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RESEARCH ARTICLE

# Combined effects of di (2-ethylhexyl) phthalate and bisphenol A on thyroid hormone homeostasis in adolescent female rats

Xuan Zhang[1] · Yuejiao Zhao[2] · Cheng Cheng[1] · Liuli Li[1] · Mingyang Xiao[1] · Guopei Zhang[1] · Xiaobo Lu[1]

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## Abstract

Phthalates and bisphenols are two typical classes of endocrine-disrupting chemicals (EDCs) which cause endocrine disorder in humans and animals. Phthalates and bisphenols are suggested to be associated with thyroid dysfunction. However, the effects of combined exposure and the detailed mechanisms are yet poorly understood. We investigated the combined effects of di (2-ethylhexyl) phthalate (DEHP) and bisphenol A (BPA) on thyroid function during puberty. Female Sprague Dawley rats were gavaged from postnatal 28 to 70 days with a single or combined exposure of DEHP (0, 150, and 750 mg/kg/day) and BPA (0, 20, and 100 mg/kg/day) according to a 3 × 3 factorial design. The thyroid weights reduced after combined exposure to the highest dose of DEHP and BPA, which noted their adverse effects on thyroid. Additionally, DEHP could increase the number of follicular epithelial cells in thyroid. Both DEHP and in combination with BPA could disturb the levels of thyroid hormones in serum, such as TT3 and TT4. Meanwhile, the possible mechanism was also discussed in the present study. DEHP treatment induced a significant increase of phosphorylation of cAMP-response element binding protein (Creb) via estrogen receptor α (Esr1), while the upregulation was nullified by the concomitant presence of BPA. In conclusion, the complex action of DEHP/BPA mixture may disturb the thyroid hormone homeostasis, which ultimately would affect the development of thyroid during puberty.

**Keywords** Di (2-ethylhexyl) phthalate · Bisphenol A · Combined effects · Thyroid hormone homeostasis · Estrogen receptor α

## Introduction

Endocrine-disrupting chemicals (EDCs) widely exist in the environment, which have drawn much attention due to their adverse effects on multiple systems. Essentially, these EDCs can disrupt hormone balance by mimicking or blocking hormones (Gore et al. 2015), which would ultimately damage physiology function, such as endocrine and reproductive systems (Riaz et al. 2016) (Ma et al. 2017).

As typical EDCs, di-2-ethylhexyl phthalate (DEHP) and bisphenol A (BPA) are utilized in a range of consumer products, including personal care products, plastics, and food packaging, leading to widespread human exposure (Guo and Kannan 2012) (Kang et al. 2006) (Welshons et al. 2006). Meanwhile, their adverse effects are also widespreadly concerned (Hogberg et al. 2008) (Guidry et al. 2015). Previous studies demonstrated that phthalate exposure was associated with an increased risk of breast cancer (Ahern et al. 2019) (Lopez-Carrillo et al. 2010). Additionally, BPA exposure has adverse effects on the endocrine and reproductive systems (Markey et al. 2001) (Bilgi et al. 2019) (Li et al. 2019) and its prepubertal exposure may also elevate the susceptibility to mammary carcinogens (Leung et al. 2017). Recently, epidemiological evidence suggested that both DEHP and BPA might contribute to increase the risks of thyroid diseases, such as thyroid cancer and benign nodule (Liu et al. 2020) (Marotta et al. 2019) (Xie et al. 2017).

Thyroid hormones (THs) are essential endocrine messengers for the growth and development of virtually all vertebrates (Mendoza and Hollenberg 2017). The synthesis and

Xuan Zhang and Yuejiao Zhao contributed equally to this work.

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✉ Xiaobo Lu
xblu@cmu.edu.cn

[1] Department of Toxicology, School of Public Health, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang 110122, Liaoning Province, People's Republic of China

[2] Cancer Hospital of China Medical University/Liaoning Cancer Hospital & Institute, Shenyang, People's Republic of China

secretion of THs are modulated by the hypothalamic-pituitary-thyroid (HPT) axis. As known thyronine (T4), triiodothyronine (T3), or thyroid stimulating hormone (TSH) may regulate the function of thyroid (Mondal et al. 2016). Accumulating evidences have shown the associations between phthalates exposure and altered TH levels (Johns et al. 2016) (Chuang et al. 2017) (Morgenstern et al. 2017) and thyroid functions (Meeker et al. 2007) (Miller et al. 2009) (Pan et al. 2017). Several studies have demonstrated that DEHP was negatively linked to the levels of total T3 (TT3), total T4 (TT4), and TSH in adults and pregnant women (Park et al. 2017) (Gao et al. 2017). Another study found that DEHP was negatively associated with the levels of free T3 (FT3) and free T4 (FT4) in children (Weng et al. 2017). Furthermore, an investigation reported that TSH was inversely correlated to the urinary BPA level across pregnancy (Aung et al. 2017). Other data indicated that BPA was positively associated with FT4 (Aker et al. 2016). While several studies demonstrated the adverse effect of DEHP or BPA single exposure on thyroid, few direct evidence of their combined effects on thyroid hormone homeostasis has been emphasized.

