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Tris(4-hydroxyphenyl)ethane (THPE), a trisphenol compound, is antiestrogenic and can retard uterine development in CD-1 mice

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ABSTRACT

Tris (4-hydroxyphenyl)ethane (THPE), a trisphenol compound widely used as a branching agent and raw material in plastics, adhesives, and coatings is rarely regarded with concern. However, inspection of *in vitro* data suggests that THPE is an antagonist of estrogen receptors (ERs). Accordingly, we aimed to evaluate the antiestrogenicity of THPE *in vivo* and tested its effect via oral gavage on pubertal development in female CD-1 mice. Using uterotrophic assays, we found that THPE either singly, or combined with 17β-estradiol (E_2) (400 μg/kg bw/day) suppressed the uterine weights at low doses (0.1, 0.3, and 1 mg/kg bw/day) in 3-day treatment of weaning mice. When mice were treated with THPE during adolescence (for 10 days beginning on postnatal day 24), their uterine development was significantly retarded at doses of at least 0.1 mg/kg bw/day, manifest as decreased uterine weight, atrophic endometrial stromal cells and thinner columnar epithelial cells. Transcriptome analyses of uteri demonstrated that estrogen-responsive genes were significantly downregulated by THPE. Molecular docking shows that THPE fits well into the antagonist pocket of human ERα. These results indicate that THPE possesses strong antiestrogenicity *in vivo* and can disrupt normal female development in mice at very low dosages.

1. Introduction

The presence of endocrine-disrupting chemicals (EDCs) in the environment has become a great concern in the past decades (Colborn et al., 1993; Matthiessen and Johnson, 2007;Mann et al., 2009; Spindola Vilela et al., 2018). Among the known EDCs, bisphenol compounds have been focally studied, and now, bisphenol A (BPA), a typical EDC, has been regulated or banned in many countries in materials or containers for food and drinks because of its estrogenic activity and adverse effects on human health (Colborn et al., 1993; Krishnan et al., 1993; Zhou et al., 2017). However, the chemicals being recognized as EDCs are only the "tip of the iceberg" (Birnbaum, 2013). Identifying all the chemicals with endocrine activities from all the chemicals used and released worldwide is a major challenge (Birnbaum, 2013). In recent years, *in vitro* high-throughput screen assays for EDCs have been developed and many chemicals have been screened as potential EDCs (Judson et al., 2015; Kim et al., 2016; Rotroff et al., 2014; Wambaugh et al., 2013). Now, verification and validation of the potential EDCs by *in vivo* toxicity tests becomes one of the key priorities of EDCs studies.

In this study, the chemical of concern is tris(4-hydroxyphenyl) ethane (THPE, CAS: 27,955-94-8, $C_{20}H_{18}O_3$), which has a similar use history as that of BPA, but has not received enough attention in environmental sciences. THPE is a trifunctional phenolic compound that has been widely used as a branching agent and raw material, usually together with BPA, in a variety of polymers, such as polycarbonates, epoxy resins, polyarylates, adhesives and coatings since the 1970s (Ihre et al., 2001; Liou et al., 2007; Margotte et al., 1976). The introduction of THPE can improve the mechanical strength and hardness, heat resistance, solvent resistance and adhesion of polymers. THPE is a high production volume chemical listed in the U.S. Environmental Protection Agency (EPA) Toxic Substances Control Act inventory (Laws et al., 2006; U.S. Environmental Protection Agency U.S. EPA, 2016; U.S. Environmental Protection Agency U.S. EPA, 2019). The annual aggregate product of THPE in the U.S. was 500,000–1,000,000 lb in 2012–2013 (U.S. Environmental Protection Agency U.S. EPA, 2016). THPE is also listed as a Persistent, Mobile and Toxic (PMT) substance and as a very Persistent, very Mobile (vPvM) substance registered under REACH, with the P_conclusion "Pot. P/vP++" and M_conclusion "vM" (EU, 2018; UBA/NGI, 2016). In terms of THPE toxicity, little information

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was available other than the acute oral 50% lethality dose (LD_{50}) of >5000 mg/kg body weight and acute dermal LD_{50} of >2000 mg/kg body weight (https://pubchem.ncbi.nlm.nih.gov/compound/93118).

Recently, THPE has been presented as an agonist/antagonist of multiple targets in the PubChem database (https://pubchem.ncbi.nlm.nih.gov). Of these targets, ERs, including ERα and ERβ, are of primary concern. The antagonistic activity values (IC_{50}) of THPE versus ERα and ERβ are 10.87 μM and 2.43 μM, respectively. In the same assays, IC_{50} values of the typical antiestrogen compounds are 24.3613 μM for raloxifene on ERα and 0.3459 μM for tamoxifen on ERβ, suggesting that THPE may have strong antiestrogenic properties. Rotroff et al. (2014) evaluated 1814 chemicals by *in vitro* screening assay and found that THPE ranked in the top 25 chemicals with an estrogen-antagonist composite score of 59.25. Thus, we aimed to test the antiestrogenic activity of THPE *in vivo* and investigate its effects on uterine development, given that other antiestrogens such as RU39411 and BHPF have been reported to prevent uterine development in some animals (Kaplan-Kraicer et al., 1996; Xiao et al., 2018; Zhang et al., 2017).

2. Materials and methods

2.1. Chemicals

THPE (>99%), E_2 (≥98%), and fulvestrant (FULV) (>98%) were obtained from Sigma-Aldrich (StLouis, MO). All solvents were of analytical grade and were distilled before use. Ultrapure water was generated using a Millipore Super-Q system (Millipore, Bedford, MA).

