Environmental Pollution 242 (2018) 1625–1632

Contents lists available at ScienceDirect

Environmental Pollution

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

Bisphenol AP is anti-estrogenic and may cause adverse effects at low doses relevant to human exposure☆

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ARTICLE INFO

Article history:
Received 29 May 2018
Received in revised form
7 July 2018
Accepted 24 July 2018
Available online 24 July 2018

Keywords:
Bisphenol AP
Bisphenol A alternative
Anti-estrogenicity
Glucose tolerance test
Anti-uterotrophic effect
Endocrine disrupting chemical

ABSTRACT

A recent increase in the use of bisphenol A (BPA) alternatives to manufacture plastics has led to safety concerns. Here, we evaluated the estrogenic and anti-estrogenic activities of bisphenol AP (BPAP), a poorly studied BPA alternative, using *in vitro*, *in vivo* and *in silico* tools. BPAP exhibited weak estrogenicity but strong anti-estrogenicity ($IC_{50} = 2.35\ \mu M$) in a GeneBLAzer™ β-lactamase reporter gene assay. BPAP, when administered alone or in combination with E_2 ($50\ \mu g\ kg^{-1}$ bw d^{-1}) for 3 d, significantly decreased the uterine weights of post-weaning CD-1 mice at doses of $10\ mg\ kg^{-1}$ bw d^{-1} and higher. When administered alone to prepubertal CD-1 mice for 10 d, BPAP significantly decreased the uterine weights at doses of $80\ \mu g\ kg^{-1}$ bw d^{-1} and higher. Toxicogenomic analysis showed that BPAP regulated an opposite patterns of gene expression than that of E_2 in mouse uteri. In a glucose tolerance test using male mice, BPAP was found to disrupt the blood glucose homeostasis at low doses relevant to human exposure (1 and $100\ \mu g\ kg^{-1}$ bw d^{-1}). Our results suggest that BPAP should be of great concern which might affect the sexual development in immature feminine and disrupt the blood glucose homeostasis at very low doses.

1. Introduction

BPA is a typical endocrine-disrupting chemical that can activate estrogen receptors and some other nuclear receptors to cause endocrine diseases and disorders and increase the incidence of endocrine-related cancers (Krishnan et al., 1993; Hejmej et al., 2011; Newbold et al., 2009; Ho et al., 2006; Lu et al., 2018). Materials synthesized using BPA has been widely used in food and beverage packaging. However, the finding that BPA can be released into foods led many countries (such as Canada, European Union, and China) to restrict or ban the use of this chemical in materials that come into contact with food, including eating and drinking utensils and particularly, bottles used to provide liquids to infants (USFDA, 2008; Krishnan et al., 1993). Although many manufacturers have since introduced other bisphenols (e.g., bisphenol S, bisphenol F, and bisphenol AF) as BPA alternatives, some of these chemicals were reported to be as estrogenic as BPA (Delfosse et al., 2012; Rochester et al., 2015; Matsushima et al., 2010). Therefore, the relative safety of BPA alternatives remains of great concern.

Bisphenol AP (BPAP), or 4,4'-(1-phenylethylidene) bisphenol (CAS No. 1571-75-1), is widely used in the synthesis of various polymers such as PC, epoxy resins, polyarylates, polyethers, polyetherimides, polyphenylene ethers, and copolymers (Noskov, 1978; USEPA, 2015; Li and Ueda, 1984). In recent years, BPAP-containing materials have been increasingly used to manufacture various products such as coatings, moldings, photosensitive resin composition film, composite matrices, electrical insulation materials, thermal papers, optical film, and structural adhesives for use in the electronics, automobile, interior decoration, aerospace, and other industries (USEPA, 2015; Mercer, 1993; Qiu et al., 2016; Sezer et al., 2011). The widespread use of these products has inevitably led to BPAP contamination of both environments and foods and subsequent human exposure. BPAP was detectable in indoor dust and in personal care products, such as body washes, hair care products, makeup, skin lotions, and toothpastes (Liao and Kannan, 2014b; Wang et al., 2015; Liao et al., 2012). In an analysis of different categories of food items, Liao and Kannan (2013) reported BPAP detection rates of 8.3% in 289 food items collected from China and

☆ This paper has been recommended for acceptance by David Carpenter.

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https://doi.org/10.1016/j.envpol.2018.07.115
0269-7491/

11.2% in 267 food items from the United States, with a maximum BPAP concentration of 127 ng g^{-1} fresh weight in vegetables (Liao and Kannan, 2013; Liao and Kannan, 2014a). Based on the measured concentrations and daily ingestion rates of foods, Liao and Kannan (2013) also estimated the daily dietary intakes of BPAP for various age groups in the United States and reported that infants, toddlers, and children comprised the most exposed groups, with estimated daily dietary BPAP intakes of 3.27, 8.43, and 3.28 ng kg^{-1} body weight (bw), respectively (Liao and Kannan, 2013). The average concentration of BPAP in food items collected from China was reported to be 0.711 ng g^{-1} fresh weight, which is over 12 times higher than the average concentration in the United States (0.059 ng g^{-1}) (Liao and Kannan, 2013; Liao and Kannan, 2014a), suggesting that there might be a high exposure burden to people living in China. Zhang et al. (2016) measured the concentrations of bisphenols in urine samples collected from Chinese people and reported that BPAP was detectable in 9% of samples from rural areas and 15% from urban areas, with a maximal concentration of 0.703 ng ml^{-1}.