To date, humans are concurrently exposed to a large number of chemicals through various routes (Suk et al. 2002). Previous studies have shown that BPA and DEHP in utero exposure exerted adverse effects on fetal male reproductive development and cord blood estradiol levels (Sunman et al. 2019), as well as phthalates and BPA exposure during in utero windows of susceptibility in relation to the testosterone concentrations and breast development in offspring girls (Watkins et al. 2017). Additionally, most data support the effects of bisphenol A and some phthalates on the development of obesity and type 2 diabetes mellitus (Stojanoska et al. 2017). Comparing with a single exposure, combined exposure to several chemicals may induce some entirely different effects. There is a concern that different EDCs act in synergy and may result in so-called cocktail effects. Anne Katchy et al. found that co-exposure to BPA and soy-based phytoestrogens could lead to additive estrogenic effects (Katchy et al. 2014). However, another study revealed that nonyl phenol (NP) and dibutyl phthalate (DBP) could disrupt the function of Sertoli cells and hormone levels in serum, while their mixture effects were mainly antagonistic (Hu et al. 2014). In concordance, other authors found that antagonism on the expression levels of genes was involved in pituitary-gonadal cross-talk after exposure to DEHP and polychlorinated biphenyls (PCBs) (Fiandanese et al. 2016). A recent study has also indicated antagonistic interactions of neurotoxicity after a combined exposure to lead (Pb) and DEHP (Li et al. 2018). Additionally, an in vitro experiment described that the combined effect of BPA and DBP might be antagonistic in the modification of TNF-α expression (Couleau et al. 2015). Cassandra D. Kinch revealed that exposure to environmental concentrations of the contaminants BPA, DEHP, nonylphenol, and fucosterol can lead to morphological defects of zebrafish embryos, which was distinct from individual contaminants, in a manner that cannot be explained by simple additive effects (Kinch et al. 2016). Besides, a mixture of phthalates and BPA presented in human amniotic fluid could disturb the human G protein-coupled receptor (RXFP2) function, and produce potential antagonistic effects that are not displayed by the compounds, individually (Suteau et al. 2020). Although DEHP could also prompt the upregulation of thyroid transcription factor-1 (TTf-1), TSHr, and the expression of NIS in thyroid tissue (Dong et al. 2017), as well as neonatal exposure to BPA disturbed the function of HPT axis in adult rats in estrus (Fernandez et al. 2018), through binding to TH receptors in a non-competitive pattern (Jung et al. 2007), no study to date has understood the deleterious impact of cocktails of DEHP and BPA in vivo on the thyroid. Accordingly, we supported the hypothesis that co-exposure to DEHP and BPA may exert an effect that differs from the ones of each disruptor alone on the thyroid, which is considered as one of the most important endocrine organs.

Collectively, in order to explore the combined effects of DEHP and BPA on thyroid hormone homeostasis in vivo, a 3 × 3 factorial design was used in the present study and several parameters such as thyroid weight, histological changes, and serum hormonal levels were investigated after exposure to DEHP and BPA in adolescent female rats. In addition, estrogen receptor pathway and other related indexes involved in the possible mechanism were also detected and analyzed. Hopefully, our current study may contribute to provide some scientific clues for evaluating the combined effects of DEHP and BPA on thyroid function, and highlight the necessity of eliminating the substance from plastic products.

## Materials and methods

### Animals

Sixty-three female Sprague Dawley rats (SPF grade) (three-week-old) were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animal Use License number of SYXK-LN 2013-0001. All experiments and surgical procedures were approved by the Animal Use and Care Committee of China Medical University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering. Rats were housed at a temperature of 24 ± 1 C with 12-h light/12-h dark cycles and humidity 50–60%. Solid-bottomed polycarbonate cages with stainless steel wire-bar

lids were used to house 2 rats per cage containing corn-cob bedding and nesting material. Food (Changsheng biotechnology, Shenyang, China) and tap water were provided ad libitum. Animals were housed for 1 week before being entered into the study.

## Design and treatments

DEHP, BPA, and corn oil were purchased from Sigma-Aldrich (St. Louis, MO, USA). The study was conducted in 9 groups of 7 rats according to a 3 × 3 factorial design, and the detailed grouping and administration are shown in Table 1. The female rats were gavaged with vehicle (corn oil) or DEHP (0, 150, 750 mg/kg/day) and BPA (0, 20, 100 mg/kg/day) which was dissolved in corn oil until adulthood (endpoint: postnatal day 70) (Boughammoura et al. 2020) by a well-trained technician. The DEHP dose 1 (150 mg/kg/day) was equal to 1/200 of the half lethal dose of DEHP for rat, and DEHP 150 mg/kg/day also based on no-observed-adverse-effect-level (NOAEL) of 30 mg/kg/day in rats (David et al. 2000). Further, the DEHP dose 2 (750 mg/kg/day) was equal to 1/40 of the half lethal dose of DEHP for rat, and the DEHP 750 mg/kg/day was also known to be able to induce adverse impact in rats without causing systemic toxicity (Shelby 2006). Similarly, BPA dose 1 (20 mg/kg/day) was equal to 1/200 of the half lethal dose of BPA for rat, and also based on NOAEL of 5 mg/kg/day in rats while dose 2 (100 mg/kg/day) was equal to 1/40 of the half lethal dose of BPA for rat (US Environmental Protection Agency 2011). The intragastric administration was performed at a fixed time (8:30–9:30 a.m.) every day. All rats' body weights were measured and recorded at the end of each week.