2.2. Antiuterotrophic assays in weaning CD-1 mice

All animal studies were approved by the Institutional Animal Care and Use Committee of Peking University, and were performed in accordance with the University's Guidelines for Animal Experiments, which meet the ethical guidelines for experiments involving animals in China. Weaning female CD-1 mice (20 days old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice with a maximal difference in body weight of 1 g were selected for the experiments and randomly assigned to either the treatment or control groups. The animals were housed five to a cage in identical environments, with ready access to food and water, a temperature of 22 °C ± 2 °C, a relative humidity of 40%–60%, artificial lighting on a 12-h/12-h light-dark cycle, and treated in the Laboratory Animal Centre of Peking University.

Mice were randomly assigned to eight experimental groups. Each group was consisted eight mice. Chemicals were dissolved in peanut oil and each mouse would receive 10 ml/kg bw/day of peanut oil vehicle. Mice treated only with 10 ml/kg bw/day of peanut oil vehicle were used as a negative control, and mice treated with 400 μg/kg bw/day E_2 were used as a positive control. THPE was administered at dosages of 0.1, 0.3, and 1 mg/kg bw/day, singly or together with 400 μg/kg bw/day E_2. Treatment by oral gavage to each mouse was performed for 3 days beginning on postnatal day (PND) 21 according to the dosage levels stipulated above. After the period of treatment, the mice were weighed and then killed by cervical dislocation 24 h after the final treatment according to the sequence of the treatments. The uteri were isolated and weighed, and the relative uterine weight, ratio of uterine weight to final body weight (bw), was calculated to evaluate the antiuterotrophic activities of chemicals. Uteri were frozen in liquid nitrogen for the next-generation sequencing-based transcriptome analyses.

2.3. Uterine development effects tests using adolescent mice

To analyze the effects of THPE on uterine development at low dosages, two 10-day exposure experiments were performed according to our previous methods (Xiao et al., 2018; Zhang et al., 2017), beginning on PND 24. In both experiments, mice were randomly assigned to either the treatment groups (n = 10) or the control group (n = 10), and those treated with 10 ml/kg bw/day peanut oil vehicle were used as control groups. In Experiment 1, mice were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) on PND 20, and operations were performed in the Laboratory Animal Centre of Peking University after 3 days' acclimatization. THPE was dosed at 3, 10, 30, and 100 mg/kg bw/day and 10 mg/kg bw/day dosage of FULV were used as a positive control. In Experiment 2, the mice used were produced by the Beijing Vital River Laboratory Animal Technology Co., Ltd., and all operations were performed at the Beijing Vital River Laboratory Animal Technology Co., Ltd. THPE was dosed at 0.1, 0.3, and 1 mg/kg bw/day as per Experiment 1. All dosages were carried out via oral gavage to each mouse for 10 days beginning on PND 24, which is close to the onset of puberty. The uteri were isolated, weighed, and fixed in 4% paraformaldehyde for histologic examination. The fixed uteri were finally embedded in paraffin and sectioned serially at 5 mm. Slides were stained with hematoxylin and eosin (H&E) and observed under a light microscope (Olympus BX53). Images of uterine cross sections for mice in control and 0.1 mg/kg bw/day THPE group of the Experiment 2 were acquired using an ImageXpress Micro 4 High-Content Imaging System (Molecular Devices, Sunnyvale, CA) and the total cell numbers of the uterine cross sections were counted through the cell nuclei.

2.4. Next-generation sequencing-based transcriptome analyses

Uteri of weaning CD-1 mice from the control group, the 400 μg/kg bw/day E_2 group and the 1 mg/kg bw/day THPE combined with 400 μg/kg bw/day E_2 group were selected for the transcriptome analyses. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from the uteri according to the manufacturer's protocol. A Dynabeads mRNA purification kit (Life Technologies) was used to isolate mRNA from total RNA. An Agilent 2100 Bioanalyzer was used to evaluate the quality of total RNA and mRNA. The qualified RNA samples were selected for RNA sequencing and real-time quantitative reverse-transcription (qRT-PCR). For each group, two samples were subjected to RNA sequencing (about 6 Gb of data per sample) at the Biodynamic Optical Imaging Centre of Peking University. RNA sequencing data were analyzed using CLC Genomics Workbench (v12) with reference to Mus_musculus GRCm38.p6. Reads per kilobase of exon model per million mapped reads (RPKM) values were determined as the relative transcript levels. Baggerley's test was used to statistically assess the RPKM value for each gene and thus determine differential expression (DE) between groups. A P value of <0.05 and an absolute fold-change of greater than 2 were used to confirm the significance of genes/transcripts. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (Jiao et al., 2012).

2.5. qRT-PCR

To verify the data from the transcriptome analysis, each of the samples subjected to RNA sequencing were chosen for gene expression measurement using qRT-PCR according to previous publications (Xiao et al., 2018; Zhang et al., 2017). Five small proline-rich proteins (SPRRs) genes were analyzed and the primers

used are listed in Table 1. Actb (actin, beta) was used as an endogenous control to normalize the data. Amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems). The relative expression was quantified using the $2^{-\Delta\Delta CT}$ method.