Our recent study found that fluorene-9-bisphenol (BHPF), a BPA alternative, exhibited strong anti-estrogenic activity and may therefore affect the sexual development in mice at doses much lower than the no-observed-adverse-effect level reported for BPA (Zhang et al., 2017). Given the molecular structural similarities of BPAP and BHPF, we expect that BPAP will exhibit a similar anti-estrogenic activity. In this study, we determined the estrogenic and anti-estrogenic activities of BPAP using multiple toxicological assays and performed a glucose tolerance test to evaluate the effects of BPAP at doses relevant to human exposure.

2. Materials and methods

2.1. Chemicals and reagents

BPAP (>99%), E_2 (≥98%), fulvestrant (FULV) (>98%), tamoxifen (TAM) (≥99%), and 4-hydoxytamoxifen (4-OHT) (≥98%) were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.).

2.2. Molecular docking

Scigress software (Ultra Version 3.0.0; Fujitsu Ltd., Tokyo, Japan) was used for *in silico* molecular docking analyses. The three-dimensional structures of the ligand-binding domains of hERα, PDB ID 1ERE (1.9 Å), and 3ERT (3.1 Å), were downloaded from the Protein Data Bank database (http://www.rcsb.org/) and used as the initial starting template for molecular docking. Automated docking of the flexible ligand into the flexible active site was generally performed as previously described to validate the docking procedure, the original ligands (E_2 and 4-OHT) in the crystal structures were drawn, energy-optimized and docked into their binding sites, respectively. The root-mean-square-distances (RMSD) between pairs of equivalent atoms of the newly calculated and the original molecules were calculated.

2.3. Yeast two-hybrid assays and GeneBLAzer™ β-lactamase reporter gene assay

The yeast two-hybrid assay with the human estrogen receptor-alpha (hERα) were used to test the estrogenic and anti-estrogenic activities of chemicals, as previously described (Zhang et al., 2011; Lv et al., 2017). GeneBLAzer™ β-lactamase reporter gene assays were performed using the SelectScreen™ cell-based nuclear receptor profiling service provided by Thermo Fisher Scientific Corporation (Madison, WI, USA). The ERα-UAS-bla GripTite™ 293 cell line was used to evaluate the agonistic and antagonistic activities of BPAP. E_2 and 4-OHT were used as positive controls for evaluating the agonistic and antagonistic activities, respectively. All experiments were performed in triplicate. Sigmoidal dose-effect curves for the estrogen agonist and antagonist properties were calculated using Prism 6 software (GraphPad Inc., San Diego, CA, U.S.A.). The IC_{50} values were calculated based on these sigmoidal dose-effect curves.

2.4. 3-D immature mouse uterotrophic assays and 10-d immature mouse anti-uterotrophic assay

All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University and were performed in accordance with the Guidelines for Animal Experiments of the university, which meet the ethical guidelines for the use of experimental animals in China. Female CD-1 mice (age: 20 postnatal days; PND), were obtained from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). After an acclimatization period of 2 d, the mice were weighed, weight-ranked, and assigned randomly to treatment or control groups using completely randomized design. The estrogenic and anti-estrogenic activities tests were performed primarily as previously described (Zhang et al., 2017). In brief, BPAP was dissolved in peanut oil (vehicle) to prepare the dose of 250, 50, and 10 mg kg^{-1} bw d^{-1} for estrogenic activity test. Mice treated with peanut oil only were used as a negative control. To test the anti-estrogenic activity of BPAP, 10 and 50 mg kg^{-1} bw d^{-1} BPAP were administered together with 50 μg kg^{-1} bw d^{-1} dose of E_2. Mice treated only with 50 μg kg^{-1} bw d^{-1} E_2 were used as a negative control, and those treated with 10 μg kg^{-1} bw d^{-1} FULV together with 50 μg kg^{-1} bw d^{-1} dose of E_2 were used as a positive control. To study the anti-estrogenic activity of BPAP at lower doses for a longer exposure period, a 10-d anti-uterotrophic assay in immature mice was performed according to our previous method (Zhang et al., 2017). Doses of 400, 80, 16, 3.2 μg kg^{-1} bw d^{-1} BPAP were prepared in peanut oil and treated mice via oral gavage for 10 d beginning on PND 24. Mice treated with peanut oil only were used as a negative control. Each group of the assay was consisted 8 mice. At 24 h after the final treatment, the mice were weighed and sacrificed, and the relative uterine weight was determined.

2.5. Next-generation sequencing-based transcriptome analysis and real time quantitative reverse transcriptase PCR (Q-RT-PCR)

Uteri of mice in the control, 50 mg kg^{-1} bw d^{-1} BPAP, 50 μg kg^{-1} bw d^{-1} E_2, and combined 50 mg kg^{-1} bw d^{-1} BPAP and 50 μg kg^{-1} bw d^{-1} E_2 groups of the 3-d immature mouse uterotrophic assays were selected for a transcriptome analysis. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from the uteri per the manufacturer's protocol. DNase I (TaKaRa Biotechnology, Dalian, China) was used to digest DNA and thus prevent genomic DNA contamination, and the total RNA was subsequently purified and recovered. 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. For each group, a mixture of equal amounts of mRNA from three samples was subjected to high-throughput sequencing at the Biodynamic Optical Imaging Center of Peking University according to the Illumina transcriptome sequencing method (Illumina, San Diego, CA, U.S.A.). RNA-Seq analyses were performed using CLC Genomics Workbench (version 9.5.2) with reference gene model annotation (Mus_musculus.GRCm.38.gtf). Reads per kilobase of exon model per million mapped reads (RPKM) values were

determined as the relative transcript levels. Baggerley's test (Baggerly et al., 2003) 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 absolute fold change >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. Finally, Q-RT-PCR with SYBR green detection was performed with an Agilent Mx3005P to validate the gene expressions. Twelve genes that were enriched in biological process of "response to estrogen" were selected for the validation. Primers were designed by Primer Express Software v3.0 (Applied Biosystems) and listed in Table 1.