## Sample collection and preparation

All animals were sacrificed under chloral hydrate anesthesia within 24 h after the last treatment. The blood samples were obtained from the aorta abdominal in each group after animals were anesthetized and then were allowed to clot. Serum was obtained by centrifugation at 3000 rpm for 15 min and then stored at − 80 C before hormonal detection. The thyroid samples were rapidly removed, weighted, and snap-frozen in liquid nitrogen or fixed in paraformaldehyde for later analysis.

## HE staining and histological evaluation

Thyroids were fixed in paraformaldehyde, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 μm, and stained with Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China) for microscopic examination.

## Enzyme-linked immunoabsorbent assay

Total T4 (TT4), total T3 (TT3), free T4 (FT4), free T3 (FT3), and TSH levels in serum of rats were measured using the rat's THs ELISA kits (Jiangsu Meilian Bioengineering Institute, China) according to the manufacturer's instructions. All samples were run in duplicate.

## Quantitative real-time PCR

Total RNA was extracted from the thyroid using a TRIzol® Reagent (Invitrogen Inc., Burlington, ON, Canada), followed by being reverse transcribed to cDNA with a HiScript II Q Select RT SuperMix for qPCR Kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Primers specific for genes were designed and synthesized by Sangon Biotech (Shanghai, China). The primers are shown in Table 2. Real-time PCR was carried out using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) on a Light Cycler 480 II (Roche, Germany) PCR detection system according to the protocol provided by the manufacturer. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as an internal reference due to previous successful application. The relative quantification of mRNA levels was performed using the comparative Ct method and formula $2^{-\Delta\Delta Ct}$ (Bustin et al. 2009).

## Immunohistochemistry

Sections of the thyroid gland were deparaffinized, rehydrated, and subjected to antigen retrieval. Antigen retrieval was performed in Tris-EDTA buffer by microwave for 15 min, slowly cooled down to room temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide, and then the sections were incubated at 4 C overnight with the primary antibodies: rabbit antibody anti-Esr1 (1:200) and anti-phospho-Creb (1:200) (Affinity Biosciences, Cincinnati,

**Table 1** 3 × 3 factorial design

| SD ♀ rats $n = 63$ (7 per group) | | BPA | | |
|---|---|---|---|---|
| | | NO | Dose 1 (20 mg/kg) | Dose 2 (100 mg/kg) |
| DEHP | NO | Ctrl | B20 | B100 |
| | Dose 1 (150 mg/kg) | D150 | B20 + D150 | B100 + D150 |
| | Dose 2 (750 mg/kg) | D750 | B20 + D750 | B100 + D750 |

**Table 2** The primer sequences used in the study

| Primer | Type | Primer sequence |
|---|---|---|
| *Esr1* | Forward | AATTCTGACAATCGACGCCAG |
| | Reverse | GTGCTTCAACATTCTCCCTC CTC |
| *Creb* | Forward | ATTGCCCCTGGAGTTGTTAT |
| | Reverse | CTGCTTCCCTGTTCTTCATTAG |
| *Gapdh* | Forward | GCTCTCTGCTCCTCCCTGTTCT |
| | Reverse | CAGGCGTCCGATACGGCCAAA |

OH, USA). After incubation with the primary antibodies, the sections were incubated with appropriate biotinylated immunoglobulin and avidin-biotin peroxidase complex. Using DAB (Maxim Biotechnologies, Fuzhou, China) complexes visualized the reaction product. The average optical density of immunohistochemistry in each sample was analyzed using Image-Pro Plus software. Each stained section was evaluated by a minimum of 5 randomly selected × 20 high-power fields for further statistical analysis.

### Bioinformatics analysis

STITCH and chEMBL databases were used to identify the potential targets for the toxicity of DEHP and BPA, and Cytoscape software was used to construct an interaction network related to the molecular targets of DEHP and BPA. Gene oncology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis were performed on potential targets of DEHP and BPA using the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

### Statistical analysis

All experiments were performed at least in triplicate, and data were presented as the mean ± standard deviation (SD). Statistical analyses of the mean were performed by one-way ANOVA and least-significant difference (LSD) using the SPSS software, version 21.0 (SPSS, Inc., Chicago, IL, USA). Data transformation or nonparametric test was performed when the data cannot meet the homogeneity of the variance. Statistical significance was defined as $P < 0.05$.

## Results

### Effects of 6-week exposure to DEHP and BPA on body weight and thyroid coefficient

To evaluate the effect of DEHP and BPA on the growth and development of rats, hair growth, mental state, and body weight were observed. We found the rats in all groups survived well and no apparent differences in living status and hair growth were observed. Although an upward trend in body weight was displayed in each rat, no significant changes occurred among all groups. Notably, after 3 weeks, DEHP-exposed groups showed an increasing tendency in body weight compared with the control. On the contrary, BPA-exposed groups showed an decreasing tendency in body weight than the control (Fig. 1a). In addition, we measured the thyroid weight of each group in the end of the experiment and found that the coefficient of thyroid organ in the DEHP 750 + BPA 100 group was significantly lower than that of the control ($P < 0.05$), and also lower than that of the DEHP 750 group ($P < 0.05$) (Fig. 1b).

