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Exposure to phthalates impaired neurodevelopment through estrogenic effects and induced DNA damage in neurons

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ABSTRACT

Phthalates are commonly used in plastic products in daily life. The endocrine-disrupting effects of phthalates have been widely reported. Accumulating evidence from human cohorts and lab animals indicate exposure to phthalates might impair neurodevelopment. However, the direct causal relationship and mechanism between phthalates with neurodevelopment and neurotoxicity have not been firmly established. We found that phthalates (*i.e.* DBP, DINP, BBP) disrupted the expression of estrogen receptors (*esr1*, *esr2a*, *esr2b*), and impaired neurogenesis in the brain of zebrafish during embryonic development. Moreover, the abnormal expression of estrogen receptors, especially *esr2a*, was partly rescued in zebrafish which exposed to phthalates, with the estrogen receptor antagonist tamoxifen. Hence, impaired neurogenesis of zebrafish exposed to phthalates was partly reversed by tamoxifen treatment. Moreover, our results show that induced pluripotent stem cells (iPSC)-derived human neurons exposed to phthalates triggered double-strand DNA breaks *in vitro*. Overall, this study demonstrates that exposure to phthalates affects neurodevelopment in zebrafish embryos and induces neurotoxicity in human neurons partly through disrupting the expression of estrogen receptors.

1. Introduction

Phthalates are a family of synthetic chemical esters of phthalic acid and first introduced in the 1920s as plasticizers to increase the flexibility, pliability and elasticity of plastics, food packages, fragranced products, polyvinyl chloride (PVC) building materials, personal care products, cosmetics, and pharmaceuticals (Ejaredar et al., 2015). It is by the weaker intermolecular forces, but not the covalent bond of phthalates binding plastic polymers, thereby phthalates in plastics products are continuously released into the environment, atmosphere, food, even into the human body from medical devices (Chen et al., 2011). It has been reported that the concentration of phthalates (*i.e.* DBP, DINP, BBP) in water can be upto ~34.4 nM (Guo et al., 2013), ~1.25 nM (Li et al., 2017), ~1.6 nM (Abtahi et al., 2019), respectively, in some area of the world. More than 4.99 million tons of phthalates are produced worldwide and ~6 million tons of them are detected in the environment every year (Chen et al., 2014; Ejaredar et al., 2015). Thus, due to widespread and heavy use of phthalates in daily consumables, humans are exposed ubiquitously to phthalate-contaminated environments mainly through diet, air inhalation, and direct dermal absorption *via* contact with plastics products (Dewalque et al., 2014; Hauser et al., 2004). Additionally, the degradation of some phthalates is not easy and they can accumulate in human tissues (Chatterjee and Karlovsky, 2010). It is widely reported that phthalates can be detected in the hair (Jones et al., 2018), urine (Qian et al., 2019; Swan et al., 2010), and blood (Minatoya et al., 2018) in humans. Moreover, phthalates can cross the blood placenta barrier, exposing the fetus to phthalates during early development (Latini et al., 2003).

It has been reported that many phthalates are endocrine disruptors which may interfere with the secretion and metabolism of natural hormones hence, affecting developmental processes (Braun et al., 2013; Kavlock et al., 1996). The numerous adverse problems of the phthalates

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which might be linked to endocrine-disrupting effects, including acute toxicity, reproductive toxicity, neurotoxicity, teratogenicity, tumorigenesis, mutagenesis and cardiac toxicity (Sun and Liu, 2017; Uren-Webster et al., 2010; Vandenberg et al., 2012), have been extensively studied. The disease costs of endocrine disruptors was attributed to 2.33 % of GDP in the USA and 1.28 % of GDP in Europe (Attina et al., 2016). In recent decades, accumulating epidemiological studies provide evidence that environmental phthalate exposure can be harmful to development in children (Ejaredar et al., 2015; Jones et al., 2018; Jurewicz and Hanke, 2011; Kougias et al., 2017). For example, maternal prenatal exposure to phthalates results in negative neurodevelopment, including lower language ability (Jones et al., 2018), lower intelligence quotient (IQ) (Factor-Litvak et al., 2014), poor cognitive and behavioral outcomes (Qian et al., 2019; Zhang et al., 2019), and social communication (Ejaredar et al., 2015; Kougias et al., 2017). It has also been reported that perinatal exposure to phthalates decreases the number of neurons and synapses in the cortex and cognitive flexibility in rats (Kougias et al., 2018). However, the mechanisms underlying phthalates adversely affecting neurodevelopment are largely unknown.