2.6. Molecular docking

Scigress software (Ultra Version 3.0.0; Fujitsu Ltd., Tokyo, Japan) was used for *in silico* molecular docking analyses primarily according to our previous methods (Xiao et al., 2018; Zhang et al., 2017). The three-dimensional structure of the ligand-binding domain of human ERα, PDB IDs 3UUC, was downloaded from the Protein Data Bank website (http://www.rcsb.org/) and used to evaluate the binding affinity of THPE in the antagonist pocket of human ERα. The structure was refined, and all water molecules except those that were considered important to the ligand-binding pocket composition were removed from the protein via Scigress-integrated procedures. The structure of THPE was drawn and energy-optimized for molecular modulation. Automated docking of the flexible ligand into the flexible active site was performed using a genetic algorithm. The docking calculations were evaluated on a 0.25 Å grid in a 15 Å × 15 Å × 15 Å box that contained the active site for the original ligand. The local search parameters included 300 maximum iterations at a rate of 0.06, and the procedure was set to run 30,000 generations with an initial population size of 50, elitism of 7, crossover of 0.8, mutation of 0.2, and convergence of 1.0. After automated docking, a geometric optimization calculation for the human ERα-THPF complex was performed using augmented MM3 parameters. The potential of mean force (PMF), a knowledge-based approach that extracts pairwise atomic potentials from structure information of known protein-ligand complexes contained in the Protein Data Bank, was used to score the binding affinity of THPF in the active site.

2.7. Data analysis

Data analyses were performed using the statistical program SPSS (v.18.0; Chicago, IL). Data are presented as means and standard deviations unless otherwise indicated. Group differences were assessed using a one-way analysis of variance and Fisher's least significant difference tests. *P* values of <0.05 were considered to indicate statistical significance. The figures were made with the R programming language.

3. Results and discussion

3.1. Antiestrogenic activity of THPE in weaning mice

The rodent uterotrophic bioassay is a useful *in vivo* screening test for estrogenic substances that has been incorporated into the U.S. EPA's screening and testing program for environmental endocrine-disrupting chemicals (EDCs) (Padilla-Banks et al., 2001). The ability of chemicals to reduce or abolish the uterotrophic response of E_2 provides a valuable extension of the uterotrophic assay for detection of antiestrogens (Odum et al., 1997; Xiao et al., 2018; Zhang et al., 2017). In this study, we performed the immature mouse uterotrophic assay to demonstrate the *in vivo* estrogenic and antiestrogenic properties of THPE and found that THPE showed no estrogenic activity but antiestrogenic activity in the weaning CD-1 mice. When singly administered to mice, THPE suppressed the uterine weights to 66.57% ± 2.76%, 94.38% ± 8.00% and 86.86% ± 2.42% that of control at dosages of 0.1, 0.3 and 1 mg/kg bw/day, respectively (Fig. 1A). When administered to mice combined with E_2, THPE counteracted the uterotrophic effects of E_2 in a dose-dependent manner, and significantly ($P < 0.05$) reduced the uterotrophic effects of E_2 (400 μg/kg bw/day) to 76.65% ± 6.81% and 65.94% ± 3.06% in the 0.3 and 1 mg/kg bw/day groups, respectively (Fig. 1B). These data indicate that THPE possesses obvious *in vivo* antiestrogenic activity.

3.2. Effects of THPE on pubertal uterine development in adolescent mice

Estrogens play important roles via ERs in a vast array of physiological processes, and disruption of ER signaling has been verified to adversely affect human health (Deroo and Korach, 2006; Jia et al., 2015). Considering that endogenous estrogens rapidly increase to high levels when female mice reach puberty, to support uterine development, we hypothesized that THPE might antagonize the endogenous estrogens and thus deleteriously affect uterine development in adolescent mice. To test this hypothesis, we performed two dosage experiments in mice via oral gavage for 10 days beginning on PND 24, which were close to the onset of puberty. As shown in Fig. 2, the relative uterine weights of THPE-treated adolescent mice were significantly decreased ($P < 0.05$) in dose-dependent patterns.

In Experiment 1, the relative uterine weights were significantly ($P < 0.05$) decreased to 70.74% ± 13.17%, 59.59% ± 15.02%, 41.19% ± 9.76%, and 45.92% ± 9.67% of that of control in mice given dosages of 3, 10, 30, and 100 mg/kg bw/day THPE, respectively, and that of FULV (10 mg/kg bw/day) group were decreased ($P < 0.01$) to 28.64% ± 3.15% of that of control (Fig. 2A). In Experiment 2, the relative uterine weights were significantly ($P < 0.05$) decreased to 58.90% ± 8.39%, 52.67% ± 7.97%, and 46.99% ± 4.24% of that of control in the mice given dosages of 0.1, 0.3, 1 mg/kg bw/day THPE, respectively (Fig. 2B).

The lower dosages of THPE produced more obvious changes on uterine weights in Experiment 2 than in Experiment 1, possibly because Experiment 2 was carried out at the premises of the Beijing Vital River Laboratory Animal Technology Co., Ltd., which was the birthplace of the mice. This meant the mice experienced no transportation or condition changes and were thus possibly better prepared for the 10-day exposure experiment.

Histologic analyses of uteri found that 0.1 mg/kg bw/day THPE decreased the endometrial thickness and increased the stromal density and that both the endometrial stromal cells and columnar epithelial cells in the uterine sections of THPE-treated mice were obviously atrophic (Fig. 3). The total cell number counted from the

Table 1
The primer list of selected genes for qRT-PCR.