### Histological changes in thyroid due to exposure to DEHP and BPA

HE staining was conducted to assess histological changes in the thyroid. The basic structure and function unit of thyroid tissue in rats is thyroid follicles, which present slightly spherical. Each follicle is composed of simple epithelial cells, termed follicular cells. A small number of larger C cells are located beside the follicle. The results showed that the

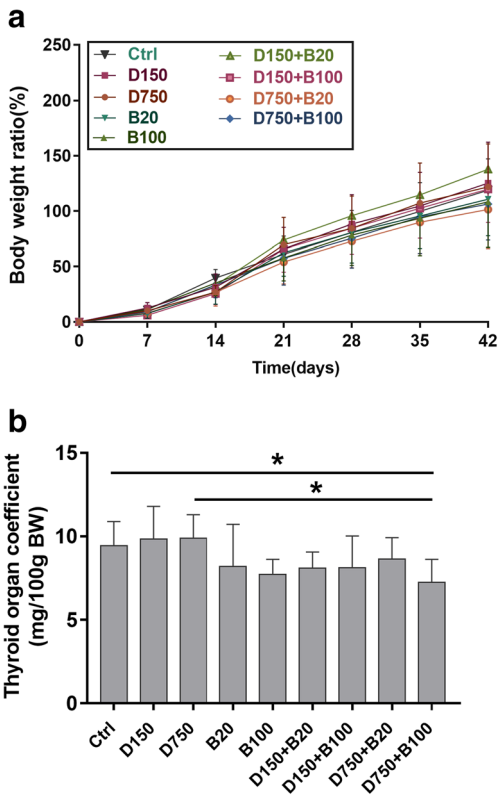


**Fig. 1** Dynamic change of body weight ratio and relative thyroid weights of rats after exposure to DEHP and BPA (mean ± SD) ($n = 7$). **a** Dynamic change of body weight ratio of rats. **b** Relative thyroid weights of rats. An asterisk indicates significantly different at $P < 0.05$

numbers of follicular epithelial cells in the thyroid tissues of rats exposed to DEHP 750 ($P < 0.05$) or BPA 100 were higher than that of the control (Fig. 2a, b). Additionally, the thyroid follicular diameters of rats in DEHP 750, BPA 100, and DEHP 750 + BPA 100 groups show a lower tendency than those in the control, but no statistical difference was found (Fig. 2a, c).

## Exposure to DEHP and BPA altered THs and TSH levels in serum

ELISA data showed that TT3 level in serum of rats exposed to DEHP 150 was slightly increased, while a significant reduction was found when combined with BPA treatment ($P < 0.05$), and that of rats exposed to DEHP 750 + BPA 20 was significantly lower than that of rats exposed to DEHP 750 ($P < 0.05$) (Fig. 3a). Compared with the control, the serum TT4 level of rats in the DEHP 750, BPA 20, BPA 100, and the combined exposure groups was significantly reduced ($P < 0.05$). Notably the combined exposure to DEHP and BPA further reduced the TT4 level of rats comparing with the single exposure to DEHP ($P < 0.05$) (Fig. 3b). FT3, FT4, and TSH levels in serum of rats in each group showed no significant changes compared with those in the control group (Fig. 3c–e).

## Potential targets and pathway enrichment analysis of DEHP and BPA

STITCH and chEMBL databases were used to identify the potential targets for the toxicity of DEHP and BPA. We obtained 46 molecular targets of DEHP and 52 molecular targets of BPA. An interaction network containing 89 nodes was established using Cytoscape software (Fig. 4a). The Venn diagram showed that 14 targets are common to both compounds (Fig. 4b), which is considered as one of the important target sets of DEHP and BPA. Remarkably, the result suggested that Esr1 may act as a mediator in the interaction between DEHP and BPA. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to analyze the GO enrichment of potential targets of DEHP and BPA based on the terms of biological process (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway enrichment. From the consensus 47 biological processes, including 36 statistically significant biological process with $P$ value < 0.05, 15 biological processes and KEGG signaling pathway with the smallest $P$ value were chosen to display (Fig. 4c, d). We noticed that some biological processes may be related to the toxicity of DEHP and BPA, such as transcription regulation and steroid hormone–mediated signaling pathways, while KEGG signal pathway included

