 (Fig. 5A and B), a transcriptional factor which is regulated mainly by TSHR signals (Mascia et al., 2002). Importantly, TSHR expression was increased significantly in the thyroid tissues of rats treated with DHEP (Fig. 5C) which corresponded with increased expression of Pax 8 even at the low dose (3 mg/kg) in dose dependent manners (Fig. 5D).

3.4. TSHR knockdown inhibited cellular proliferation and γH2AX expression

To verify the role of TSHR pathway in DEHP-induced cellular proliferation and γH2AX expression in thyroid cells, siRNA of TSHR was transfected to 8505C cells. As a result, increased cell viability (Fig. 6A) and BrdU incorporation (Fig. 6B) by DHEP treatment was abolished by knockdown of TSHR. Increased cell cycle proteins such as cyclin E, pERK, and pAKT by DHEP treatment was also inhibited by TSHR knockdown as well as γH2AX and Pax8 expression (Fig. 5C) supporting the pivotal role of TSHR signaling in DEHP-induced thyroid proliferation and DNA damages.

4. Discussion

Here we demonstrated that DEHP induces cell proliferation and DNA damages in the thyroid *in vitro* and *in vivo*. These effects were shown at relatively low level of DEHP. *In vitro* experiments showed that DEHP can induce DNA damages and cell proliferation in low micromolar ranges and *in vivo* data suggested that DEHP can influence thyroid tissues at low doses from 0.3 mg/kg, which is not far-fetched from the reported human exposure ranges of 0.003–0.03 mg/kg/day in adult and 0.23–0.34 mg/kg in children. These effects appear to stem from the disruption of normal thyroid hormone regulation as evidenced by upregulation of TSHR and activation of down-stream pathways, indicating the thyrotropic effects of DEHP.

TSHR is a member of the glycoprotein hormone receptors that is pivotal to the development and function of the thyroid. The main physiological stimulus regulating thyroid function is administered by TSH released from the pituitary gland. TSH exerts its functions through TSHR mediated pathways (Goel et al., 2011). TSH-TSHR pathway regulates the expression of Pax8, a transcriptional factor which involves in the regulation of thyroid-specific genes such as thyroglobulin, TSHR and sodium/iodide symporter (De Felice et al., 2004). Pax8 also has a critical role in thyroid cell survival and proliferation (Di Palma et al., 2013). In this study, even low doses of DHEP activated TSHR downstream signaling pathways as evidence by p-ERK and p-AKT with increased cellular proliferation and DNA damage response. Of note, knockdown of TSHR through siRNA attenuated these DEHP-induced events, suggesting that DHEP may directly upregulate TSHR signaling pathways, which may result in increased cellular proliferation and DNA damage response in the thyroid.

Most importantly TSHR-dependent cell proliferation and DNA damages occurred simultaneously *in vitro* and *in vivo*, suggesting a potential risk of thyroid carcinogenicity of phthalates. The potential risk of carcinogenicity of phthalates in human has been illuminated previously. DEHP is classified as a 'possible carcinogens' by the International Agency for Research on Cancer (IARC). DEHP has been suspected to be associated with diverse types of cancers such as breast cancer (Lopez-Carrillo et al., 2010; Zhang et al., 2016), pancreatic cancer (Selenskas et al., 1995; Ventrice et al., 2013) and uterine leiomyomas (Fu et al., 2017). The mechanism underlying the carcinogenic potential of DEHP has been suggested as the activation of androgen receptors, suppression of p53, and stimulation of peroxisome proliferator receptors (Hsieh et al., 2012) and aryl hydrocarbon receptor (Del Pup et al., 2016). Genotoxicity of DEHP was also observed as DNA damages, and chromosomal aberration (Caldwell, 2012). Here we demonstrated that DEHP can stimulate thyroid cell proliferation and DNA damages through the activation of TSHR pathway. DNA damages accompanied the cell proliferation, which may promote the risk of carcinogenicity of DEHP.

