Estrogen Disrupting Effect and Ecological Risk Assessment of PHCZs By Multi-model

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Introduction

Polyhalogenated carbazoles (PHCZs), as a novel type of environmental organic contaminations, have attracted more and more attention for its frequent detection and structural similarity with dioxin[1]. Despite its complex natural and anthropogenic sources[2], studies on the potential health and ecological risks of PHCZs are still scarce.

Materials and Methods

In this study, dual-luciferase reporter gene assayS via estrogen reporter (ER), H295R steroidogenesis assay, uterotrophic assay, and molecular docking were combined to evaluate the estrogenic effects and possible mechanisms of three PHCZs. Then, the developmental toxicity of 2,7-dibromocarbazole (2,7-DBCZ) was examined in zebrafish and the teratogenic effects of 2,7-DBCZ was evaluated in transgenic zebrafish larvae. In addition, RNA-sequencing (RNA-Seq) with bioinformatic analyses including GO function and KEGG pathway enrichment analysis was performed to investigate the functional pathways of the differentially expressed genes, and to explore the potential mechanisms of toxicity.

Results

Results of reporter gene showed that 2,7-DBCZ, 3-bromocarbazole (3-BCZ), and 2-bromocarbazole (2-BCZ) exhibited ER agonistic activities with the REC20 values of 6.17×10^{-7} M, 1.44×10^{-6} M, and 1.47×10^{-6} M, respectively. The H295R steroidogenesis assay showed that three PHCZs up-regulated the expression of *3βHSD2*, *17βHSD*, *CYP17*, and *CYP19*, and slightly induced the synthesis of E2. The in vivo experiment results showed 2,7-DBCZ and 3-BCZ caused significant increase in relative uterine weight, epithelial cell height, uterine gland, and serum estradiol level. Molecule docking showed that hydrogen bond, the position and the number of bromine substitutions, and hydrophobic interaction are very important to the estrogenic activities of PHCZs. The 96-h LC_{50} of 2,7-DBCZ was 1.79 μM and the 96-h EC_{50} for the occurrence of concentration-dependent pericardial edema was 0.62 μM. Transcriptomic analyses revealed that 90 genes were differently expressed with 51 genes up-regulated and 39 genes down-regulated in zebrafish. Pathway enrichment analysis indicated aryl hydrocarbon receptor (AhR) activation and downstream modification of retinoid or heme homeostasis may play a role in the developmental toxicity of 2,7-DBCZ.

Table 1. Uterine index of immature female rats administered by three PHCZs and E_2 for 7 days.

Chemicals	Oil	2,7-BCZ	3-BCZ	2-BCZ	E_2
Dose	Control	100 mg/kg/day	100 mg/kg/day	100 mg/kg/day	100 μg /kg/day
Relative uterine weight (% of body weight)	0.053±0.080	0.064±0.002	0.064±0.003	0.060±0.006	0.167±0.021
Wet uterine weight (g)	0.043±0.007	0.048±0.001	0.051±0.003	0.048±0.007	0.137±0.002
Serum estradiol (pg/mL)	106.86±12.599	167.535±23.513	147.040±20.998	126.822±16.837	191.473±24.133
Epithelium cell height (μm)	10.09±1.15	15.77±1.89	14.77±3.37	12.55±0.74	36.22±4.47

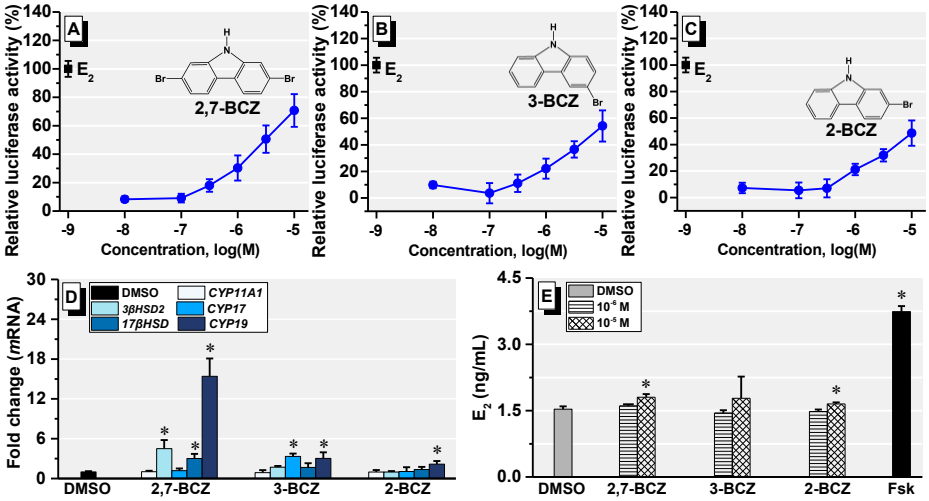

Figure 1. A-C: The agonistic activities of test chemicals in ERα mediated reporter gene assays. CHO cells transfected with *rERα/pCI*, *pERE-AUG-Luc*, and *phRL-tk*, and then the cells were incubated with different concentrations of test chemicals (0.01 μM to 10 μM). One nM E_2 was used as a positive control for the interaction with ERα. The luciferase activity was set at 100%. **D**: The five steroidogenic genes mRNA expression levels treated by 2,7-BCZ, 3-BCZ, and 2-BCZ in H295R cells. **E**: Effects of production of E_2 in H295R cells treated by test chemicals. Data are shown as mean ± SD of at least 3 independent experiments. *Statistically significantly different from DMSO (0.1%) ($p < 0.05$).