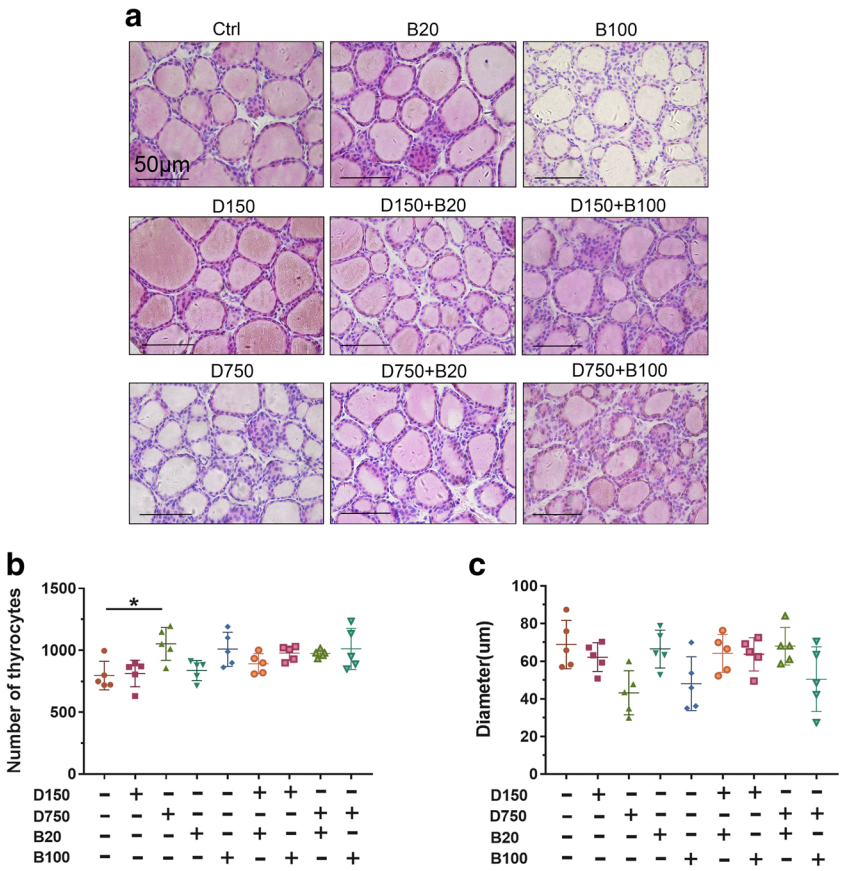


**Fig. 2** Effects of DEHP and BPA on the histology of thyroids ($n$ = 7). **a** Hematoxylin-eosin staining (H&E staining) of the thyroid gland (original magnification, × 40). Scale bar = 50 μm. **b** Quantification of thyrocytes staining in each group. **c** Quantification of thyroid follicular cavity diameter in each group. An asterisk indicates significantly different at $P < 0.05$

steroid hormone biosynthesis, VEGF, estrogen receptor, thyroid hormone, prostate cancer, FoxO, and tumor and PI3K-AKT signaling pathway.

### Effects of DEHP and BPA on Esr1-mediated transcriptional activation

Protein and mRNA levels of *Esr1* were measured by immunohistochemical staining and RT-PCR in the study. Immunohistochemical results showed that Esr1 expression in the thyroid tissues of rats exposed to DEHP 750 and BPA 100 was higher, and DEHP 750 was significantly higher than that of the control ($P < 0.05$), while the expression level of Esr1 in the DEHP 750 + BPA 100 exposure group was significantly lower than that in the DEHP 750 and BPA 100 exposure group ($P < 0.05$) (Fig. 5a, b). Meanwhile, the *Esr1* mRNA levels of rats exposed to DEHP 750 + BPA 100 were significantly lower than that of rats exposed to DEHP 750 ($P < 0.05$) (Fig. 5c). After the change of Esr1 was observed, the expression of its downstream p-Creb was further detected. Compared with the control, the expression of p-Creb in the DEHP 750 and BPA 100 exposed groups showed an increased way, and the DEHP 750 treatment was significant ($P < 0.05$) (Fig. 5d, e), while the mRNA levels of *Creb* in each group were not significantly changed (Fig. 5f).

## Discussion

The present study focuses on the combined effects induced by two endocrine disruptor compounds, DEHP and BPA in female rats during puberty. Firstly, both DEHP and BPA have the potential to disrupt thyroid hormone homeostasis, while BPA showed a greater impact on serum TH levels than DEHP. Moreover, both DEHP and BPA individually could increase the number of follicular epithelial cells of thyroid. The combined effect of DEHP and BPA may depend on Esr1, further leading to the alteration of phosphorylation of