2.6. Glucose tolerance test in mice

To study the effects of BPAP at low doses relevant to human exposure, a glucose tolerance test using male mice was perform for BPAP at doses of 1 and 100 μg kg^{-1} bw d^{-1} primarily as previously described (Alonso-Magdalena et al., 2006). Male CD-1 mice (6 weeks old) were obtained from Experimental Animal Tech Co. of Weitonglihua (Beijing, China) and acclimatized in the experimental environment for 2-week with a temperature of 22 °C ± 2 °C, relative humidity between 40% and 60%, and artificial lighting in a 12 h/12 h light-dark cycle. Peanut oil were used as solvent control. Each group of the assay was consisted 10 mice using completely randomized design. Intraperitoneal administration was performed for each of the mice. From 8 h after the intraperitoneal administration, the mice were fasted overnight for 16 h with *ad libitum* drinking of water, and then fasting blood glucose levels were measured by sampling blood from the tail vein. Soon afterwards, animals were injected intraperitoneally with 2 g kg^{-1} bw of glucose, and blood samples were obtained from the tail vein. Blood glucose was measured in each sample immediately after sampling using an Accu-Check Performa glucometer (Roche Diagnostics, Indianapolis, IN, USA).

2.7. Data analysis

Data analyses were performed using the statistical program SPSS (v.18.0; Chicago, IL, U.S.A.). Data are presented as means and standard deviations unless otherwise indicated. Differences between groups were assessed using a one-way analysis of variance and Fisher's least significant difference tests. P values of <0.05 were considered statistically significant.

3. Results

3.1. The binding predictions for BPAP toward estrogen receptor α

Molecular docking was developed to understand the structural basis for the observed estrogenic and anti-estrogenic properties of BPAP. As is shown in Fig. 1A and B, both E_2 and 4-OHT were well docked back into their binding sites with the RMSD values of 0.175 Å for E_2 in agonist pocket (PDB ID 1ERE) and 0.545 Å for 4-OHT in antagonist pocket (PDB ID 3ERT). The low RMSD values indicated the reliability of this *in silico* method. By this method, we found that BPAP could fit into both agonist pocket (1ERE) and antagonist pocket (3ERT) of estrogen receptor α, with PMF values of -48.64 kcal mol^{-1} for binding with agonist pocket and -56.77 kcal mol^{-1} for binding with antagonist pocket (Fig. 1C–F). The important features of this interaction include the formation of hydrogen bonds between a phenolic hydroxyl group of BPAP with the carboxylate of Glu-353 and guanidinium group of Arg-394 in the pocket, as well as the van der Waals forces between the phenyl rings and alkyl group of BPAP and the core hydrophobic moiety provided by Phe404, Leu349, Ala350, Trp383, Leu384, Leu387, Met388, Leu391, Val418, Leu428, Met517, Leu525, Met342, Met343, Met421, Ile424, and Leu346.

3.2. Estrogenic and anti-estrogenic activities of BPAP in vitro

The estrogenic and anti-estrogenic activities of BPAP were evaluated using a yeast two-hybrid assay and GeneBLAzer™ β-lactamase reporter gene assay. As shown in Fig. 2A and C, the slight increases in β-galactosidase activities in both assays suggests that BPAP may exert very weak estrogenic activity. However, when combined with 1×10^{-9} M E_2, BPAP obviously inhibited the β-galactosidase activities induced by E_2 in concentration-dependent manners in both assays (Fig. 2 B and D). The observed IC_{50} of BPAP in the yeast two-hybrid assay was 32.15 μM, or 2.37-fold higher than that of TAM (13.56 μM). In the GeneBLAzer™ β-lactamase reporter gene assay, the IC_{50} of BPAP was 2.35 μM, or 656-fold higher than that of 4-OHT (3.58 nM). TAM is a well-known selective estrogen-receptor modulator with strong anti-estrogenic activity, and 4-OHT is a metabolite of TAM with an ER affinity 30–100 times stronger than that of TAM itself (Ahmad et al., 2010). The finding that the anti-estrogenic activity of BPAP was similar to that of TAM suggests that BPAP should be considered a strong anti-estrogen.

3.3. Anti- uterotrophic effect of BPAP in mice

The *in vivo* estrogenic and anti-estrogenic activities of BPAP were evaluated in immature CD-1 mice using a 3-d uterotrophic

Table 1
Primers of selected genes for quantitative real-time PCR.