While most of studies regarding endocrine disrupting activities of phthalates have been focused on the reproductive system, several studies have reported that DEHP can affect thyroid hormone regulation. Liu et al. (2015) and Dong et al. (2017) reported that T3, T4 thyroid hormone and thyrotropin releasing hormone (TRH) were reduced in rats exposed to DEHP at high doses (150–750 mg/kg). In these studies, proliferative changes in the thyroid tissues were also noted but due to extremely high doses compared to realistic human exposure level, a relevance to human health effects was questionable. Recently, we found that even at lower doses of DEHP (30 mg/kg or higher) could induce the cell proliferation and alter the gene expression in thyroid (Kim et al., 2018b). Here we demonstrated that even lower doses of DEHP (as low as 3 mg/kg) *in vivo* can induce proliferative responses as determined by increased expression of cyclin E and D. Of note, Pax8, a member of the Paired-box gene family which is a critical regulator required for proper development and differentiation of thyroid follicular cells (Di Palma et al., 2013; Pasca di Magliano et al., 2000) was increased by DEHP treatment. Interestingly, Pax8 is known to increase in thyroid carcinomas (Dupain et al., 2016) further supporting the potential risk of thyroid carcinogenicity of DEHP.

In conclusion, we demonstrated that DEHP induces DNA damage and cellular proliferation in thyroid *in vitro* and *in vivo*, which appear to

depend on TSHR activation. Of note, these effects were shown at low doses of DEHP with significant effects appearing even at low micromolar *in vitro* and submilligram per kg *in vivo*.

Conflicts of interest

All authors have no conflict of interests to declare for this study.

Acknowledgement

This research was supported by grants from Ministry of Food and Drug Safety in 2015 (15162MFDS631), Basic Science Research Program through National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2058657) and the Korea government (MSIT) (No. 2018R1A5A2025286).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.010.

