Novel insights into di-(2-ethylhexyl)phthalate activation: Implications for the hypothalamus-pituitary-thyroid axis

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Abstract. Di (2-ethylhexyl) phthalate (DEHP), an environmental pollutant, is widely used as a plasticizer and causes serious pollution in the ecological environment. As previously reported, exposure to DEHP may cause thyroid dysfunction of the hypothalamic-pituitary-thyroid (HPT) axis. However, the underlying role of DEHP remains to be elucidated. The present study performed intragastrical administration of DEHP (150, 300 and 600 mg/kg) once a day for 90 consecutive days. DEHP-stimulated oxidative stress increased the thyroid follicular cavity diameter and caused thyrocyte oedema. Furthermore, DEHP exposure altered mRNA and protein levels. Thus, DEHP may perturb TH homeostasis by affecting biosynthesis, biotransformation, bio-transportation, receptor levels and metabolism through disruption of the HPT axis and activation of the thyroid-stimulating hormone (TSH)/TSH receptor signaling pathway. These results identified the formerly unappreciated endocrine-disrupting activities of phthalates and the molecular mechanisms of DEHP-induced thyrotoxicity.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; D1, deiodinases 1; D2, deiodinases 2; D3, deiodinases 3; FT3, free triiodothyronine; FT4, serum free thyroxine; GSH, glutathione; MDA, malondialdehyde; NIS, sodium iodide symporter; PAX-8, paired box protein 8; PDS, pendrin protein; SOD, superoxide dismutase; T3, triiodothyronine; T4, tetraiodothyronine; T-AOC, total antioxidant capacity; TC, total cholesterol; Tg, thyroglobulin; TG, triglyceride; TH, thyroid hormone; TPO, thyroperoxidase; TRH, thyrotropin-releasing hormone; TRHr, thyrotropin-releasing hormone receptor; TSAb, thyroid-stimulating antibody; TSHr, thyroid-stimulating hormone receptor; TSH-β, thyroid-stimulating hormone β; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2; TTR, serum transthyretin protein

Key words: hormone homeostasis, di-(2-ethylhexyl)phthalate, thyroid, hypothalamus-pituitary-thyroid axis, thyroid-stimulating hormone/thyroid-stimulating hormone receptor signaling

Introduction

Thyroid hormones (THs), synthesized and released by the thyroid, are vital in physiological systems, including growth, development and basal metabolism (1). Thyroid dysfunction is one of the leading endocrine disorders in the world (2). Endocrine disturbance, including disruption of the TH system, has been studied in western countries since 1996 (3). Iodine deficiency and autoimmune diseases are the pivotal causes of thyroid dysfunction and numerous studies indicate that genetic and environmental factors interfere with endocrine signaling by affecting TH levels, thereby causing thyroid disease (4-7). The use of chemicals has increased and pollutants have consequently become a major health problem worldwide, especially those that alter the function of the thyroid gland and secretion of THs (8).

Phthalates are widely used as plasticizers and softeners in various commercial products such as pharmaceutical devices, food packaging, make-up and cosmetics (9-13). Di-(2-ethylhexyl)phthalate (DEHP), one of the most common phthalates, is added to plastics to make them flexible, but it is an environmental endocrine disruptor (14-16). DEHP binds non-covalently to plastic matrices that allow it to leach from end-products, hence it readily pollutes air, food and water (17-20). DEHP exposure is mainly by inhalation of polluted air, ingestion of contaminated water or food and contacted with the skin (21). DEHP is a toxic chemical and following absorption, toxic effects occur when it is metabolized to dangerous metabolites such as monoethylhexyl phthalate (22-27). Thus, human exposure to DEHP is a worldwide concern.

The hypothalamus-pituitary-thyroid (HPT) axis regulates the thyroid endocrine system and all the evidence indicates that the thyroid is vulnerable to DEHP disrupting the endocrine system through its effects on TH biosynthesis, transport, secretion and metabolism (28). The hypothalamus secretes thyrotropin-releasing hormone (TRH), which stimulates the pituitary to secrete TSH and THs regulate TSH secretion

through a negative feedback loop in mammals (29). The biosynthesis and secretion of THs relies on iodine and iodine absorption by thyroid follicular cells (30). TSH regulates these kinase pathways, which mediate the expression of genes associated with thyroid gland development, including the actions of paired box protein 8 (PAX-8), sodium iodide symporter (NIS), thyroid transcription factor 1 (TTF-1), iodide transporter pendrin (PDS) and thyroid peroxidase (TPO) (31,32). In animal experiments, including DEHP as a contaminant in rat feed, decreased the plasma concentration of tetraiodothyronine (T4) and its metabolites and induced histological changes in the thyroid gland (33,34). Cell culture experiments revealed that changes in TH activity and iodide uptake by thyroid follicular cells following exposure to DEHP may be associated with its antagonistic activity (35,36). Recent epidemiological studies have indicated that there is a correlation between DEHP exposure and THs, in which levels of free thyroxine (FT4) and triiodothyronine (T3) in serum were negatively correlated with the concentration of DEHP (37-39). TH activation and inactivation is mediated by the deiodinase family of enzymes. When T3 and T4 enter the cell through the thyroid hormone transporter, the concentration of thyroid hormone is controlled by three iodothyroxine deiodinases [deiodinases 1 (D1), deiodinases 2 (D2) and deiodinases 3 (D3)]. D1 and D2 convert thyroxine T4 into active T3, while D3 prevents T4 from activating and terminates T3 operation (40). DEHP disrupts the stability of THs and the HPT axis by altering the expression of genes, hormone levels and enzyme activities. These alterations can be reliably used to evaluate the effects of chemicals used on the thyroid endocrine system.

Despite encouraging progress, our understanding of DEHP toxicity remains poor and the underlying mechanisms remain to be elucidated. The present study reported the risk of thyroid diseases from exposure to DEHP and explored the potential of mechanisms by which DEHP affects the HPT axis and the thyroid-stimulating hormone (TSH)/TSH receptor (TSHr) pathways.

Materials and methods

Chemicals and reagents. DEHP (C24H38O4, CAS: 117-81-7, purity 99.0%) was purchased from the National Institute of Standards and Technology. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), blood glucose (GLU), urea nitrogen (BUN), creatinine (CREA), total cholesterol (TC), triglycerides (TG) reagent kits were obtained from Maccura Biotechnology Co., Ltd. Total antioxidant capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and glutathione (GSH) contents were measured by using assay kits (Jiancheng Haihao Biotechnology Co., Ltd.). Serum transthyretin protein (TTR; cat. no. GD-S0712-A), TRH (cat. no. GD-S2054-A) and thyroid-stimulating antibody (TSAb; cat. no. GD-S1768-A) levels were measured using ELISA kits (Shanghai Guduo Biological Technology Co. Ltd.). Serum T4, T3, free triiodothyronine (FT3), FT4 and TSH levels were measured using radioimmunoassay kits (Beijing North Institute of Biotechnology Co., Ltd.). Anti-NIS polyclonal (cat. no. ab83816), anti-TSHr polyclonal (cat. no. ab202960), anti-thyroglobulin (Tg) monoclonal (cat. no. ab156008), TPO (cat. no. ab203057), anti-GAPDH (cat. no. ab8245) and anti-α tubulin (cat. no. ab7291) antibodies were obtained from Abcam. TTF-1 (D2E8 rabbit mAb cat. no. 12373) and PAX-8 (D2S2I rabbit mAb cat. no. 59019) were purchased from Cell Signaling Technology, Inc. TSH-β antibody (cat. no. MAB4507) was purchased from R&D Systems Inc. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (cat. no. ZB-2301), AP-conjugated rabbit anti-goat IgG (cat. no. ZB-2306) and AP-conjugated rabbit anti-mouse IgG (cat. no. ZB-2305) secondary antibody were purchased from ZSGB-BIO.