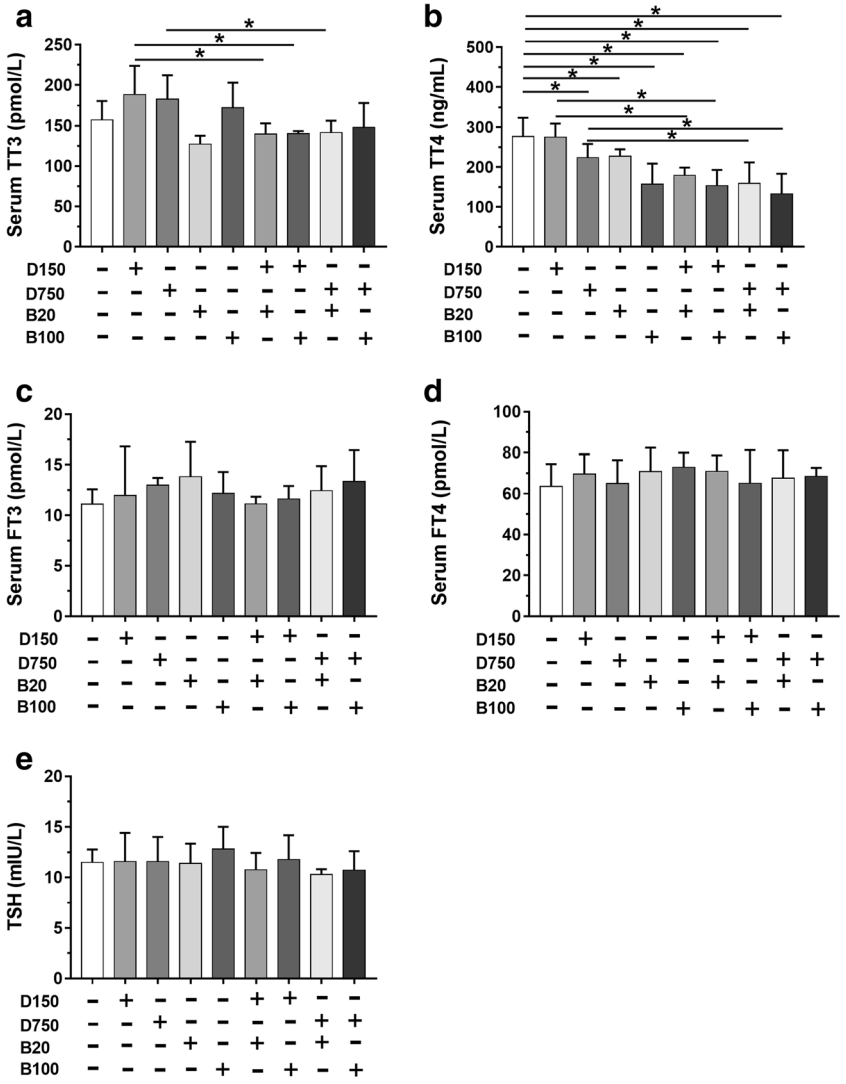


**Fig. 3** Comparation of THs and TSH levels in serum in each group ($n = 7$). **a** Total T3. **b** Total T4. **c** FT3. **d** FT4. **e** TSH. An asterisk indicates significantly different at $P < 0.05$