Gene	Ensembl Gene ID	Forward primer (5′-3′)	Reverse primer (5′-3′)
Actb	ENSMUSG00000029580	AGATGACCCAGATCATGTTTGAGA	CACAGCCTGGATGGCTACGT
Sprr2a1	ENSMUSG00000078664	GTATTCCTGGTACTCAAGCATTGGT	CGGCACGGCTGATTGC
Sprr2a2	ENSMUSG00000068893	GGTCACTGCTGTTTCATTTCCT	ATTAGACCATCACCAAAGGGG
Sprr2b	ENSMUSG00000050092	GTGTCCACCCAAGAATAAATGAG	AGGACAGGCGTTCAAAGGAG
Sprr2f	ENSMUSG00000050635	ATGGGTCTTGTTCCATTGTTCA	AACAGTAACAACTACCCTGCTCAAG
Sprr2g	ENSMUSG00000046203	CTAGTAGATGTCCCTCAGTGCCTT	AGCAAATGGAACATCCGTGA

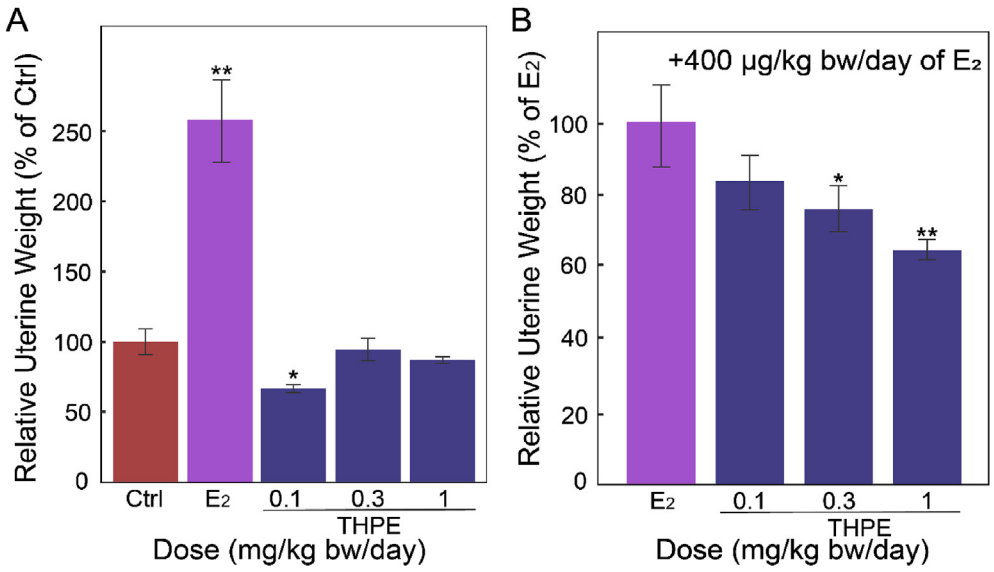

Fig. 1. Antiuterotrophic effects of THPE in weaning CD-1 mice orally gavaged for 3 days beginning on PND 21. (A) Animals were treated with THPE or E_2 singly; data are expressed as percentages of control. (B) Animals were treated with THPE in combination with 400 μg/kg bw/day of E_2; data are expressed as percentages of E_2 group. Each group was consisted 8 mice using completely randomized design. The error bars indicate standard deviations (SDs) from the average values. $^*P < 0.05$; $^{**}P < 0.01$.

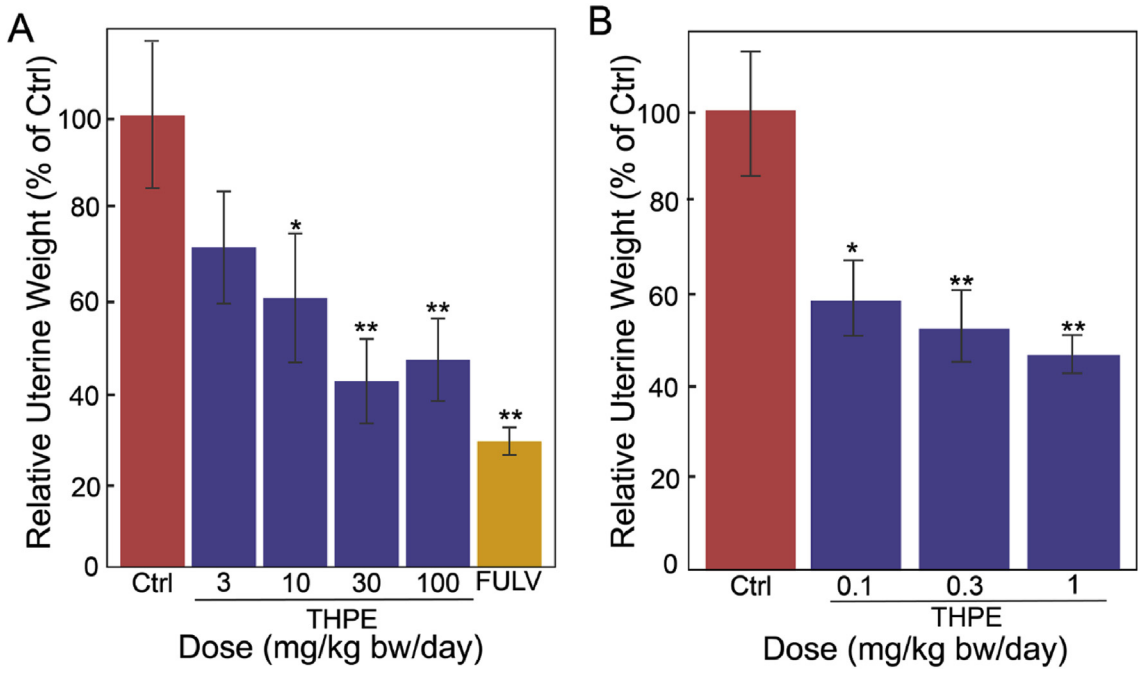

Fig. 2. Decreased uterine weights caused by THPE in adolescent CD-1 mice. Animals were treated via oral gavage for 10 days beginning on PND 24. Mice in the control group were treated only with peanut oil vehicle, and FULV was used as positive control to validate the method. Ten mice per group were used for the assays. Data are expressed as percentage of the vehicle control and error bars indicate SDs from the average values. $^*P < 0.05$; $^{**}P < 0.01$.