Animals and experimental design. A total of 40 healthy 2-week-old male Wistar rats (70±10 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. All animals were housed under a constant temperature (23±1˚C) and humidity (50-60%) and a 12-h light/dark cycle. All rats were provided with distilled water and standard AIN-93M diet *ad libitum*. Rats were randomly assigned to experimental groups (n=10): A control group, a 150 mg/kg/day DEHP group [~ five times the no-observed-adverse-effect level (NOAEL)], 300 mg/kg/day DEHP group (~10 times the NOAEL) and a 600 mg/kg/day DEHP group (~20 times the NOAEL). The NOAEL was obtained from a 104-week study on the chronic toxicity of DEHP in rats using a previously described method (41). Based on the investigation of DEPH properties in different experiments, a concentration of 600 mg/kg/day, which is 1/40 of the half lethal dose of DEHP for rats, was used in subsequent experiments (42). DEHP was administered to rats via gavage and control animals received peanut oil without phthalate. DEHP was intragastrically administered to rats daily for 90 consecutive days. All protocols were performed in accordance with the Regulations of the Ethical Committee for Research on Laboratory Animals, as assessed and approved by the Ministry of Health of China and the Institute of Zoology Animal and Medical Ethics Committee of Harbin Medical University. Subsequent experiments were conducted under the strict principles of Good Laboratory Practice to ensure good quality *in vivo* toxicology studies.

Sample collection and measurement of biochemical indices. The body weight of the rats was measured weekly. On day 89, the rats were transferred to the metabolic cage, and urine samples were collected for 24 h. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (35 mg/kg). Euthanasia was performed by abdominal aortic bleeding. Blood samples (~5 ml/per rat) were collected from the abdominal aorta. Thyroid, pituitary, hypothalamus, liver, kidney and testis tissue samples were obtained and frozen in liquid nitrogen, and then were transferred to a -80˚C refrigerator for storage until analysis. Serum biochemical indicators were detected using an automatic biochemical analyzer (Hitachi High-Tech Corporation) according to the manufacturer's instructions. Measurements of ALT, AST, TC and TG were performed according to the manufacturer's protocol.

Determination of oxidative stress indices. The content of oxidation products and the activity of antioxidant enzymes are indicators of the level of oxidation and therefore the degree of oxidative damage (43). Antioxidant enzyme activities of SOD and CAT and antioxidant levels of MDA, GSH and T-AOC in

serum were measured according to the manufacturer's protocols.

Effects of DEHP exposure on THs. Serum T3, T4, FT3, FT4 and TSH levels were obtained using radioimmunoassay kits (Beijing North Institute Of Biotechnology Co., Ltd.) according to the instructions supplied with the Automatic Gamma Counter (Wallac Wizard-2 2470 Automatic Gamma Counter; PerkinElmer, Inc.).

Serum TTR, TRH and TSAb were measured according to the ELISA kit instructions. A BioTek 3 MFD instrument (BioTek Instruments, Inc.) was used to repeatedly measure standards solution and samples.

The urine iodine (UI) determination method was performed according to the principle of Ascerium-Cerium Catalytic Spectrophotometric Determination of Urine Iodine (WS/T 107-2006) and the analysis standard of iodine in urine (GBW09109h; National Institute of Standard Substance, Beijing, China) was based on the reference analysis provided by the National Iodine Deficiency Reference Laboratory (44,45). A TU-1901 double beam ultraviolet visible-visible spectrophotometer (Beijing Puxi General Instrument Co., Ltd.) was used to determine the iodine content in standard and diluted samples.

Histology and histopathology. The thyroid, liver, kidney and testes samples from the four groups were fixed in 10% paraformaldehyde for 12 h, dehydrated and trimmed and embedded in paraffin blocks at room temperature. Multiple 4-μm thick sections were deparaffinized with xylene, stained with hematoxylin and eosin (H&E;1:5) for 5 min at 25˚C and representative samples were analyzed by light microscopy (magnification, x200). The number of follicular epithelial cells and the diameter of the follicular cavity were measured using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc.) to quantitatively assess histological changes in glands.

Total RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA of tissues was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and treated with genomic DNA wiper. cDNA was synthesized from 1 g total RNA (65˚C for 5 min and rapid cooling on ice) using ReverTra Ace qPCR RT kit (cat. no. FSQ-201; Toyobo Life Science). RT was performed at 37˚C for 15 min and 98˚C for 5 min. RT-qPCR was performed using THUNDERBIRD SYBR® qPCR Mix with 50X ROX reference dye (cat. no. QPS-201T; Toyobo Life Science) according to the manufacturer's instructions. Expression levels were measured for NIS, PAX-8, Tg, TSHr, TTF-1, TTF-2, PDS, TPO, TSH-β, TRHr, D1, D2 and D3. The relative expression levels of target genes were calculated using $2^{-\Delta\Delta Cq}$ method (46). The primer sequences (Table I) were designed according to the cDNA sequence from GenBank. All reactions were run in triplicate. A melting curve was generated during amplification to verify the absence of primer dimers or incorrectly paired products. All primers were synthesized by Sangon Biotech, Co., Ltd.

Western blot analysis. Samples were processed to determine tissue (thyroid, liver, kidney and testis) protein concentrations as previously described (47). The protein concentrations were measured by the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology.). Equal amounts of protein (20 μl per lane) were separated by electrophoresis on a 10% SDS polyacrylamide gel under reducing conditions and the separated proteins were transferred onto a polyvinylidene fluoride membrane. The polyvinylidene fluoride membranes were then blocked with 1% bovine serum albumin (cat. no. V900933; Sigma-Aldrich; Merck KGaA) in TRIS-buffered saline with 0.05% Tween-20 (TBST) at room temperature for 1 h, then incubated with NIS (1:1,000 dilution), TSHr (1:1,000 dilution), Tg (1:1,000 dilution), TPO (1:1,000 dilution), TTF-1 (1:1,000 dilution), PAX-8 (1:1,000 dilution), TSH-β (1:500 dilution) and GAPDH (1:2,000 dilution) overnight at 4˚C. The next day, membranes were rinsed three times (10 min each) with 1% TBST and incubated with AP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000 dilution), AP-conjugated rabbit anti-goat IgG (1:1,000 dilution) or AP-conjugated rabbit anti-mouse IgG (1:1,000 dilution) for 1-2 h at room temperature. After washing six times in TBST buffer, a chemiluminescence detection system (Tanon Science and Technology Co., Ltd.) was used to detect the resultant signals. The individual protein bands were quantified by using ImageJ software (v1.50; National Institutes of Health). Each western blot analysis was repeated ≥3 times.