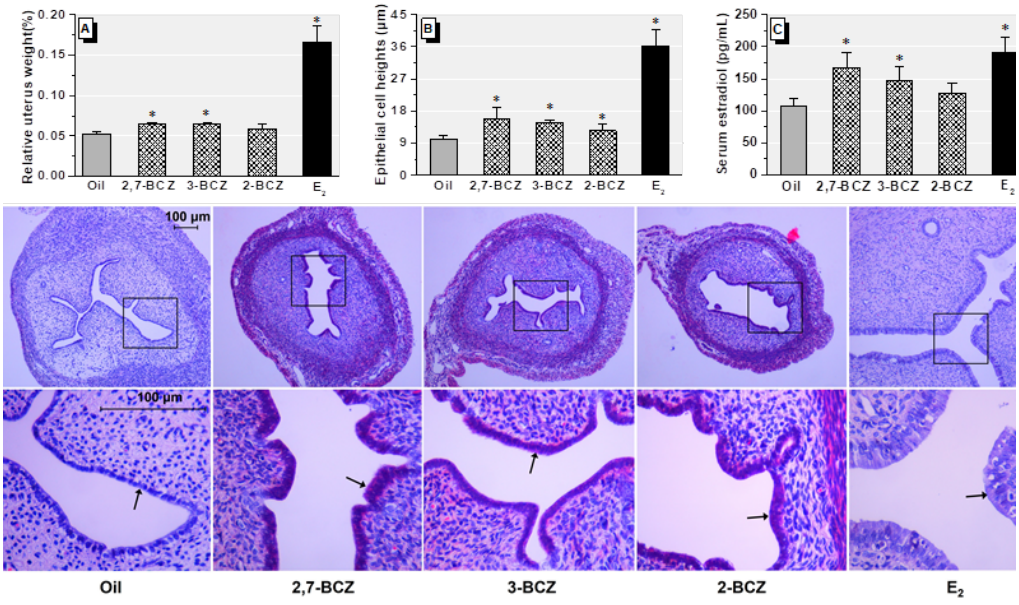

Figure 2. A: The relative uterine weights (% of body weight) increased by 2,7-BCZ, 3-BCZ, and 2-BCZ, respectively. **B**: Uterine epithelium cell heights increased after the treatment of 2,7-BCZ, 3-BCZ and 2-BCZ, respectively. **C**: Serum estradiol level increased after the treatment of 2,7-BCZ, 3-BCZ, 2-BCZ and E_2, respectively. **D**: HE staining showing transverse sections of uterus (original magnification 100×, bar =100 μm, black box: **Figure 2D** was shrunk by 4 times). **E**: Black box areas of **Figure 2C** are enlarged to show the uterine luminal epithelium in uterine sections (original magnification 400×, bar =100 μm, black arrow: luminal epithelium). *Statistically significantly different from oil control ($p< 0.05$).

Table 2. Acute toxicity of 2,7-DBCZ, 3,6-DBCZ, and 3,6-DCCZ to *Danio rerio* embryos.

Group	Mortality (%)
Blank control	5.56 ± 2.41
Solvent control	6.94 ± 2.41
2,7-DBCZ	93.06 ± 6.36
3,6-DBCZ	5.56 ± 2.41
3,6-DCCZ	4.17 ± 4.17

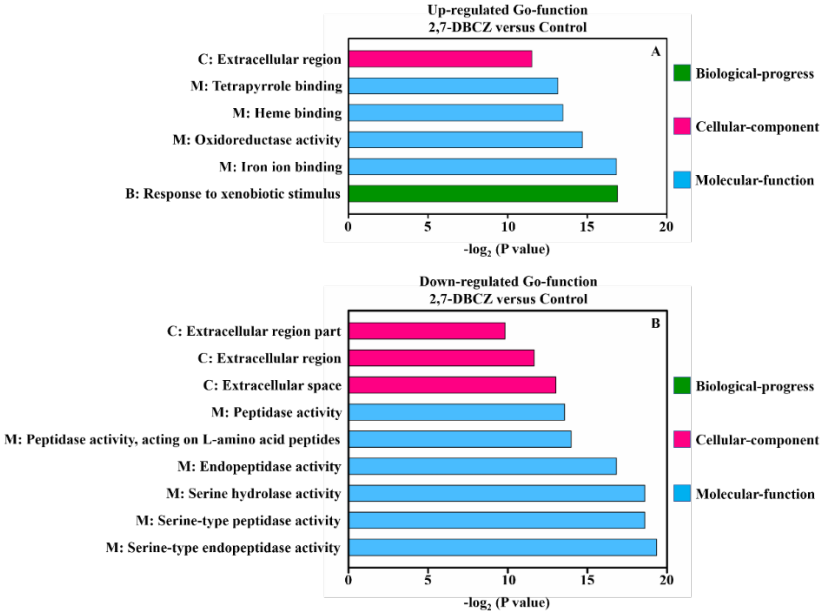

Figure 3. Significantly enriched GO terms of differentially expressed genes. The y-axis represents the significantly enriched GO terms. The x-axis denotes the number of genes in a category. **A**: Significantly enriched GO terms of up-regulated genes of 2,7-DBCZ state versus control state. **B**: Significantly enriched GO terms of down-regulated genes of 2,7-DBCZ state versus control state.