Gene symbol	Accession number	Sequence of forward primer (5′ to 3′)	Sequence of reverse primer (5′ to 3′)
Grn	NM_008175	ACACCACGGATCTCCTGACCAA	TGTTGAGGCGGCAGCAGGTATA
Aldh1a2	NM_009022	AGGCAAAGGGCTGGGAAGGAA	TGGCAATCCGCATGTCATCAGT
Sprr2b	NM_011469	GTGTCCACCCAAGAATAAATGAG	AGGACAGGCGTTCAAAGGAG
Esr1	NM_007956	TGCTCCTAACTTGCTCCTGGACAG	TTCATCATGCGGAACCGACTTGAC
Sprr2a	NM_001164787	GGTCACTGCTGTTTCATTTCCT	ATTAGACCATCACCAAAGGGG
Sprr2g	NR_003548	CTAGTAGATGTCCCTCAGTGCCTT	AGCAAATGGAACATCCGTGA
Msx2	NM_013601	CACGCACACCCTTCACCACATC	CCGCTCTGCTATGGACAGGTACT
Hnrnpd	NM_001077265	AAGGAAGAGGAGCCAGTGAAGAAGA	TGCTGCTGCTGCTGATACTGTTC
Oxtr	NM_001081147	CGCTCGCCGTCTACATTGTACC	GCCGTCTTGAGTCGCAGATTCT
Ccna2	NM_009828	AGAGGCAGCCAGACATCACTAACA	ACCTCAACCAGCCAGTCCACAA
Ptgs2	NM_011198	TCTGGTGCCTGGTCTGATGATGTAT	GGATGCTCCTGCTTGAGTATGTCG
Actb	NM_007393	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC
Gapdh	NM_008084	TGCCCCCATGTTTGTGATG	TGTGGTCATGAGCCCTTCC

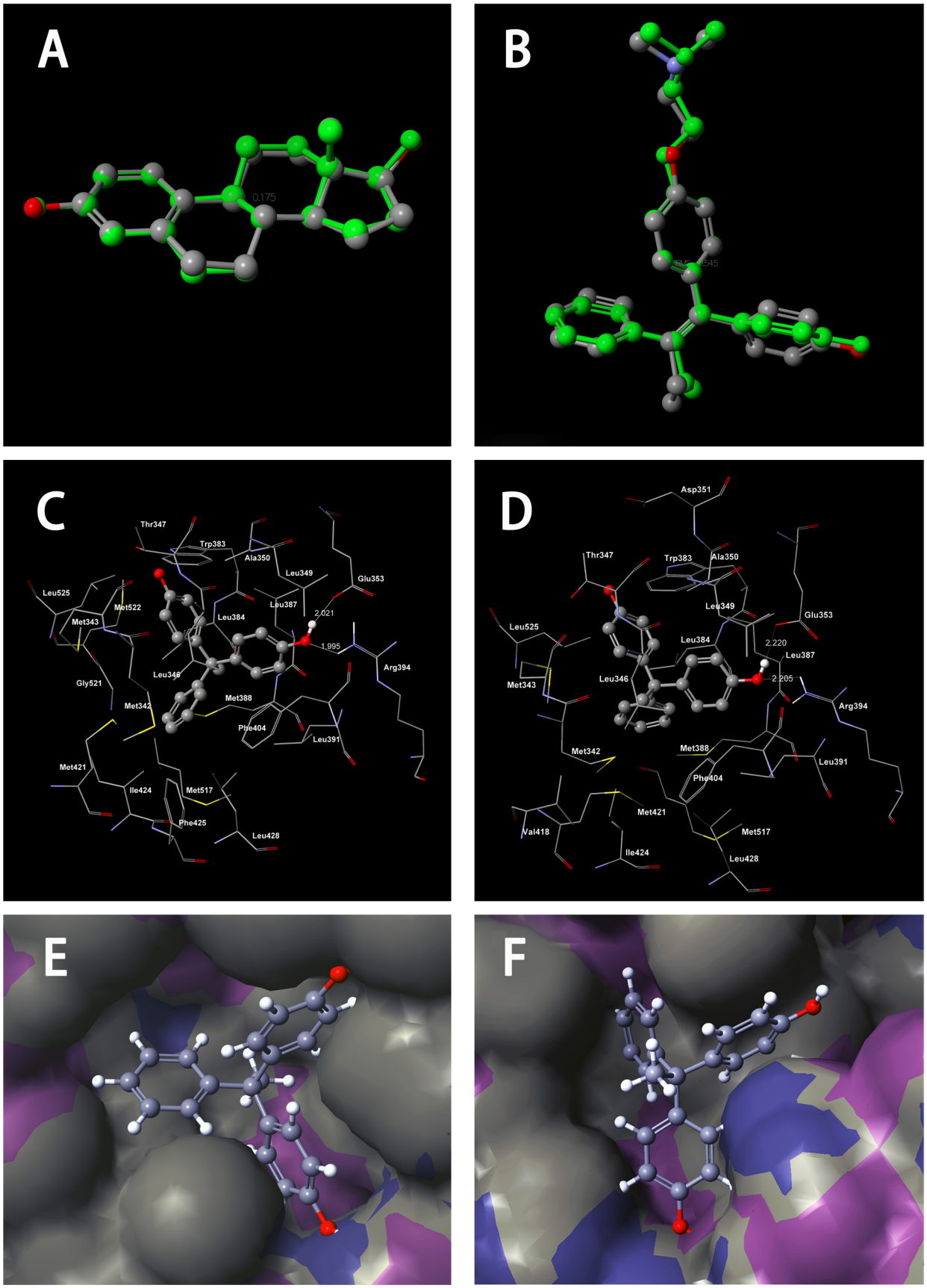

Fig. 1. *In silico* molecular docking analysis of the estrogenicity and anti-estrogenicity of bisphenol AP (BPAP). (A) Validation of the docking of 1ERE with E_2 (the docked ligand shows in green); (B) Validation of the docking of 3ERT with 4-OHT (the docked ligand shows in green); (C) Agonist interaction between BPAP and hERα active site (1ERE); (D) Antagonist interaction between BPAP and hERα active site (3ERT); (E) Simulated binding position of BPAP in the agonist pocket of hERα (1ERE); (F) Simulated binding position of BPAP in the antagonist pocket of hERα (3ERT). Dotted lines indicate hydrogen bonds between BPAP and receptor amino acid residues.