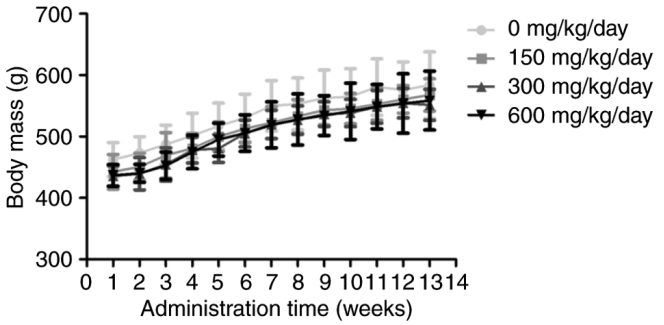

Figure 1. Body mass of rats in each group at each timepoint. Data are presented as the mean ± standard error of the mean (n=10 rats per group).

Statistical analysis. Each experiment was performed ≥3 times. Statistical analysis was performed using SPSS version 20.0 (IBM Corp.). All data are expressed as the mean ± standard error of the mean. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni's test. Graphs presenting the results were produced using GraphPad Prism 5.0 (GraphPad Software, Inc.) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in bodyweight and general physical status. No rats succumbed during the treatments and rats in each group gained body mass normally over time. There was no difference in the physiological parameters of the rats in each group compared with time-matched control groups at all time-points (Fig. 1).

Table I. Primers used in the present study.

Primers	Type	Primer sequence	GenBank
NIS	Forward	5'-CAGTTCTGGAATGGACACGG-3'	NM_052983.2
	Reverse	5'-TCTTGGTCACAGCAGGGATG-3'	
PAX-8	Forward	5'-AGCAGCAGTAGTGGTCCTCG-3'	NM_031141.2
	Reverse	5'-TTTATGGCGTAGGGTGAATGA-3'	
Tg	Forward	5'-GCCCTAACTCATCCGTCCA-3'	NM_030988.2
	Reverse	5'-TGTTGATAAGCCCATCGTCCT-3'	
TTF-1	Forward	5'-GCACTTGGAGTAAGGCAGAAA-3'	XM_006224320.2
	Reverse	5'-ACCCCACGATACACGAACC-3'	
TTF-2	Forward	5'-CGAGTGAAGCCATTGACGA-3'	NM_001106454.2
	Reverse	5'-AAGCGGGGCAGACGATT-3'	
PDS	Forward	5'-TCCCAAAATACCGAGTCAAGG-3'	NM_019214.1
	Reverse	5'-TCAGAACAACGGACCCCAC-3'	
TPO	Forward	5'-ATGAGGCTGTGACTGAAGATGA-3'	NM_019353.2
	Reverse	5'-GTGGTCCGTGAGGAGTTTGA-3'	
TSH-β	Forward	5'-TACTGCCTGACCATCAACACC-3'	NM_013116.2
	Reverse	5'-GGTAGGAGAAATAAGGAGCAACAT-3'	
TSHr	Forward	5'-GTGGGAATAAGCAGCTACGC-3'	NM_012888.1
	Reverse	5'-GGATTTCGGACGGTGATGT-3'	
TRHr	Forward	5'-AGGAGTCAGACCGCTTTAGCA-3'	NM_013047.3
	Reverse	5'-GAACTGGGTCCATTCTTCTCG-3'	
D1	Forward	5'-GTGGTGGTGGACACAATGCAG-3'	NM_021653.3
	Reverse	5'-TTGTAGTTCCAAGGGCCAGGTTTA-3	
D2	Forward	5'-GCTCTATGACTCGGTCATTCTGCTC-3'	NM_031720.3
	Reverse	5'-GACACGTGCACCACACTGGA-3	
D3	Forward	5'-CGTGTCAGCGCAGCAAGAGTA-3'	NM_017210.3
	Reverse	5'-TGCCGCTCTGGATGACGTAG-3	
β-actin	Forward	5'-CCGTAAAGACCTCTATGCCAACA-3'	NM_013116.2
	Reverse	5'-GGGGCCGGACTCATCGTA-3'	

NIS, sodium iodide symporter; PAX-8, paired box protein 8; Tg, triglyceride; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2; PDS, pendrin protein; TPO, thyroperoxidase; TSH-β, thyroid-stimulating hormone β; TSHr, stimulating hormone receptor; TRHr, thyrotropin-releasing hormone receptor; D1, deiodinases 1; D2, deiodinases 2; D3, deiodinases 3.

Effects of exposure to DEHP on relative organ weight. To validate the role of DEHP in organs, relative organ weight [(organ weight/body weight) x 10,000] were calculated (Fig. 2). Relative organ weight for thyroid, epididymis and testicular fat did not significantly differ among treatment and control groups ($P<0.05$). However, relative organ weights for liver, kidney were increased in DEHP treatment groups compared with the control group; relative organ weights for testis were decreased in DEHP 600 mg/kg/day treatment group compared with the control group.

Effects of exposure to DEHP on oxidative stress. To further clarify the potential effects of DEHP-induced oxidative stress, several oxidative stress-associated parameters were measured. The findings demonstrated that MDA levels were significantly increased following DEHP treatment, while the activities of T-AOC, SOD, CAT and GSH in DEHP-exposed rats were decreased compared with the control group (Fig. 3).

Effects of exposure to DEHP on biochemical indices. The effects of exposure to DEHP on biochemical indices are presented in Fig. 4. The results of routine blood and urine metabolic indicators ALT, AST, TG and UI revealed similarities among the four groups. Unexpectedly, compared with the 300 mg/kg/day dose group, there were significant differences in TC levels after treatment with 150 and 600 mg/kg/day.

Effects of exposure to DEHP on THs. As revealed in Fig. 5, no significant difference was observed in levels of THs (FT4,