uterine cross sections of mice in 0.1 mg/kg bw/day THPE group was decreased to 66.23% that of control, of which the number of endometrial stromal cells were reduced to 63.30% that of control, while the number of uterine myocytes (95.63%) are closed to that of control. These phenomena are consistent with previous findings that the antiestrogens EM-800 and BHPF reduced uterine weights and caused atrophy of the endometrium in adolescent mice (Deroo and Korach, 2006; Zhang et al., 2017). Because the uterine stromal cells play a critical role in pregnancy-associated neovascularization, atrophic endometria have been considered a cause of the adverse pregnancy outcomes observed in mice exposed to antiestrogens (Kaplan-Kraicer et al., 1996; Laws et al., 2008; Zhang et al., 2017).

The incidence of adverse pregnancy outcomes in humans has increased in many countries in recent decades, and exposure to environmental pollutants has been proposed as a cause (Kaplan-Kraicer et al., 1996; Kramer, 2003; Laws et al., 2008). Antiestrogens may be one of these pollutants and thus play a role in this increase. This possibility has serious implications for future human reproductive health.

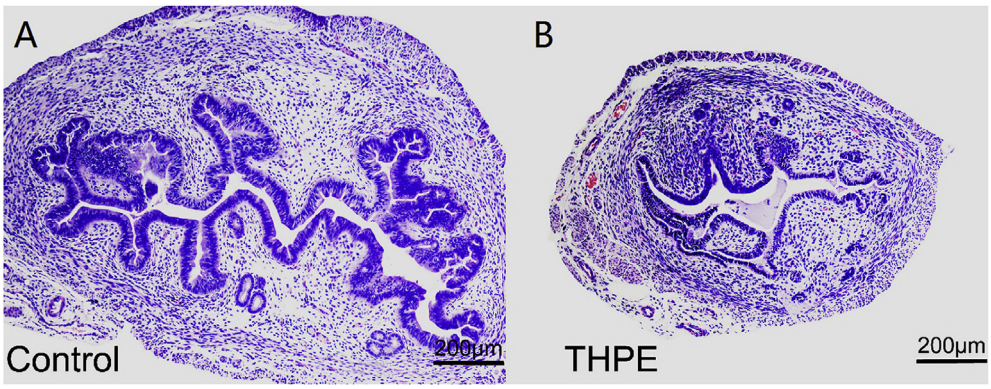

Fig. 3. Photomicrographs of uterine sections stained with H&E. (A) Uterine section of mice in control group that was treated with peanut oil only. (B) Uterine section of mice in 0.1 mg/kg bw/day THPE group. Animals were treated via oral gavage for 10 days beginning on PND 24.

3.3. *Antiestrogenicity of THPE demonstrated by transcriptome analyses and qRT-PCR*

Uteri of mice in groups from the antiuterotrophic assays that used weaning CD-1 mice were selected for transcriptome analyses (Fig. 4). In comparison with that of control, the expressions of 628 genes were significantly altered by treatment, with 69.7% upregulated and 30.3% downregulated in mice treated with 400 μg/kg bw/day E_2. In the uteri of mice treated with 400 μg/kg bw/day E_2 in combination with 10 mg/kg bw/day THPE (THPE/E_2 combination group), only 147 genes had significantly altered expression, with 53.7% upregulated and 46.3% downregulated (P value < 0.05 and absolute fold change >2) (Fig. 4A).

Comparison of the expression profile of genes in the uteri of mice in THPE/E_2 combination group with that of mice treated with only E_2, showed that expressions of 566 genes were significantly modified by THPE, with 33.6% upregulated and 66.4% downregulated. Clearly, the presence of THPE neutralized the E_2-dependent gene regulation, and most of the differentially expressed genes (DEGs) showed expression patterns opposite to that induced by E_2 (Fig. 4B). Of particular note is the small proline-rich protein 2 family (Sprr2) that are known as a cluster of genes positively regulated by estrogenic compounds via nuclear ERs in the mouse uterus (Hong et al., 2010). In this study, Sprr2a1, Sprr2a2, Sprr2b, Sprr2f, and Sprr2g were found to be clearly upregulated by E_2, and this upregulation was significantly offset by the presence of THPE. Fig. 5 shows the expression changes of Sprr2a1, Sprr2a2, Sprr2b, Sprr2f, and Sprr2g measured by qRT-PCR. In addition, opposite regulations of gene transcription by E_2 and THPE were also observed in Gja1, Ptgis, Gstm5, Olfm1, Ldha, Pgk1, Eno1b, Tpi1,