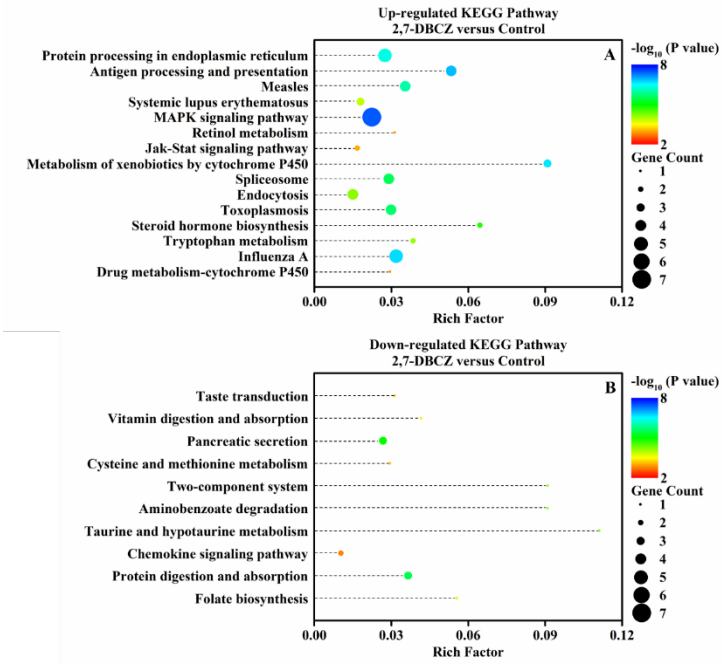

Figure 4. KEGG pathway enrichment of differentially expressed genes. The y-axis represents the significantly enriched KEGG pathway. The x-axis denotes the rich factor of differentially expressed genes. The color of the dot represents the -log10 (P value) and the size of the dot represents the gene count. **A**: KEGG enrichment analysis performed on the up-regulated DEGs of 2,7-DBCZ state versus control state. **B**: KEGG enrichment analysis performed on the down-regulated DEGs of 2,7-DBCZ state versus control state.

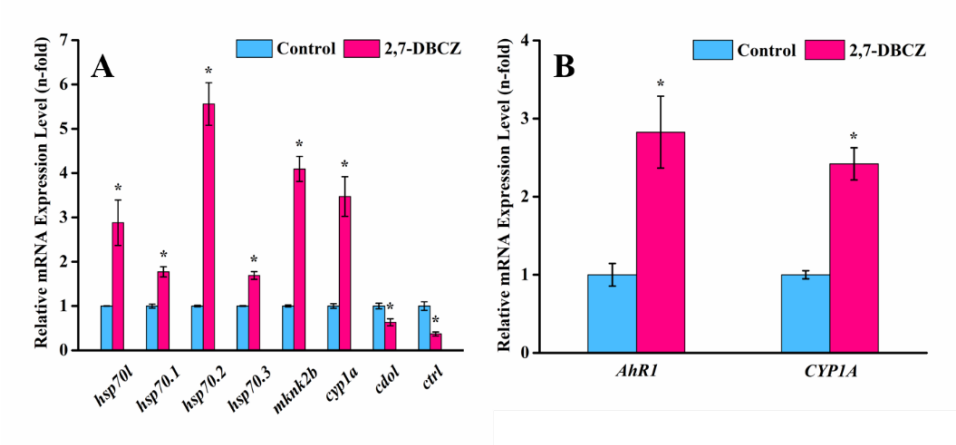

Figure 5. Relative mRNA expression level of related genes. **A**: Relative mRNA level of the selected eight genes with different expression profiles from the results of RNA-seq. Results are expressed as the fold relative to the value of control group. Each value represents the mean ± SD (n=three samples, *p<0.05). **B**: Relative mRNA expression level of genes related to AhR activation. Results are expressed as the fold relative to the value of control group. Each value represents the mean ± SD (n=three samples, *p<0.05).

Acknowledgements:

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References:

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2. Guo, J.; Li, Z.; Ranasinghe, P.; Bonina, S.; Hosseini, S.; Corcoran, M. B.; Smalley, C.; Rockne, K. J.; Sturchio, N. C.; Giesy, J. P., Spatial and Temporal Trends of Polyhalogenated Carbazoles in Sediments of Upper Great Lakes: Insights into Their Origin. *Environ. Sci. Technol.* 2016, 51, (1), 89-97.