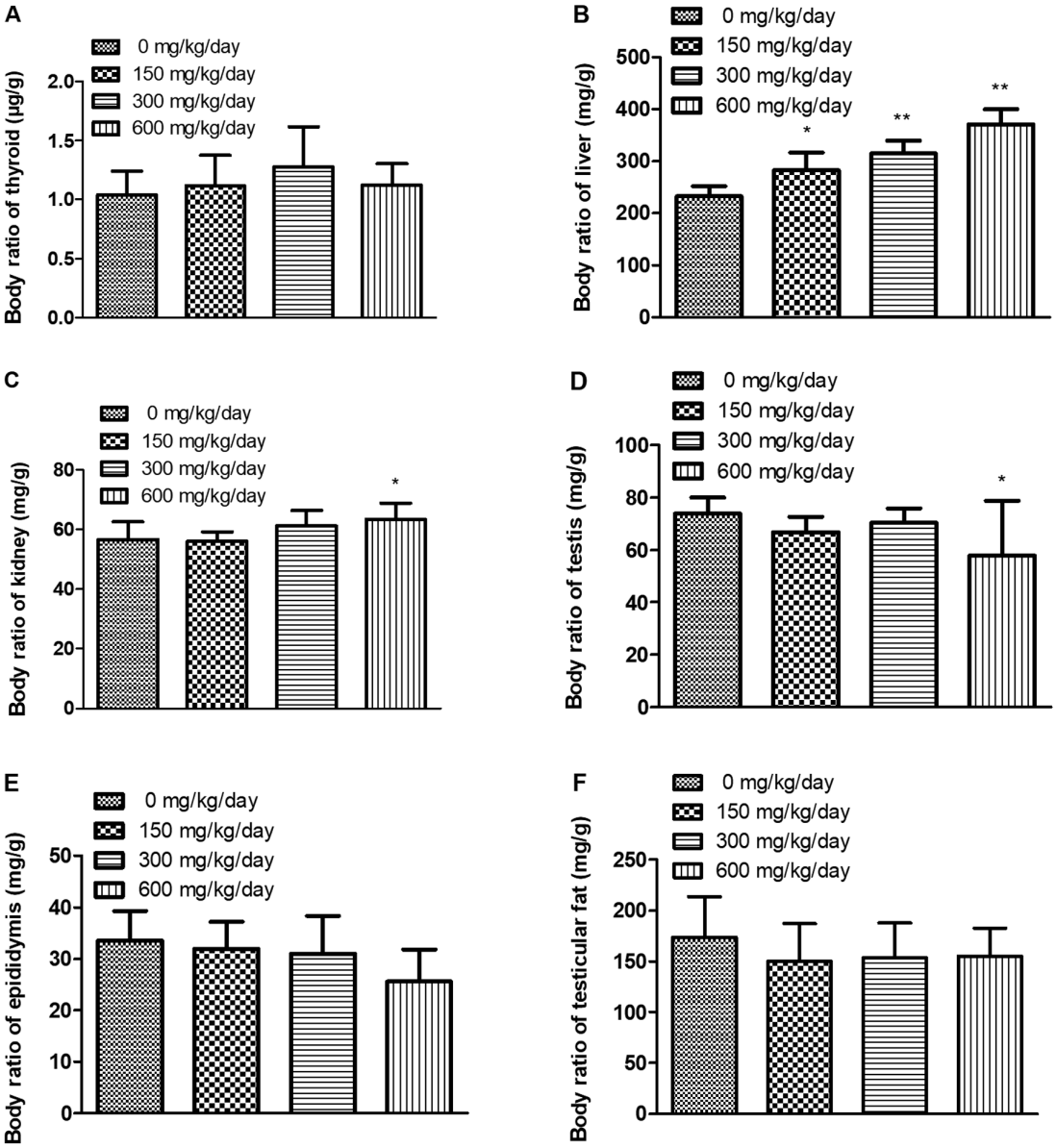

Figure 2. Relative organ weight of rats in each group at the end of the experiment. The body ratio of the (A) thyroid, (B) liver, (C) kidney, (D) testis, (E) epididymis and (F) testicular fat. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05, **P<0.01 vs. the control group; (n=10 rats per group).

TRH, TTR and TSAb) in DEHP treatment compared with the control group (P>0.05). No changes were observed with 150 mg/kg/day DEHP (a no effect level) and that for FT3 and T4, the effects were not dose-dependent. However, levels of T4 and FT3 in the 300 mg/kg/day dose group were significantly decreased compared with that of the control group (P<0.05). Levels of T3 and TSH in the 600 mg/kg/day dose group were also significantly different compared with the control group (P<0.05).

DEHP exposure leads to histological changes. Analysis of histological changes revealed altered TH levels following DEHP exposure. H&E staining demonstrated that the follicular epithelium was cube-shaped in the control group, colloids were uniform, most of the thyroid gland was normal, only some epithelium was shed and inflammatory cell infiltration was evident (Fig. 6A). In the low-dose group, the follicular epithelium was also cube-shaped, part of the epithelial cytoplasm was loose and epithelial shedding was evident (Fig. 6B). In the medium dose group, part of the follicular epithelium exhibited signs of necrosis, follicular collapse was observed along with inflammatory cell infiltration and residual follicular epithelial vacuolar degeneration was apparent. In addition, the cytoplasm appeared foamy and vacuolated and enlarged follicular epithelial cells were noticeable (Fig. 6C). In the 600 mg/kg/day dose group, most of the thyroid follicles were collapsed and disappeared, inflammatory cell infiltration was advanced, part of the filter follicle epithelium was shed and follicle epithelium vacuolar degeneration was even more pronounced. Additionally, the nucleus was deformed while the nuclear membrane was shrunken and chromatin was aggregated. The expansion of the rough endoplasmic reticulum was also evident, along with the appearance of vacuoles (Fig. 6D).

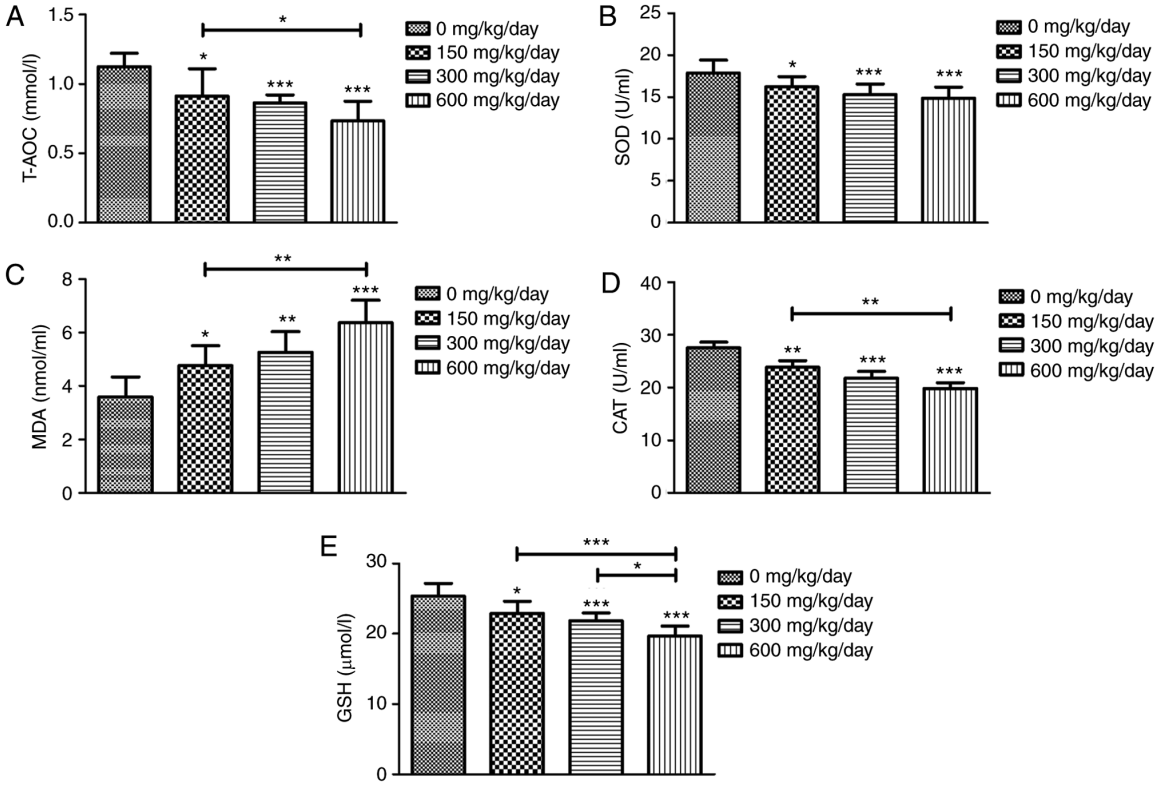

Figure 3. Antioxidant factors associated with oxidative stress in different groups. (A) T-AOC; (B) SOD; (C) MDA; (D) CAT and (E) GSH. Significant differences from control groups are presented. *P<0.05, **P<0.01, ***P<0.001 vs. the control group; (n=10 rats per group). T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; GSH, glutathione.