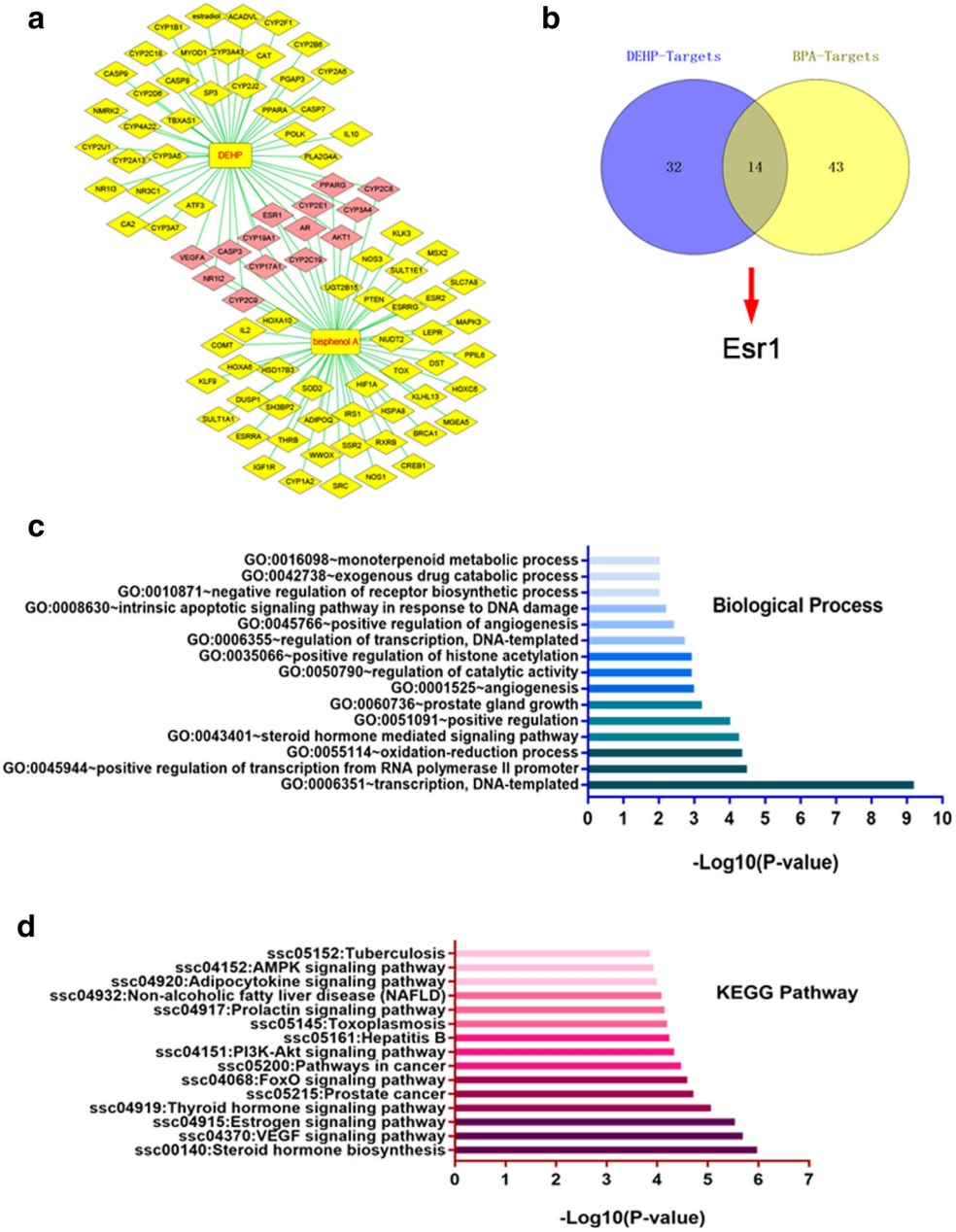


**Fig. 4** Target analysis of DEHP and BPA. **a** Chemicals-targets interaction network. **b** Venn diagram of potential targets of DEHP and BPA. **c** Biological process enrichment analysis of DEHP and BPA targets. **d** Analysis of KEGG signaling pathway enrichment of DEHP and BPA targets

Creb, and disturb the development of thyroid. No study to date has reported the interactions between DEHP and BPA on thyroid function indicated by in vivo experiment. In the present study, a 3 × 3 factorial design is used to analyze the complex effects of multiple EDCs on thyroid function, which can provide the opportunity to detect the interactions among multi-components (Collins et al. 2014). Since a 42-day in vivo experiment during puberty was used to clarify the toxicity of DEHP and BPA on thyroid development, a higher dose than human exposure was necessary. Hopefully, as a gross assessment of the short-term combined effects of DEHP and BPA on thyroid function, the present study may provide some references for our subsequent research, which contributes to our understanding of the adverse effect of EDCs mixture on human health during puberty.

Although the sequence of events that leads from puberty chemical exposure to altered dysfunction and even cancer is not completely understood, a weight of evidence is emerging that exposure to these chemicals during developmental periods produces persistent changes in growth, and functions in that tissue over the lifespan. Exposure to BPA and DEHP during puberty is of concern, based on numerous epidemiological studies reporting a range of effects associated with DEHP and BPA exposure, such as the effects related to neurobehavior, growth and development, and reproductive tissue dysfunction (Gore et al. 2015). In humans, thyroid