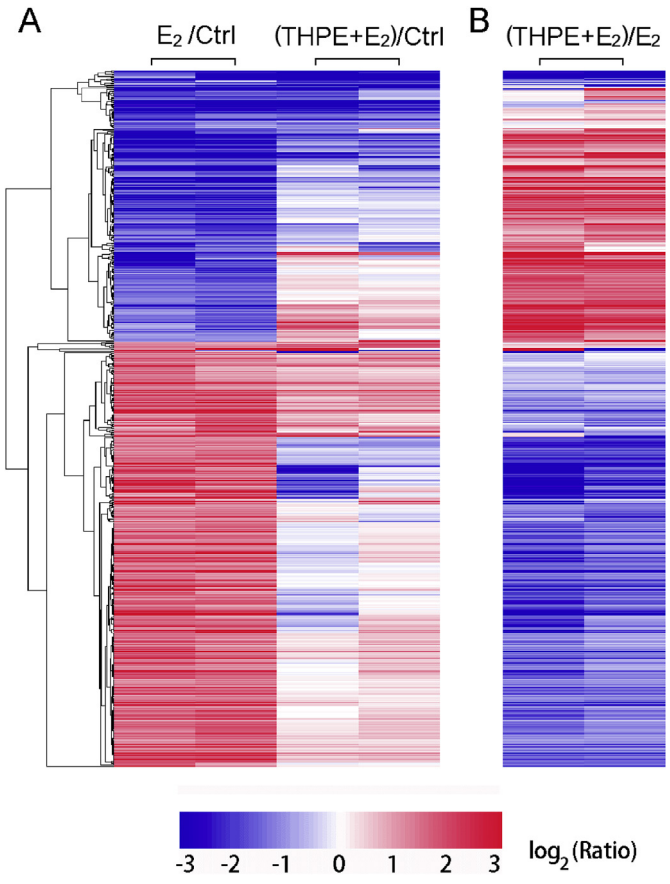

Fig. 4. Hierarchical cluster analyses of the differentially expressed genes (DEGs) based on log ratio RPKM data. (A) Ratios data of DEGs RPKM of E_2 (400 μg/kg bw/day) group and THPE/E_2 combination group (THPE + E_2) to that of control; (B) Ratio data of DEGs RPKM of THPE + E_2 group to that of E_2 group. Mice treated with peanut oil only were used as a control. Animals were treated via oral gavage for 3 days beginning on PND 21.

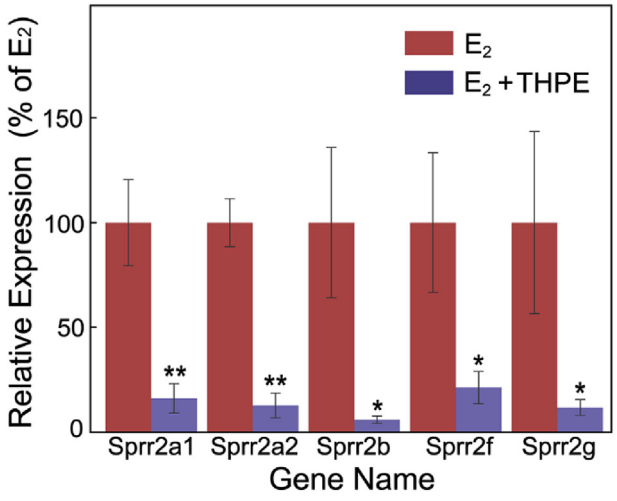

Fig. 5. Relative expressions of Sprr2 family genes in the uteri of the mice treated with E_2 (400 μg/kg bw/day) or E_2 + THPE (400 μg/kg bw/day E_2 combined with 1 mg/kg bw/day THPE) determined by qRT-PCR. The data are expressed as a percentage of the E_2 group. Error bars indicate the SD from the average. *P < 0.05, **P < 0.01. Animals were treated via oral gavage for 3 days beginning on PND 21.

Ceacam1, Atp1b3, Slc34a2, Mmp2, Dtymk, Car2, Igf1, Igfbp5, Igfbp2, etc. These genes were reported to be significantly and consistently regulated by E_2 (Naciff et al., 2002; Kiyama et al., 2014; Karim et al., 2016; Suzuki et al., 2007). These results indicated that THPE has an antiestrogenic effect on gene expression by blocking the ERs in the uteri of mice.

We also performed GO and KEGG pathway analyses for the DEGs in this study. GO analyses showed that the DEGs in mice treated with 400 μg/kg bw/day E_2 were significantly enriched in 269 items in biological process (BP), 99 items in cellular component (CC), and 96 items in molecular function (MF) (Fig. 6A). DEGs in mice treated with 400 μg/kg bw/day E_2 combined with 1 mg/kg bw/day THPE, compared with that only treated with E_2, were enriched in 163 items in BP, 48 items in CC and 43 items in MF categories (Fig. 6B), and biological processes of "response to estradiol" (GO:0032,355, $P = 1.57 \times 10^{-5}$) and "response to estrogen" (GO:0043,627, $P = 9.66 \times 10^{-4}$) were annotated in both the E_2 group and the THPE/E_2 combination group. In the E_2 group, "response to estradiol" term accumulated 28 genes, among them, 7 genes were down regulated and 21 genes were up regulated. And in the THPE/E_2 combination group, "response to estradiol" term accumulated 16 genes and among them 10 genes were down regulated while 6 genes were up regulated. In the KEGG pathway analyses, "metabolic pathways", "pathways in cancer", "osteoclast differentiation", "chemokine signaling pathway", "complement and coagulation cascades", which related to metabolism and immunity (Kovats, 2015; O'Mahony et al., 2012) showed upregulation in E_2 group, and the upregulation trends were reduced or abolished by adding THPE (Fig. 7).