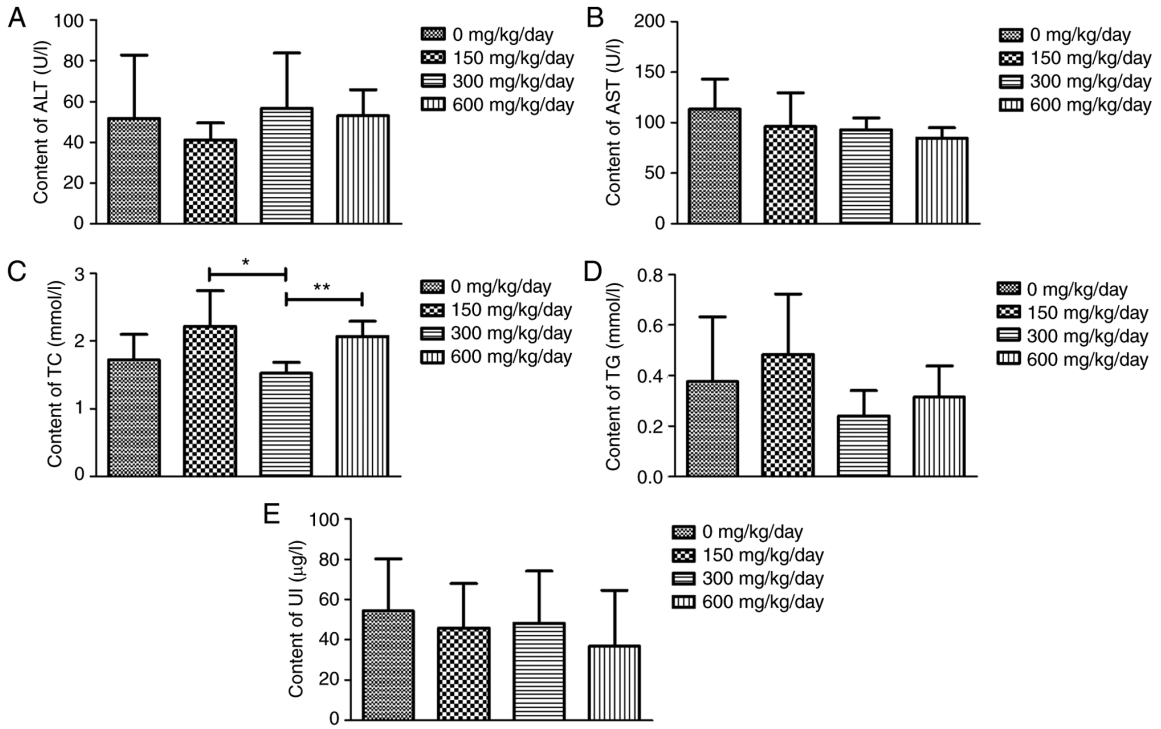

Figure 4. Effects of DEHP exposure on biochemical indices. (A) ALT, (B) AST, (C) TC, (D) TG and (E) UI. *P<0.05, **P<0.01 vs. the control group; n=10 rats per group. DEHP, di (2-ethylhexyl) phthalate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; UI, urine iodine.

In Fig. 6E the liver tissue of the control group is presented. In the 150 mg/kg/day dose group, liver lobular structure is clear, hepatocytes are arranged neatly, but partly bulked (Fig. 6F). In 300 mg/kg/day dose, a small amount of fatty liver

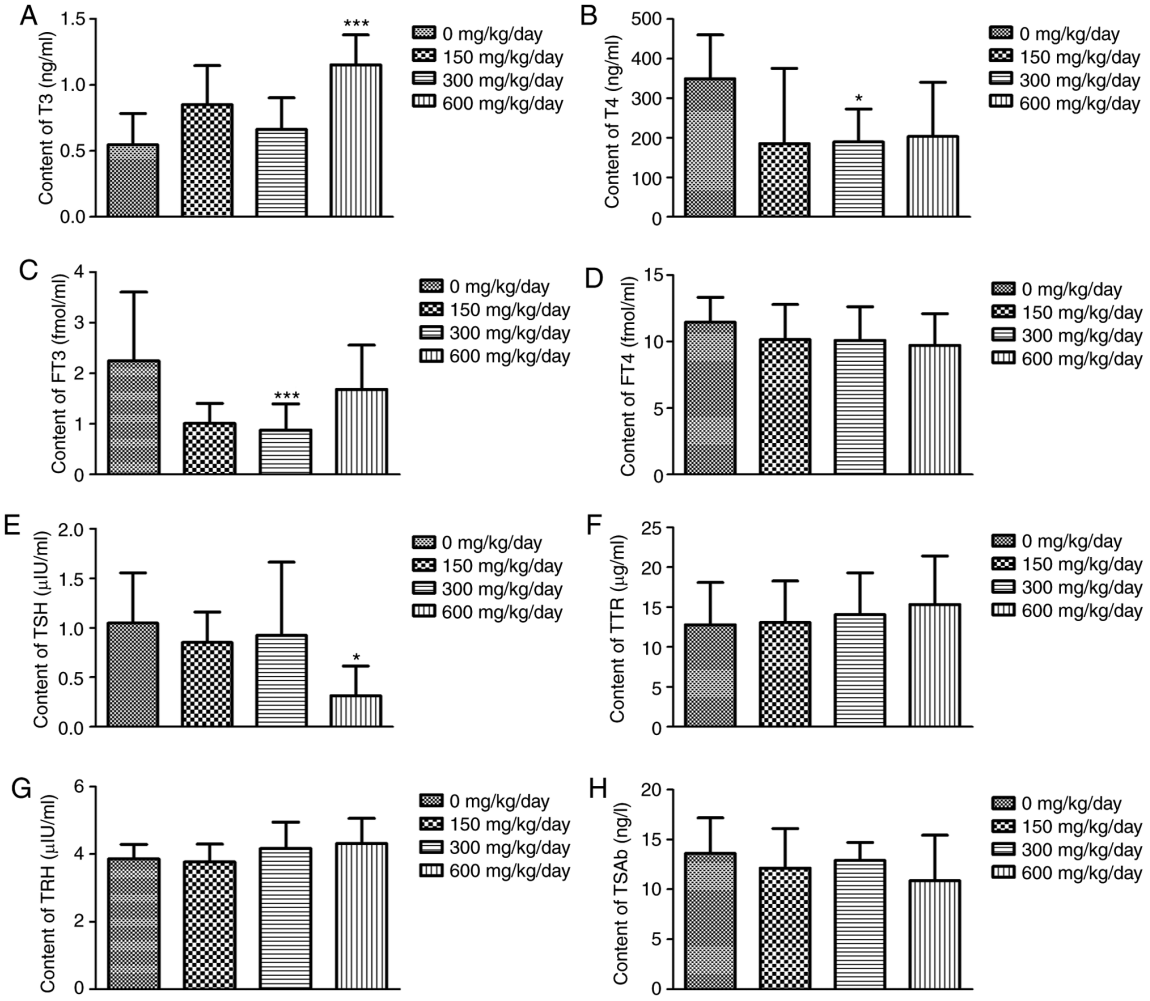

Figure 5. Changes in THs in different groups. Data are presented as the mean ± standard error of the mean of three independent experiments. (A) T3, (B) T4, (C) FT3, (D) FT4, (E) TSH, (F) TTR, (G) TRH and (H) TSAb. *P<0.05, ***P<0.001 vs. the control group; n=10 rats per group. THs, thyroid hormones; T3, triiodothyronine; T4, tetraiodothyronine; FT3, free triiodothyronine; FT4, serum free thyroxine; TSH,-stimulating hormone; TRH, thyrotropin-releasing hormone; TTR, serum transthyretin protein; TSAb, thyroid-stimulating antibody.

cells and punctate necrosis of the hepatocytes occasionally occurred (Fig. 6G). In the 600 mg/kg/day dose group, focal necrosis of hepatocytes, vacuum, hepatic sinusoidal dilation, obvious hydrodegeneration and occasional apoptotic hepatocytes were observed (Fig. 6H).