References

ATSDR, 2002. Toxicological Profile for Di (2-ethylhexyl) Phthalate. US Department of Health Human Services.
Boas, M., et al., 2006. Environmental chemicals and thyroid function. Eur. J. Endocrinol. 154, 599–611.
Calafat, A.M., McKee, R.H., 2006. Integrating biomonitoring exposure data into the risk assessment process: phthalates [diethyl phthalate and di(2-ethylhexyl) phthalate] as a case study. Environ. Health Perspect. 114, 1783–1789.
Caldwell, J.C., 2012. DEHP: genotoxicity and potential carcinogenic mechanisms-a review. Mutat. Res. 751, 82–157.
Campioli, E., et al., 2011. The endocrine disruptor mono-(2-ethylhexyl) phthalate affects the differentiation of human liposarcoma cells (SW 872). PLoS One 6, e28750.
De Felice, M., et al., 2004. Minireview: thyrotropin receptor signaling in development and differentiation of the thyroid gland: insights from mouse models and human diseases. Endocrinology 145, 4062–4067.
Del Pup, L., et al., 2016. Carcinogenetic mechanisms of endocrine disruptors in female cancers (Review). Oncol. Rep. 36, 603–612.
Deutschle, T., et al., 2008. A controlled challenge study on di(2-ethylhexyl) phthalate (DEHP) in house dust and the immune response in human nasal mucosa of allergic subjects. Environ. Health Perspect. 116, 1487–1493.
Di Palma, T., et al., 2013. Pax8 has a critical role in epithelial cell survival and proliferation. Cell Death Dis. 4, e729.
Dong, X., et al., 2017. Effects of long-term in vivo exposure to di-2-ethylhexylphthalate on thyroid hormones and the TSH/TSHR signaling pathways in wistar rats. Int. J. Environ. Res. Publ. Health 14.
Dupain, C., et al., 2016. Induction of TTF-1 or PAX-8 expression on proliferation and tumorigenicity in thyroid carcinomas. Int. J. Oncol. 49, 1248–1258.
Feige, J.N., et al., 2007. The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis. J. Biol. Chem. 282, 19152–19166.
Fu, Z., et al., 2017. Association between urinary phthalate metabolites and risk of breast cancer and uterine leiomyoma. Reprod. Toxicol. 74, 134–142.
Gao, H., et al., 2017. Effects of prenatal phthalate exposure on thyroid hormone concentrations beginning at the embryonic stage. Sci. Rep. 7, 13106.
Goel, R., et al., 2011. A signaling network of thyroid-stimulating hormone. J. Proteonomics Bioinf. 4.
Hsieh, T.-H., et al., 2012. Phthalates induce proliferation and invasiveness of estrogen receptor-negative breast cancer through the AhR/HDAC6/c-Myc signaling pathway. Faseb. J. 26, 778–787.
Kim, J., et al., 2018a. Molecular mechanism of atopic dermatitis induction following sensitization and challenge with 2,4-dinitrochlorobenzene in mouse skin tissue. Toxicol. Res. 34, 7–12.
Kim, M., et al., 2018b. Low dose exposure to di-2-ethylhexylphthalate in juvenile rats alters the expression of genes related with thyroid hormone regulation. Biomol. Ther. (Seoul). 26, 512–519.
Kim, S., et al., 2018c. Submicromolar bisphenol A induces proliferation and DNA damage in human hepatocyte cell lines in vitro and in juvenile rats in vivo. Food Chem. Toxicol. 111, 125–132.
Latini, G., 2005. Monitoring phthalate exposure in humans. Clin. Chim. Acta 361, 20–29.
Latini, G., et al., 2003. In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. Environ. Health Perspect. 111, 1783–1785.
Liu, C., et al., 2015. DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. Environ. Sci. Pollut. Res. Int. 22, 12711–12719.
Liu, J., et al., 2018. Di(2-ethylhexyl) phthalate (DEHP) influences follicular development in mice between the weaning period and maturity by interfering with ovarian development factors and microRNAs. Environ. Toxicol. 33, 535–544.
Lopez-Carrillo, L., et al., 2010. Exposure to phthalates and breast cancer risk in northern Mexico. Environ. Health Perspect. 118, 539–544.
Martino-Andrade, A.J., Chahoud, I., 2010. Reproductive toxicity of phthalate esters. Mol. Nutr. Food Res. 54, 148–157.
Mascia, A., et al., 2002. Hormonal control of the transcription factor Pax8 and its role in the regulation of thyroglobulin gene expression in thyroid cells. J. Endocrinol. 172, 163–176.
Meeker, J.D., et al., 2007. Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. Environ. Health Perspect. 115, 1029–1034.
Meeker, J.D., Ferguson, K.K., 2011. Relationship between urinary phthalate and bisphenol A concentrations and serum thyroid measures in U.S. adults and adolescents from the National Health and Nutrition Examination Survey (NHANES) 2007-2008. Environ. Health Perspect. 119, 1396–1402.
Miao, Y., et al., 2017. Lifetime cancer risk assessment for inhalation exposure to di(2-ethylhexyl) phthalate (DEHP). Environ. Sci. Pollut. Res. Int. 24, 312–320.
Mylchreest, E., et al., 1999. Disruption of androgen-regulated male reproductive development by di (n-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol. Appl. Pharmacol. 156, 81–95.
Nazaroff, W., et al., 2012. Intake to production ratio: a measure of exposure intimacy for manufactured chemicals. Environ. Health Perspect. 120, 1678–1683.
Ohtani, H., et al., 2000. Effects of dibutyl phthalate as an environmental endocrine disruptor on gonadal sex differentiation of genetic males of the frog Rana rugosa. Environ. Health Perspect. 108, 1189.
Pasca di Magliano, M., et al., 2000. Pax8 has a key role in thyroid cell differentiation. Proc. Natl. Acad. Sci. U. S. A 97, 13144–13149.
Selenskas, S., et al., 1995. Pancreatic cancer among workers processing synthetic resins. Am. J. Ind. Med. 28, 385–398.
Takatori, S., et al., 2004. Determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in human serum using liquid chromatography-tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 804, 397–401.
Takeshita, A., et al., 2006. The endocrine disrupting chemical, diethylhexyl phthalate, activates MDR1 gene expression in human colon cancer LS174T cells. J. Endocrinol. 190, 897–902.
Ventrice, P., et al., 2013. Phthalates: european regulation, chemistry, pharmacokinetic and related toxicity. Environ. Toxicol. Pharmacol. 36, 88–96.
Weuve, J., et al., 2006. Exposure to phthalates in neonatal intensive care unit infants: urinary concentrations of monoesters and oxidative metabolites. Environ. Health Perspect. 114, 1424–1431.
Wu, M.T., et al., 2013. Intake of phthalate-tainted foods alters thyroid functions in Taiwanese children. PLoS One 8, e55005.
Zhang, S., et al., 2016. Promotion of breast cancer cells MDA-MB-231 invasion by di(2-ethylhexyl)phthalate through matrix metalloproteinase-2/-9 overexpression. Environ. Sci. Pollut. Res. Int. 23, 9742–9749.