In the previous study, using transgenic medaka embryos, we have shown estrogenic activity of phthalates (*i.e.* DBP, DINP, BBP) *in vivo* (Chen et al., 2014). Furthermore, it is widely studied that estrogen affects neuroplasticity and neurogenesis in the brain *via* estrogen receptor signaling pathways (Sheppard et al., 2019). However, the physiological roles of estrogen in neurons are controversy and varies with concentration, such that both neuroprotection and reduction of neurogenesis has been documented (Baez-Jurado et al., 2018; Lim et al., 2018; Sa et al., 2018). Nevertheless, estrogen receptors, especially *esr2a*, regulate neurogenesis in zebrafish during development (Tingaud-Sequeira et al., 2004). Additionally, recent studies have shown that estrogen receptors modulate neurogenesis in the brain of zebrafish and rats (Diotel et al., 2013; Lim et al., 2018). However, it is unclear whether neurotoxicity of phthalates is related to estrogen receptor signaling pathways.

Here, we found that phthalates (DBP, DINP, and BBP) disrupted the expression of estrogen receptors (*esr1*, *esr2a*, *esr2b*), hence reduced the brain size and the number of proliferating neurons in the brain of zebrafish embryos. Besides, estrogen receptor antagonist tamoxifen increased the expression of *esr2a* in zebrafish embryos exposed to phthalates, and partly rescued the brain size and proliferating neurons. Further, *in vitro* experiments results showed that exposure to phthalates induced double-strand DNA breaks in iPSC-derived human neurons. The present study suggests that exposure to phthalates affects neurodevelopment in zebrafish embryos and induces neurotoxicity in human neurons partly through disrupting the expression of estrogen receptors.

2. Materials and methods

2.1. Chemicals

Di n-butyl phthalate DBP, 524,980, Diisononyl phthalate DINP, 376,663, Butyl benzyl phthalate BBP, 308,501 and tamoxifen T9262 used in this study were from Sigma. 17-β-Estradiol (E2) standard was from the China National Standard Material Centre (Beijing, China). The stock of 30 mM DBP, 30 mM DINP, 30 mM BBP, 0.1 mM tamoxifen and 0.1 mM E2 were prepared with methanol and stored at -20 ℃.

2.2. Zebrafish maintenance and zebrafish embryo collection

Wild type zebrafish AB strain was acquired from the Zebrafish International Resource Center (ZIRC; University of Oregon, Eugene, OR, USA). The zebrafish were maintained and the embryos were collected in a controlled environment as previously described (économiques, 2013).

2.3. Embryo exposure

Healthy zebrafish embryos of 4–128 cell stage (~2 h-post fertilization, hpf) were used for the experiment. In acute toxicity experiment of figure S1, zebrafish embryos were exposed to 1.25, 2.5, 5, 10, and 20 μM of DBP, DINP and BBP, and 0.07 % methanol as solvent control, 20 embryos for each concentration. In addition, in the previous study (Chen et al., 2014), we have shown that estrogenic activity of 5 μM of BBP was approximately equivalent to 10 nM E2, and 5 μM of DBP or DINP revealed enhanced-estrogenic activity in the presence of low dose estrogen; therefore, zebrafish embryos were exposed to 5 μM of DBP, DINP, and BBP, respectively; in addition to exposed to 10 nM E2 as the positive control in the present study of molecular experiment. Embryos were exposed to 5 μM DBP, 5 μM DINP, 5 μM BBP, and 10 nM E2, and to 0.02 % methanol as the solvent control. In the tamoxifen rescue experiment, zebrafish embryos were exposed to 10 nM tamoxifen alone, 5 μM DBP + 10 nM tamoxifen, 5 μM DINP + 10 nM tamoxifen, 5 μM BBP + 10 nM tamoxifen, 10 nM E2 + 10 nM tamoxifen, and 0.03 % methanol as the solvent control. The exposure was performed in 96-well plates, with one embryo per well and incubated at 28 ± 0.5℃. The results were checked after 3 days exposure.