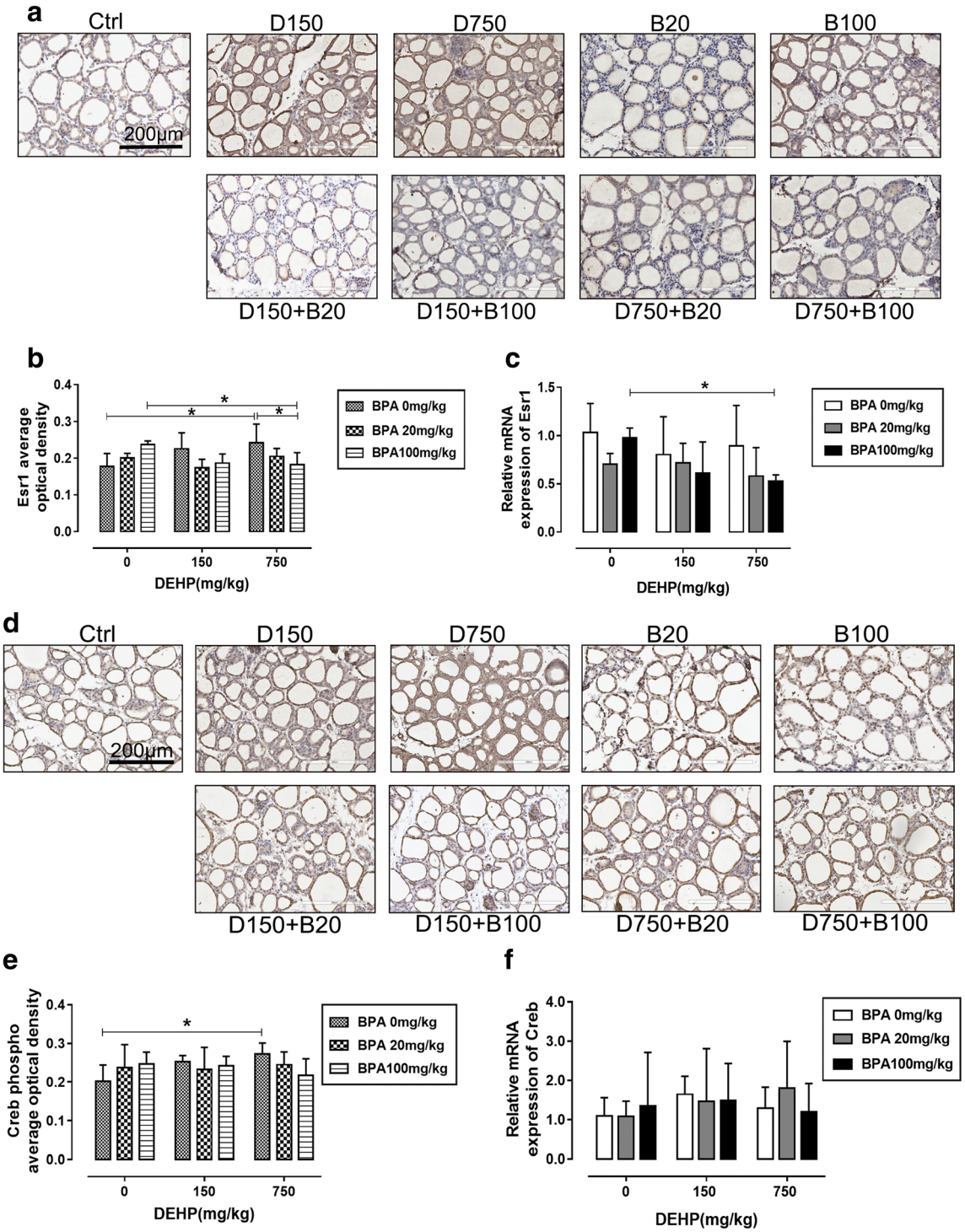


**Fig. 5** Effects of DEHP and BPA on relative gene expression in the thyroid ($n = 7$). **a** Immunohistochemistry for Esr1 in the thyroid (original magnification, × 20). Scale bar = 200 μm. **b** The average optical density of Esr1 in the thyroid. **c** The mRNA expression of *Esr1*. **d** Immunohistochemistry for phosphorylation of Creb in the thyroid (original magnification, × 20). **e** The average optical density of phosphorylation of Creb in the thyroid. **f** The mRNA expression of *Creb*. An asterisk indicates significantly different at $P < 0.05$

hormone is important for normal development of the central nervous system, cardiovascular system, and other organs (Bernal 2007). Kim and Park reviewed that BPA can interfere with thyroid hormone synthesis, transport, and metabolism, and may affect thyroid function through several possible mechanisms of action (Kim and Park 2019). Furthermore,

laboratory results found that neonatal exposure to BPA decreased the serum T4 and increased TSH levels in female rats (Fernandez et al. 2018); Dongjing et al. also reported a decreased tendency for total T4 levels and increased TSH levels in offspring with DEHP-treated pregnant female rats, while no change was found in total T3 (Dong et al. 2019). Other studies showed significantly reduced thyroid hormones (T3, T4) after DEHP exposure, whereas TSH was not affected (Liu et al. 2015). In the present study, along with the increasing dosage of DEHP or BPA, total T4 levels also decreased, especially at higher dose of BPA (100 mg/kg). When co-exposure to toxicants decreased the levels of total T3 and T4 in contrary to the single EDCs, the main effect analysis showed that BPA may have a greater impact on TH levels in serum than DEHP. TSH levels in serum were unaffected by DEHP and BPA which is consistent with the previous studies.

It is well known that DEHP and BPA may mimic or antagonize the effects of estrogens in target tissue, which could mediate main activities through the estrogen receptors (Casals-Casas and Desvergne 2011) (Tucker et al. 2018). Different compounds may induce the expression of different target genes via the same estrogen receptors (Katchy et al. 2014). Remarkably, women make up the majority of thyroid patients, and thyroid cancer is the most common malignancy tumor of the endocrine system, whose incidence is 2.9-times more common in women than in men (Rahbari et al. 2010). Increasing evidences suggested that estrogen may play an important role in the development and progression of thyroid cancer through estrogen receptors (Huang et al. 2014) (Fan et al. 2015). Similarly, our findings also suggested that thyroid gland may become a sensitive target of BPA and DEHP, and their exposure can affect the development of pubertal thyroid via disturbing the function of Esr1. Additionally, overall less is known about the exact mechanism of how DEHP and BPA affect the development of thyroid, bioinformatic analysis provided clues that Esr1 may act as an interaction mediator of DEHP and BPA, and compound-target-pathway enrichment analysis prompted us to focus on the estrogen signaling pathway in the present study. A significant reduction of mRNA expression of *Esr1* was measured in the DEHP750 + BPA100–treated rats in comparison with BPA100 treatment. Then, the protein levels of Esr1 were detected, which showed a slight but not significant increase after DEHP or BPA alone treatment. Interestingly, the upregulation after exposure to DEHP or BPA was nullified by the concomitant presence of the other. It is clear that phosphorylate activation of the Creb through Esr1 has already been described in several models (Belcher et al. 2005) (Bouskine et al. 2009). Collectively, our data demonstrated that DEHP and BPA may change the levels of Esr1, further altered the levels of phosphorylated Creb, and ultimately effected the development of thyroid in adolescent female rats.

## Conclusion

Together these data suggested that both DEHP and BPA have the potential to disrupt the thyroid hormone homeostasis, which might ultimately affect the thyroid function. Although the interaction of combined exposure is complex and not a simple summation of their single effect, our findings will deepen our understanding of the combined effects of DEHP and BPA on the thyroid development in adolescent female rats. Whether phthalates and bisphenols can induce the expression of related genes via a similar mechanism will be an interesting subject for the future exploration. Considering that humans are exposed to thousands of chemicals, a thorough analysis of combined effects of mixtures in humans needs to be undertaken to create a more reliable risk assessment of EDCs.

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## Compliance with ethical standards

All experiments and surgical procedures were approved by the Animal Use and Care Committee of China Medical University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering.

**Conflict of interest** The authors declare that they have no conflict of interest.

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