Histological studies of the kidneys in the control group revealed normal glomeruli and tubules (Fig. 6I). In the 150 mg/kg/day group, the glomeruli were contracted slightly and the tubular epithelial cells expanded (Fig. 6J). The glomerular epithelium of the 300 and 600 mg/kg/day group swelled, some of the renal tubules disappeared and protein components were visible in the tubes (Fig. 6K and L).

In the 0 and 150 mg/kg/day DEHP-induced group, the testes tissue was well organized with an intact epithelium (Fig. 6M and N). Exposure to DEHP at 300 and 600 mg/kg/day caused the seminiferous tubules at all levels of the seminiferous epithelium to be disorderly arranged with few layers, disintegration of germinal epithelial and reduction of round spermatozoa in the seminiferous tubules (Fig. 6O and P).

DEHP influences the mRNA expression levels of TSH/TSHR signaling pathway-related genes. Results for thyroid gene expression are presented in Fig. 7. The mRNA levels of PDS, NIS, PAX-8, TPO, TTF-1 and Tg were significantly increased compared with the control group after DEHP treatment. mRNA levels of TSHr, D1 and TTF-2 significantly decreased compared with the control following DEHP treatment (P<0.05). Exposure to DEHP caused an initial decrease in mRNA expression of pituitary TSH-β and significant differences between 150 and 600 mg/kg/day groups (P<0.05) and 150 and 300 mg/kg/day groups (P<0.05). The mRNA levels of hypothalamus TRHr were increased after DEHP treatment, with significant differences between 600 mg/kg/day and the control group (P<0.01) and between 150 and 600 mg/kg/day groups (P<0.01).

DEHP influences the abundance of proteins associated with TSH/TSHR signaling pathways. Compared with that of the control group, the protein levels of NIS, PAX-8, Tg, TTF-1 and TPO were significantly increased after DEHP treatment (Fig. 8). TSHr protein levels significantly decreased compared with that of the control group following DEHP treatment (P<0.05). The protein levels of pituitary TSH-β were increased after DEHP treatment and significant differences between 150

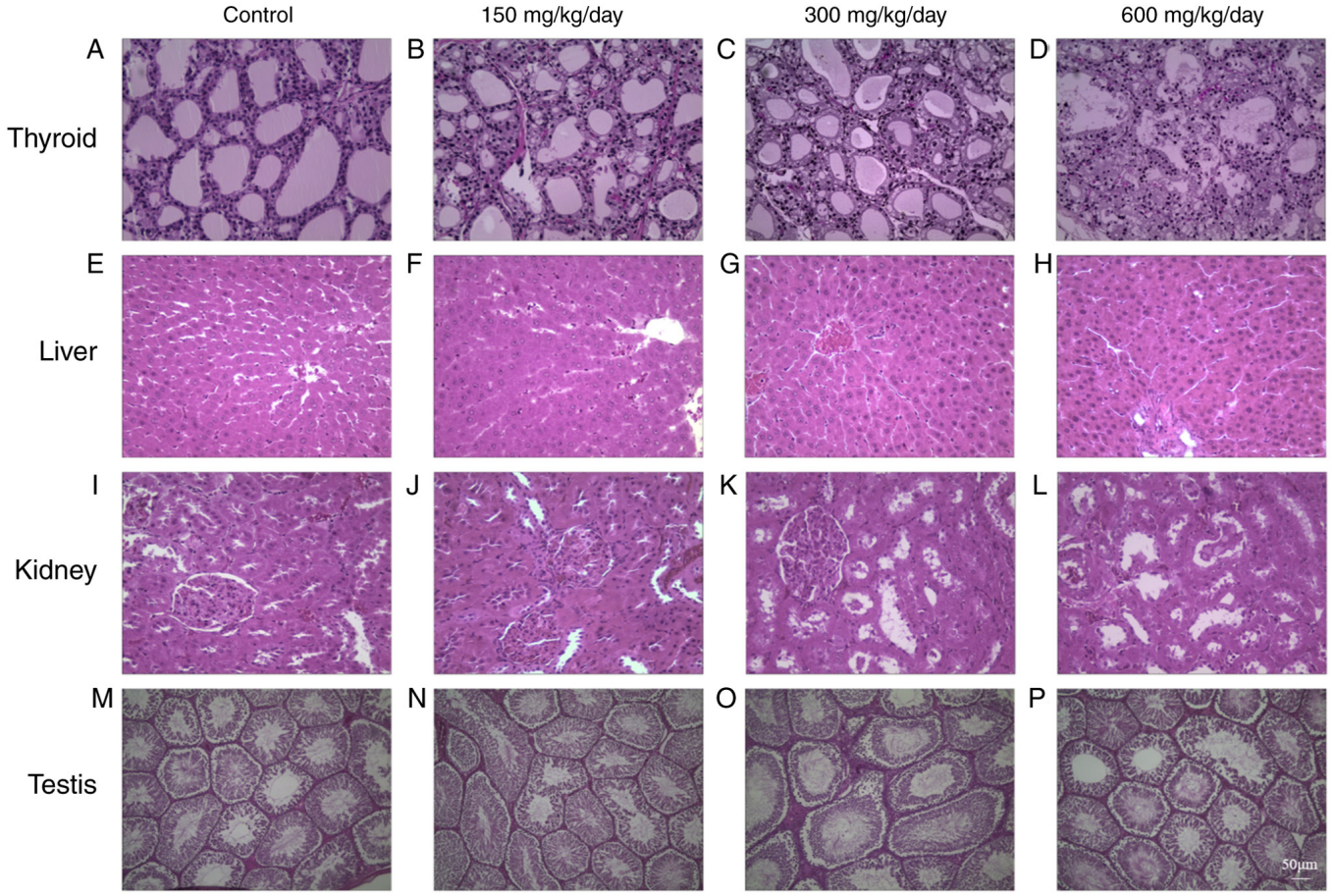

Figure 6. Effects of DEHP on the histology of (A-D) thyroid, (E-H) liver, (I-L) kidney and (M-P) testis. (A, E, I and M) 0 mg/kg/day DEHP; (B, F, J and N) 150 mg/kg/day DEHP; (C, G, K and O) 300 mg/kg/day DEHP; (D, H, L and P) 600 mg/kg/day DEHP. Scale bar, 50 μm; magnification, x200. DEHP, di (2-ethylhexyl) phthalate.

and 600 mg/kg/day groups ($P<0.05$) and between 300 and 600 mg/kg/day groups ($P<0.05$) were observed.