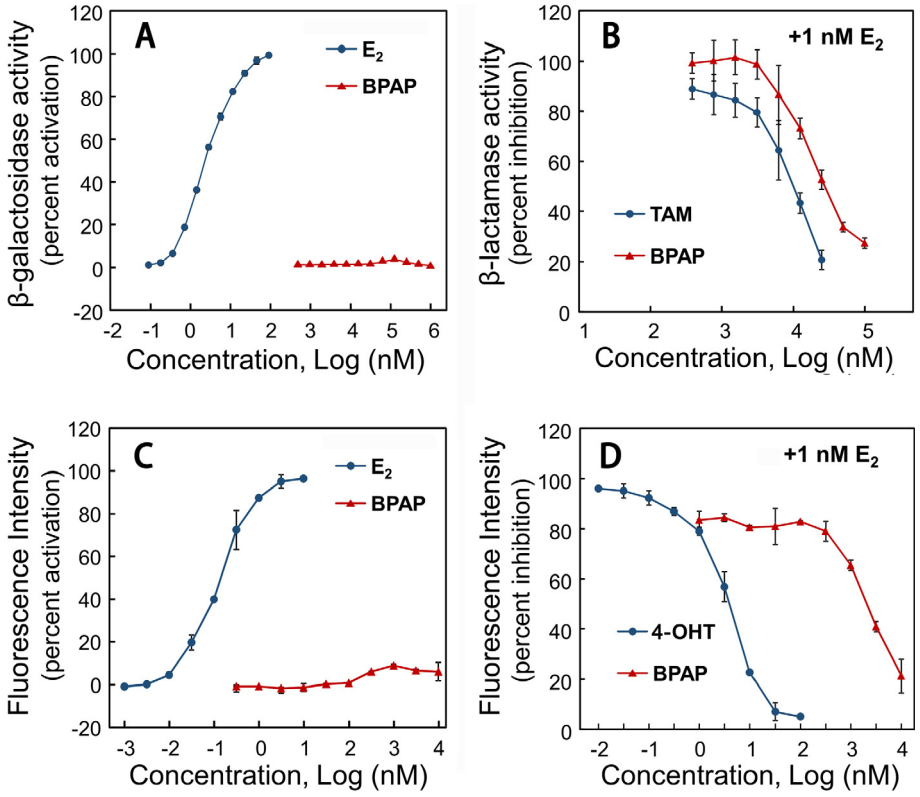

Fig. 2. Estrogenic and anti-estrogenic activities of bisphenol AP (BPAP) *in vitro*. (A) Estrogenic and (B) anti-estrogenic activities determined using yeast two-hybrid assays. (C) Estrogenic and (D) anti-estrogenic activities determined using GeneBLAzer™ β-lactamase reporter gene assays.

assay. No mortality was observed before final sampling in the experiment. As shown in Fig. 3A, treatment with BPAP alone at doses of 10, 50, and 250 mg kg^{-1} bw d^{-1} led to significant decreases in the wet uterine weights to 72.89%, 69.43%, and 55.70% of the control, respectively ($P < 0.05$), suggesting that BPAP exerts anti-estrogenic, rather than estrogenic effects *in vivo*. Combined treatment with E_2 further demonstrated the *in vivo* anti-estrogenic effects of BPAP. As shown in Fig. 3B, when administered together with 50 μg kg^{-1} bw d^{-1} E_2, BPAP significantly inhibited the uterotrophic effect of E_2 to yield wet uterine weights of only 60.57% and 62.40% in mice treated with 10 and 50 mg kg^{-1} bw d^{-1} BPAP relative to those treated with E_2 alone. These decreases were similar to those observed in mice receiving combined treatment with FULV and E_2, and further support the strong anti-estrogenic effect of BPAP *in vivo*.

Considering that endogenous estrogens are increasingly produced to meet the need of reproductive system development in female mice when they reach puberty, a 10-d anti-uterotrophic assay had been developed in our previous study to evaluate the anti-estrogenic activity of chemical using female CD-1 mice at a beginning age of 24 PND. Using the assay, we found that the BPAP could significantly decrease the relative uterine weight in mice given doses of 80 and 400 μg kg^{-1} bw d^{-1}, of which the relative uterine weights were only 59.29% and 56.30% that of control, respectively (Fig. 3C).