3.4. Docking of THPE into active sites of human ERα

Using automated docking of the flexible ligand into known active sites, we found that THPE could fit well into the antagonist pocket of human ERα (PDB IDs 3UUC) (Fig. 8A). The important feature of the interaction was the formation of hydrogen bonds between the phenolic hydroxyl group of THPE and the guanidinium group of Arg-394, the carboxylate of Glu-353, and the indolepropionic acid of Thr-347 in the pocket (Fig. 8B). Moreover, the phenyl ring and alkyl group of THPE had van der Waal interactions with the

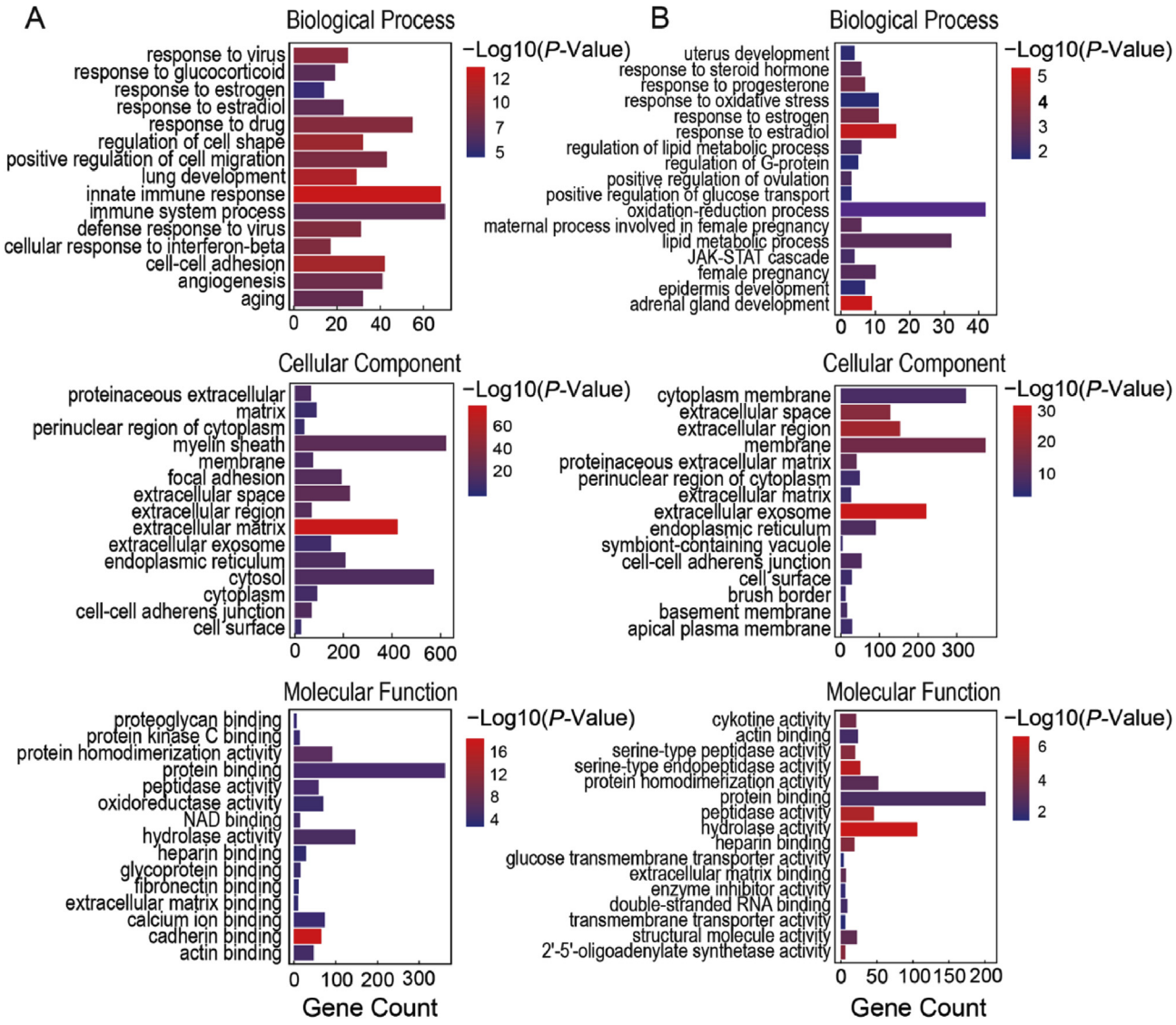

Fig. 6. Histograms of Gene Ontology (GO) enrichment of DEGs. (A) GO Classification of DEGs in E_2 (400 μg/kg bw/day)-treated group. (B) GO Classification of DEGs in E_2 + THPE (400 μg/kg bw/day E_2 combined with 1 mg mg/k bw/day THPE)-treated group. Animals were treated via oral gavage for 3 days beginning on PND 21.