Discussion

The normal thyroid function of the human body is able to maintain the function of the thyroid itself while establishing a properly functioning HPT axis (48). Thyroid indicators are intricate and interrelated and one or several indicators may be up- or downregulated following interference by exogenous chemicals (49). DEHP can interfere with the HPT axis at varying levels and thereby alter thyroid function through numerous potential mechanisms, including inducing thyroid oxidative injury, disrupting TH homeostasis, damaging hormone receptors and modifying the mechanisms of transporter proteins and cellular uptake (50).

In view of the morphological and histological changes, the results of the present study suggested that DEHP may damage the follicles and nuclei. In addition, various organelles of cells may also be damaged. The exposure to DEHP was dose-dependent and under different doses, follicular cell division increased, the nucleus became more confined and some functional organelles gradually disappeared. These findings indicated the disintegration of thyroid follicular cells, which may lead to reduced absorption of iodide from the blood, phenolic coupling, iodination of proteins and endocytosis, resulting in altered signal transduction and disturbance to biosynthesis and bio-transportation of THs (51).

In vertebrates, numerous biological processes are carefully regulated by THs and the HPT axis and serve an essential role in hormone biosynthesis and release (52). For example, a potential interaction between the thyroid and stress systems in the context of fetal brain development has been previously reported (53). A biologically based dose-response-HPT axis model is linked with physiologically based pharmacokinetic models for thyroid-active chemicals in the adult rat (54). TRH (secreted from the hypothalamus) binds to the TRHr and is not only important in the TH synthesis and stimulation of TSH (secreted from the pituitary) release, but also serves an essential role in the TH feedback loop of the HPT axis in animal models (55). During the oxidation of iodine in the follicular lumen, tyrosine [3-monoiodotyrosine (MIT); 3,5-diiodotyrosine (DIT)] is iodized and iodotyrosine is coupled to the tyrosine residues of Tg, eventually leading to the effects of T3 and T4 synthesis (56). Changes in deiodinase mRNA levels can be used as sensitive markers for detecting TH disruptions (57). D1 has a considerable influence on iodine recovery and TH degradation; D2 exclusively catalyzes outer-ring deiodination of T4 into active T3, consequently controlling the intracellular concentration of T3 (58). D3 has different catalytic properties, including inactivating enzymes, preventing T4 activation and

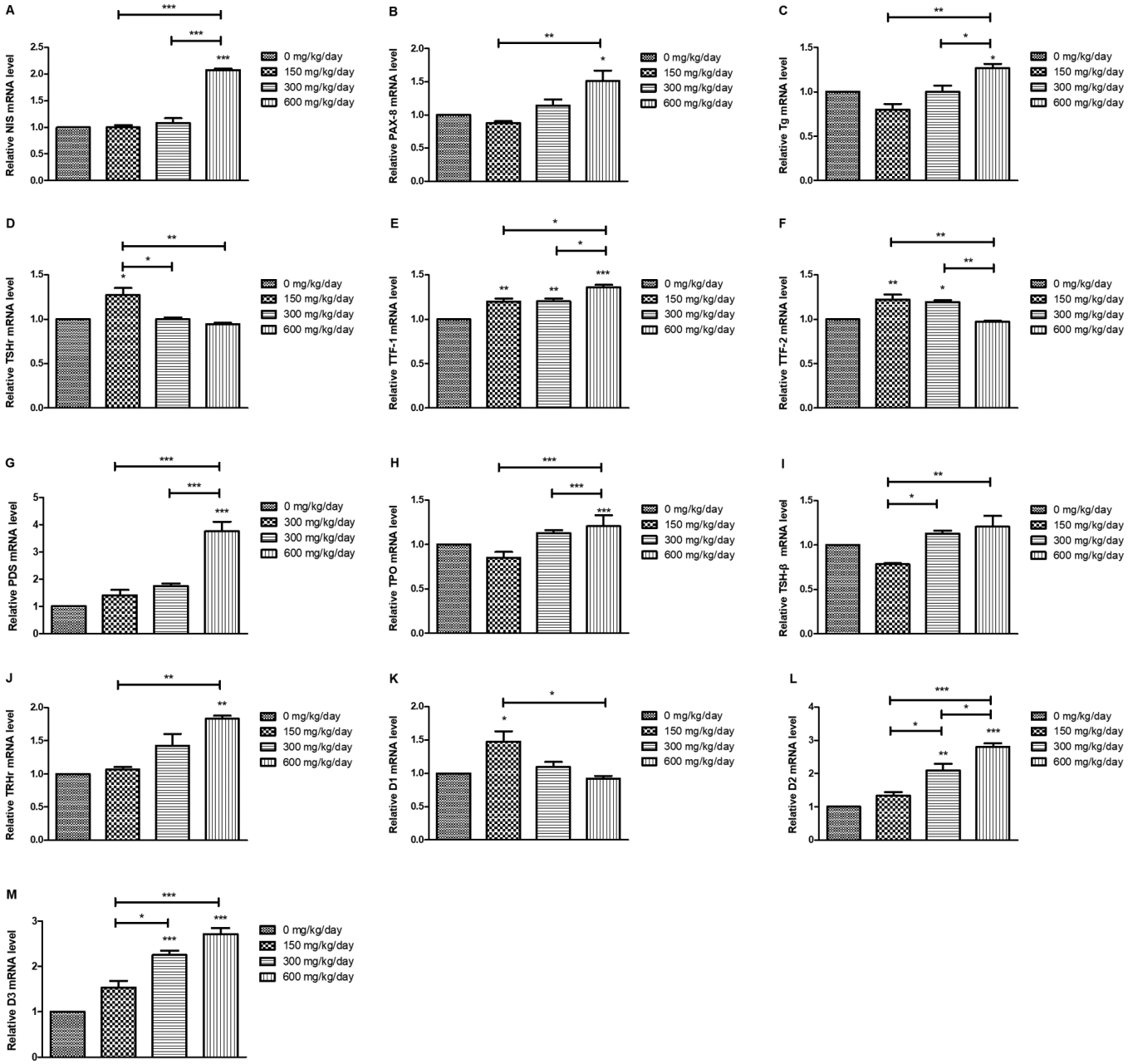

Figure 7. Thyroid gene expression levels in rats after DEHP treatment. (A) NIS, (B) PAX-8, (C) Tg, (D) TSHr, (E) TTF-1, (F) TTF-2, (G) PDS, (H) TPO, (I) TSH-β, (J) TRHr, (K) D1, (L) D2 and (M) D3. *P<0.05, **P<0.01, ***P<0.001 vs. the control group. DEHP, di (2-ethylhexyl) phthalate; NIS, sodium iodide symporter; PAX-8, paired box protein 8; Tg, thyroglobulin; TSHr, stimulating hormone receptor; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2; PDS, pendrin protein; TPO, thyroperoxidase; TSH-β, thyroid-stimulating hormone β; TRHr, thyrotropin-releasing hormone receptor; D1, deiodinases 1; D2, deiodinases 2; D3, deiodinases 3.

terminating T3 action (48). Hence, changes of deiodinase mRNA levels have been revealed to coincide with deiodinase enzyme activities (59).