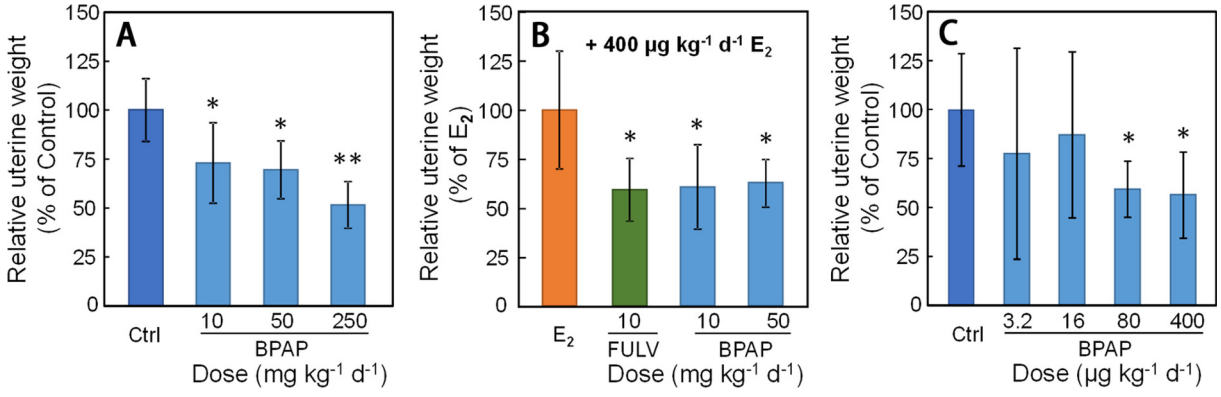

Fig. 3. Anti-estrogenic effects of bisphenol AP (BPAP) in immature CD-1 mice. (A) Anti-uterotrophic effects observed in mice treated with BPAP alone for 3 d (n = 8); (B) Anti-uterotrophic effects observed in mice treated with BPAP or FULV in combination with 50 μg kg^{-1} bw d^{-1} E_2 for 3 d (n = 8); (C) Anti-uterotrophic effects observed in mice treated with BPAP alone for 10 d (n = 8). Data are expressed as percentages of the control, and error bars indicate standard deviations from the average values. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.4. Toxicogenomic analysis of the anti-estrogenic effects of BPAP on immature mice

Next-generation sequencing (NGS) was used to analyze global gene expression profiles in the uteri of immature mice treated with 50 μg kg^{-1} bw d^{-1} of E_2, 50 mg kg^{-1} bw d^{-1} of BPAP, and 50 mg kg^{-1} bw d^{-1} of BPAP in combination with 50 μg kg^{-1} bw d^{-1} E_2 for 3 d relative to those of control mice. In mice treated with 50 μg kg^{-1} bw d^{-1} E_2, 2629 Ensembl transcript IDs exhibited significantly modified expression ($P < 0.05$, at least two-fold relative to control), with upregulation and downregulation accounting for 78.5% and 21.5%, respectively. A GO analysis showed that 1513 transcript IDs were enriched in 442 biological process categories, including cell-cell adhesion, cellular response to interferon-beta, RNA splicing, immune system process, response to estradiol, and response to estrogen. Pathway enrichment analyses showed that 620 transcript IDs were enriched in 107 pathways, including ribosome, proteoglycans in cancer, pathways in cancer, small cell lung cancer, osteoclast differentiation, and estrogen signaling pathway.

In mice treated with 50 mg kg^{-1} bw d^{-1} BPAP, 1202 Ensembl transcript IDs exhibited significantly modified expression ($P < 0.05$, at least two-fold relative to control), with upregulation and downregulation accounting for 67.5% and 33.7%, respectively. A GO analysis showed that 622 transcript IDs were enriched in 199 biological process categories, including cell-cell adhesion, cellular response to interferon-beta, RNA splicing, response to estrogen, and response to estradiol. Pathway enrichment analyses showed that 345 modified transcript IDs were enriched in 72 pathways, including ribosome, proteoglycans in cancer, small cell lung cancer, osteoclast differentiation, and estrogen signaling pathway. In other