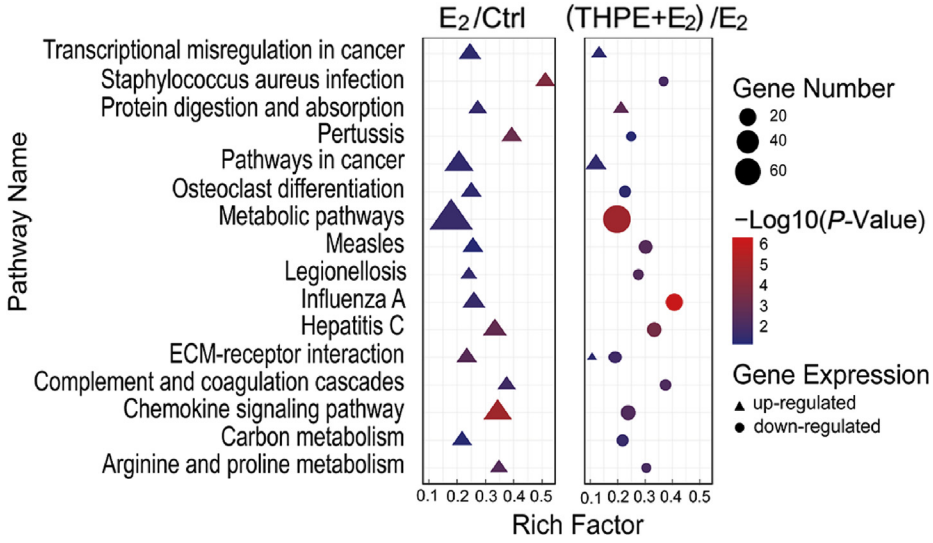

Fig. 7. Scatterplot of enriched KEGG pathways for DEGs in uteri of mice treated with E_2 (400 μg/kg bw/day) and E_2 + THPE (400 μg/kg bw/day E_2 combined with 1 mg/kg bw/day THPE). Animals were treated via oral gavage for 3 days beginning on PND 21.

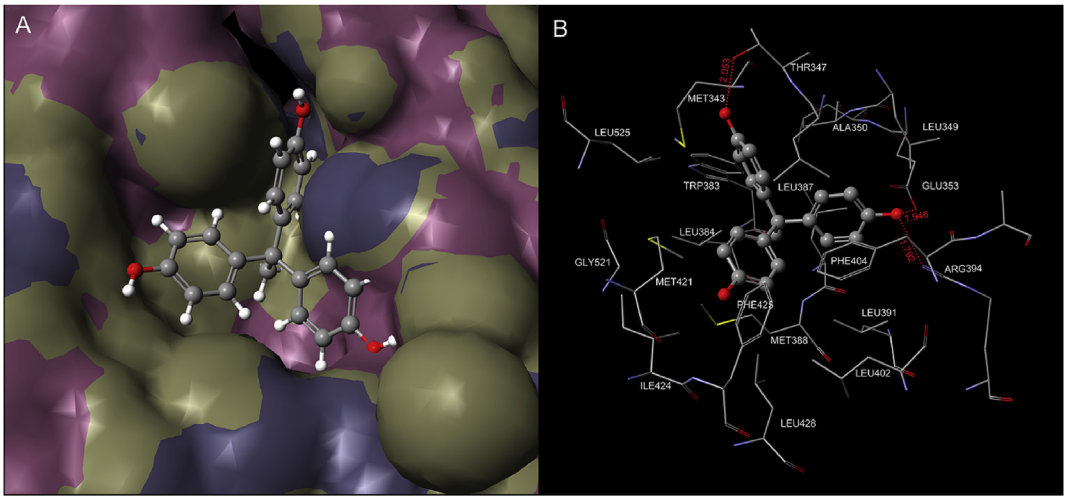

Fig. 8. *In silico* molecular docking analysis of THPE into the antagonist binding site of human ERα structure (PDB IDs 3UUC). (A) Simulated binding position of THPE in the antagonist pocket; (B) Interaction between THPE and the active site. Dotted lines indicate hydrogen bonds between THPE and receptor amino acid residues.

core hydrophobic region generated by Met-343, Leu-525, Ile-424, Met-421, Phe-404, Leu-391, Met-388, Leu-384, Leu-387, and Leu-349 (Fig. 8B). We also observed π-π interactions between the phenyl ring of THPE and those rings in Phe-425 and Phe-404 of the pocket. The PMF value of THPE binding with the active site of human ERα was calculated to be −64.98 kcal/mol, which was lower than that of BPAP (−56.77 kcal/mol), an antagonist of human ERα (Xiao et al., 2018).

Bisphenols have been increasingly used since the 1970s for synthesizing plastics and are now one of the chemical classes of most concern because of their endocrine-disrupting activities and potential adverse effects to human health (Colborn et al., 1993; Delfosse et al., 2012; Diamanti-Kandarakis et al., 2009; Krishnan et al., 1993; Leusch et al., 2019; Matsushima et al., 2010; Rochester and Bolden, 2015; Xiao et al., 2018; Zhang et al., 2017). However, although the usage history of THPE is similar to that of BPA, it has been little studied. To our knowledge, this study is the first to demonstrate the strong antiestrogenic activity *in vivo* and the adverse developmental effects of THPE. At dosage of 0.1 mg/kg bw/day and higher, THPE antagonized the activity of endogenous estrogens and clearly retarded uterine development in pubertal mice. At a dosage of 1 mg/kg bw/day, THPE could significantly reduce or abolish the response of gene expression to 400 μg/kg bw/day E_2 treatment in uteri of weaning mice. Estrogens play a critical role in maintaining the development of female reproductive organs and the course of pregnancy by regulating estrogen-responsive genes via ERs. Some documents concluded that the incidence of adverse pregnancy outcomes has increased in many countries and reported that there is emerging evidence for adverse reproductive outcomes from exposure to EDCs (Diamanti-Kandarakis et al., 2009; Kortenkamp et al., 2012; Birnbaum, 2013). Several studies have showed that antiestrogenic compounds may retard the uterine development and thereby resulting in adverse reproductive outcomes. This study provided supporting evidence for this contention and revealed the underlying mechanism of the toxicity of the antiestrogens. Because no environmental exposure levels have been reported for THPE, we believe that our data suggest a compelling need for investigation of THPE levels in the

environment and in humans.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.113962.

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