The present study measured gene expression and protein abundance associated with the synthesis and secretion of THs. Iodine is an essential constituent regulating THs (60). NIS, a plasma membrane glycoprotein, is a special active transporter that mediates I^- accumulation and breakdown and serves a crucial function in the initial biosynthesis of THs (61). Since iodine is acted on by TPO, the results of the present study revealed abnormal urine iodide following DEHP exposure (62). In the HPT axis, TSH-β serves an important role and assessment of TSH-β gene transcription can be used to evaluate DEHP-induced thyroid dysfunction (63). The present study measured both protein and mRNA levels for TSH-β, TTF-1, NIS, TRHr and TSH following DEHP exposure, but in some instances 150 mg/kg/day appeared to exhibit no notable effect on the levels, which suggested that the HPT axis was activated at higher concentrations of DEHP (64). Hence, all biosynthesis and release steps are stimulated, including Tg iodination, with increased serum TSH levels (65). Additionally, TTF-1 can regulate Tg iodination in the follicular cavity, leading to upregulation of TSHr and PAX-8. These changes in thyroid tissue confirm that DEHP alters the follicle sensitivity of

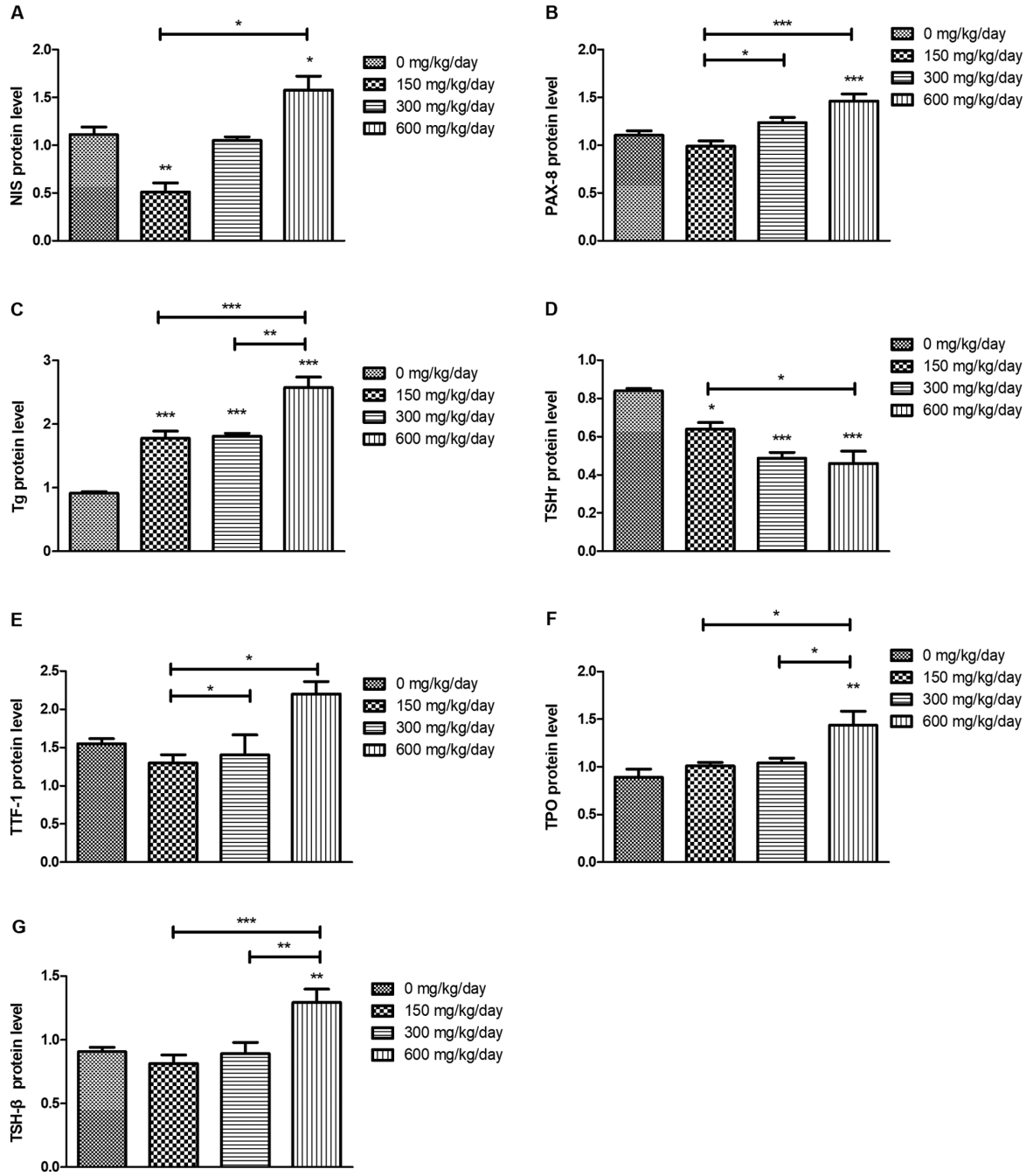

Figure 8. Abundance of proteins associated with the HPT axis following DEHP treatment. (A) NIS, (B) PAX-8, (C) Tg, (D) TSHr, (E) TTF-1, (F) TPO and (G) TSH-β. *P<0.05, **P<0.01, ***P<0.001 vs. the control group. HPT, hypothalamic-pituitary-thyroid; DEHP, di (2-ethylhexyl) phthalate; NIS, sodium iodide symporter; PAX-8, paired box protein 8; Tg, thyroglobulin; TSHr, stimulating hormone receptor; TTF-1, thyroid transcription factor 1; TPO, thyroperoxidase; TSH-β, thyroid-stimulating hormone β.

TSH/TSHR signalling (66). Collectively, these results indicate that DEHP induces morphological and physiological changes in the thyroid. Thus, DEHP disrupts the bio-transportation of THs through TSH/TSHR signaling.

The present study demonstrated that DEHP regulated the redox status of biological systems, biotransformation, biotransport and receptor levels of THs and thereby disrupted the delicate balance of the HPT axis. In addition, the findings of the present study contributed to an improved understanding of thyrotoxicity caused by phthalates.

It is unclear whether certain endocrine disruptors affect thyroid function by altering thyroid growth, or by interacting with the production of anti-thyroid antibodies and other substances that are important in thyroid metabolism (67). In addition, individuals are simultaneously exposed to various endocrine disruptors, hence mixed effects of certain endocrine disruptors may differ from effects of exposure to DEHP alone on thyroid function (68). DEHP could contribute to an environmental risk factor for changes of the endocrine system and pathogenesis of thyroid dysfunction. Thus, further research is required to elucidate the mechanisms by which DEHP disrupts hormone homeostasis and to emphasize the importance of evaluating DEHP-induced environmental risks.

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Availability of data and materials

The data sets that are used and/or analysed in the current study can be reasonably obtained from the corresponding authors.

Authors' contributions

XN, HW and WZ designed the experiments. HW and WZ performed the experiments and the data analysis. YZ, ZK and XM contributed to data collection and analysis. HW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols were performed in accordance with the Regulations of the Ethical Committee for Research on Laboratory Animals, as assessed and approved by the Ministry of Health of China and the Institute of Zoology Animal and Medical Ethics Committee of Harbin Medical University, which complies with National Institutes of Health Guidelines. Subsequent experiments were conducted under the strict principles of Good Laboratory Practice to ensure good quality *in vivo* toxicology studies.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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