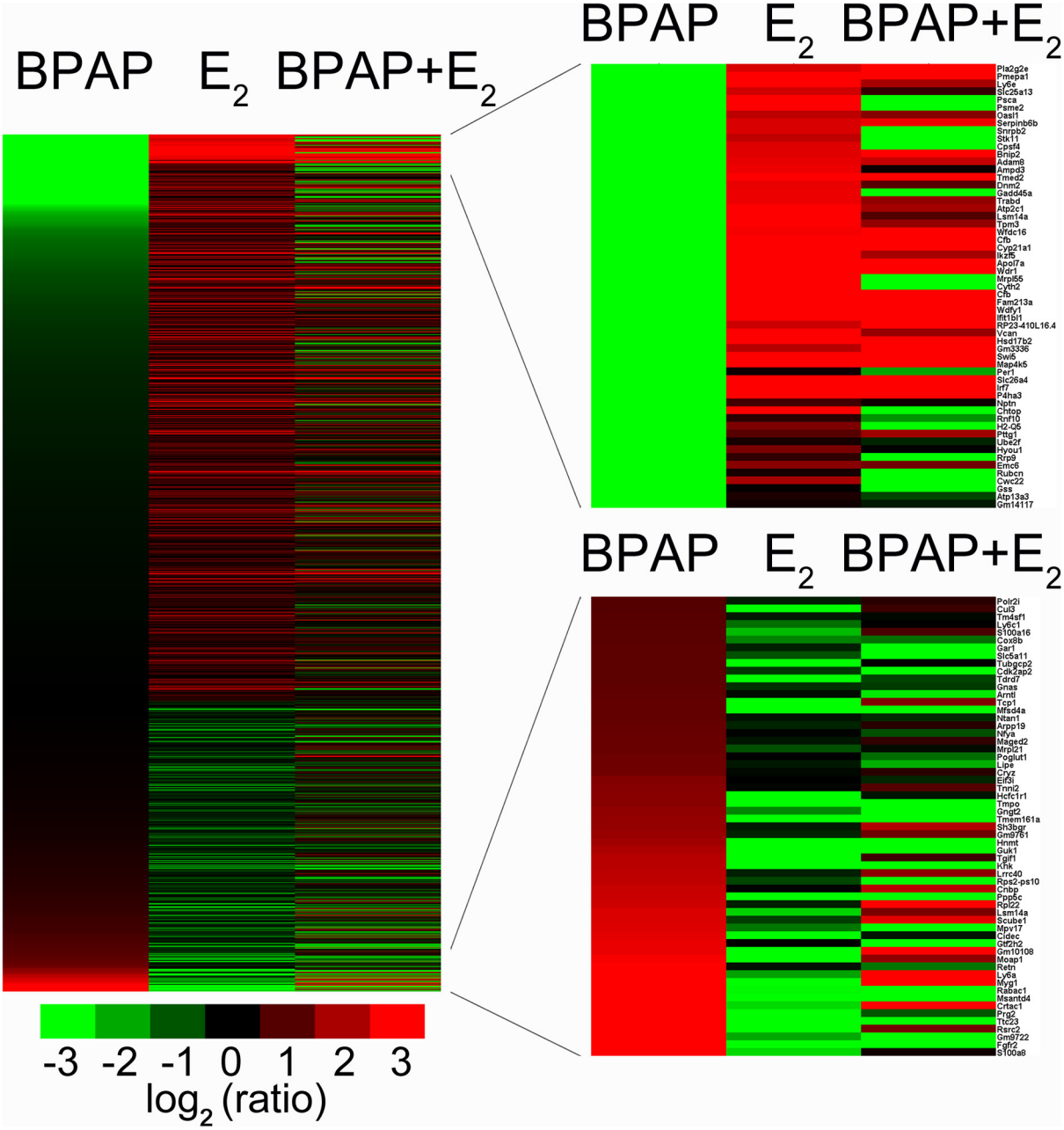

Fig. 4. Opposite patterns of gene regulation mediated by bisphenol AP (BPAP) and E_2 in mouse uteri. A total of 1275 genes that were significantly regulated by treatment with 50 mg kg^{-1} bw d^{-1} of BPAP, 50 μg kg^{-1} bw d^{-1} of E_2, or a combination thereof for 3 d are depicted using hierarchical clustering.

Table 2
Selected gene expression changes validated by quantitative real-time RT-PCR in uterus of mice. Gapdh was used as endogenous control and relative expression was evaluated by the $2^{-\Delta\Delta CT}$ method.

Gene symbol	50 μg kg^{-1} bw d^{-1} E_2		50 mg kg^{-1} bw d^{-1} BPAP		50 mg kg^{-1} bw d^{-1} BPAP combined with 50 μg kg^{-1} bw d^{-1} E_2	
	Q-RT-PCR	NGS	Q-RT-PCR	NGS	Q-RT-PCR	NGS
Grn	3.12	2.17	−3.03	−4.89	−4.58	−2.45
Aldh1a2	3.69	2.32	−2.17	−1.74	4.03	2.72
Sprr2a	43.21	21.03	3.60	−6.61	10.63	13.41
Sprr2g	32.35	30.99	−3.38	−1. 57	79.62	138.6
Esr1	3.89	5.13	−1.97	−3.85	−1.04	1.85
Sprr2b	10.36	6.02	−2.79	−4.03	9.12	13.60
Msx2	1.97	1.53	−2.17	−2.03	−1.10	−2.69
Hnrnpd	−6.25	−2.31	2.85	1.65	−2.39	3.03
Oxtr	1.98	3.94	−1.54	−2.14	1.25	2.18
Ccna2	6.32	2.30	1.47	−1.57	5.87	6.14
Ptgs2	11.36	10.81	−2.93	−2.54	14.88	14.07
Actb	1.21	1.18	1.07	−1.06	1.16	1.13

Q-RT-PCR, Quantitative real-time RT-PCR.
NGS, Next Generation Sequencing.

words, the results were similar to those observed in mice treated with 50 μg kg^{-1} bw d^{-1} E_2. As shown in Fig. 4, 1275 genes that were significantly upregulated or downregulated in at least one treatment group were oppositely regulated by BPAP and E_2, and many appeared to have been neutralized in the combination group. These results further suggest that BPAP possesses anti-estrogenic activity *in vivo*. The gene expression changes observed by NGS are validated by Q-RT-PCR (Table 2).

3.5. Anti-estrogenic effects of BPAP on blood glucose level in mice at doses relevant to human exposure

BPA and E_2 are reported to disrupt blood glucose homeostasis and alter glucose tolerance at very low doses because of their estrogenicities (Alonso-Magdalena et al., 2006; Nadal et al., 2009). In this paper, to study the effect of BPAP at low doses relevant to human exposure, a glucose tolerance test using male mice was perform for BPAP at doses of 1 and 100 μg kg^{-1} bw. No mortality was observed in treatment groups, and the mean body weight of mice treated with BPAP did not differ from that of controls. As shown in Fig. 5, blood glucose was decreased to a lower levels in BPAP −treated mice in comparison with that of vehicle-treated after a single treatment. The administration of 1 μg kg^{-1} bw BPAP provoked a more significant effect on blood glucose than that of 100 μg kg^{-1} bw. At 15-min after intraperitoneal injection with 2 g kg^{-1} bw of glucose, blood glucose in control group rose to 20.82 ± 2.21 mM, while they were only 17.13 ± 2.71 mM and 18.49 ± 2.44 mM in the 1 and 100 μg kg^{-1} bw BPAP groups, respectively.

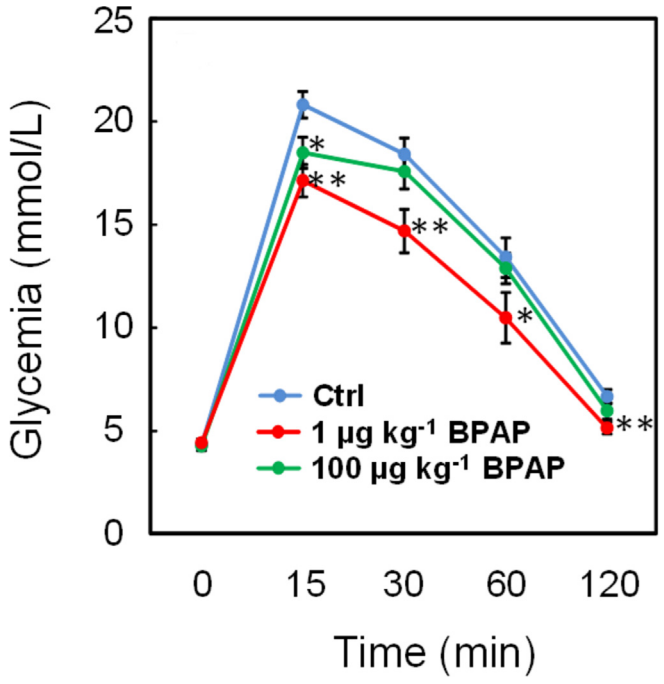

Fig. 5. Disrupted blood glucose homeostasis in 6-week old male CD-1 mice treated with BPAP for one day. Data are expressed as percentages of the control, and error bars indicate standard deviations from the average values (n = 10). *$P < 0.05$; **$P < 0.01$.

4. Discussion

Studies in previous decades have focused on the estrogenic properties of chemicals, leading to the designation of many man-made estrogenic substances as environmental estrogens. By contrast, few environmental anti-estrogens have been reported. Recently, however, several researchers have begun to investigate the anti-estrogenic properties of bisphenols. For example, Delfosse et al. (2012) studied the binding mechanisms of BPA, bisphenol AF, and bisphenol C (BPC) to estrogen receptors and demonstrated that these chemicals could act as partial agonists of ERs, with activities ranging from weak agonism (BPA) to antagonism (BPC). Furthermore, Delfosse and colleagues found that BPC displayed almost full antagonistic activity in the presence of E_2 and observed an antagonist conformation of BPC in the ERα, similar to that of the 4-OHT−binding structure (3ERT) (Delfosse et al., 2012). Zhuang et al. (2014) also indicated that structural variations in BPA analogs result in different interactions with ERα LBD and might cause distinct agonistic/antagonistic toxic effects. BPAP have been described as an estrogenic chemical with a lower estrogenic potency than that of BPA (Coleman et al., 2017). In this study, although the very low estrogenic activity of BPAP was confirmed, we observed a very high level of anti-estrogenic activity, with an IC_{50} of 2.35 μM in the GeneBLAzer™ β-lactamase reporter gene assay. Furthermore, the results of the immature mouse uterotrophic/anti-uterotrophic assays, as well as the toxicogenomic analysis, showed that BPAP had anti-estrogenic effect. Stossi et al. (2014) used a MCF-7 cell proliferation assay to study the proliferative effects and potencies of BPA analogs, and found that whereas most analogs could induce cell proliferation in a manner similar to E_2, BPAP appeared to lack the ability to enhance MCF-7 cell proliferation. Taken together, these *in vitro* and *in vivo* data demonstrated the anti-estrogenicity of BPAP.

Because estrogens play a fundamental role in the physiology of the reproductive, cardiovascular, skeletal, and central nervous systems (Mauvais-Jarvis et al., 2013), anti-estrogenicity of environmental chemicals might be considered an adverse property. Anti-estrogenic drugs, such as TAM and U39411, have been reported to prevent pregnancy, reduce litter size and weight, and induce embryonic absorption in animals (Kaplan-Kraicer et al., 1996). Our recent study found that ant-estrogenic chemicals, BHPF and TAM, could cause atrophic endometria and adverse pregnancy outcomes such as stillbirth, embryonic absorption, and low birth weights in mice (Zhang et al., 2017). So, the anti-estrogenicity of BPAP may cause similar adverse effects as those of BHPF and TAM. As shown in the results of animal experiments performed in this study, BPAP obviously slowed down the uterus development at very low doses.

Zhang et al. (2016) reported that BPAP was detectable in some urine samples collected from Chinese people, with a maximal concentration of 0.703 ng ml^{-1}. To study the effect of BPAP at environmentally relevant dose, we performed a glucose tolerance test. We found BPAP could significantly disrupt blood glucose homeostasis in mice after a single treatment at dose of 1 and 100 μg kg^{-1} bw (Fig. 5); it decreased the blood glucose levels after the intraperitoneal administration with 2 g kg^{-1} bw of glucose in comparison with control. Under the same conditions, BPA and E2 were reported to raise blood glucose levels at doses of 1 μg kg^{-1} bw d^{-1} and higher because of their estrogenic activities (Alonso-Magdalena et al., 2006; Nadal et al., 2009). Hypoglycemia in the pediatric population may cause brain injury. So the possible effects of BPAP should be of concern in the future. Considering that the endogenous estrogen changes during the estrus cycle might affect the blood glucose levels, 8-week old male mice were used for the test.

5. Conclusions

Overall, this study demonstrated the anti-estrogenic properties of BPAP by *in vitro*, *in vivo* and *in silico* tools. BPAP significantly decreased the uterine weights of post-weaning CD-1 mice at doses of 10 mg kg^{-1} bw d^{-1} and higher for 3 d and those of prepubertal mice at doses of 80 μg kg^{-1} bw d^{-1} and higher for 10 d, indicating that BPAP could delay the uterus development at very low doses. At doses relevant to human exposure (1 and 100 μg kg^{-1} bw d^{-1}), BPAP could disrupt the blood glucose homeostasis in male mice and showed an opposite tread of regulation than that of E_2 and BPA.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (21777003, 41330637, and 21377007), the International S&T Cooperation Program of China (2016YFE0117800), the National Key Technology R&D Program of China (2017YFF0211202), and the undergraduate student research training program of the Ministry of Education.

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