2.4. Human iPSC and differentiation of iPSCs into neurons

Induced pluripotent stem cells (iPSCs) were generated from human fibroblasts. The iPSC line carrying duplications in the gene for Amyloid Precursor Protein was provided by Dr. Lawrence Goldstein at the University of California, San Diego (Israel et al., 2012; Raja et al., 2016). iPSCs were prepared on matrigel coated plates and transduced with lentivirus expressing rtTA and Ngn2-GFP according to published protocols with modifications (Lin et al., 2018b; Zhang et al., 2013). One day after viral transduction, the culture media was replaced with DMEM/F-12 (12634010, Thermo Fisher Scientific) containing 1 × N-2(17502001, Thermo Fisher Scientific) and 5 μg/mL doxycycline (D1822, Sigma-Aldrich) to induce Tetracycline resistance O (TetO) gene expression. Subsequently, culture media was changed with Neurobasal medium (21103049, Thermo Fisher Scientific) containing 1 × GlutaMAX (35050061, Thermo Fisher Scientific), 0.2 μg/mL laminin (AG56 P, Millipore), 5 μg/mL doxycycline and 1 μg/ml puromycin (540222, Millipore). One half-plate volume of media was replaced with new media every 3 days until cells were ready for experimental assays.

1 nM phthalates (DBP, DINP, and BBP) were freshly prepared with culture media. Neurons were transient exposed to 1 nM phthalates and the solvent control (0.001 % methanol) containing culture media for 30 min. After exposure, the neurons were used for RNA extraction and immunostaining.

2.5. Quantitative real-time PCR (qRT-PCR)

For zebrafish, 12 embryos of 3 dpf were pooled together and total RNA was extracted using a Trizol reagent (9109; Takara Bio. Inc.). Subsequently, the genomic DNA was removed with RQ1 RNA-free DNase (M6101; Promega) and 1 μg total RNA was used to synthesize cDNA using a PrimeScript reverse transcription (RT) reagent kit (6210B; Takara) according to the manufacturer's instructions. The mRNA transcript of *esr1*(accession numbers: NM_152959), esr2a (accession numbers: NM_180966) and *esr2b* (accession numbers: NM_174862) were assessed by qRT-PCR using the SYBR Premix Ex Taq (RR402A; Takara Bio. Inc.) and *β-actin* was used as the reference gene as previously described (Xu et al., 2020). Briefly, the qRT-PCR reaction system was pre-denatured at 95 ℃ for 30 s for one cycle, and 40 cycles at 95 ℃ for 5 s followed 60 ℃ for 30 s. Subsequently, the dissociation program was performed at 95 ℃ for 15 s, 60 ℃ for 1 min and then 95 ℃ for 15 s.

Neurons were transient exposed to 1 nM phthalates and the solvent control, subsequently, total RNA was extracted using TRIzol reagent

Table 1
Primer sequence for qRT-PCR.

Gene	primer sequence	Tm (℃)
esr1 F	CAGGACCAGCCCGATTCC	59.0
esr1 R	TTAGGGTACATGGGTGAGAGTTTG	58.8
esr2a F	CTCACAGCACGGACCCTAAAC	59.4
esr2a R	GGTTGTCCATCCTCCCGAAAC	59.6
esr2b F	CGCTCGGCATGGACAAC	57.9
esr2b R	CCCATGCGGTGGAGAGTAAT	58.7
β-actin F	GCTGACAGGATGCAGAAGGA	58.9
β-actin R	TAGAAGCATTTGCGGTGGAC	57.7
c-fos Fh	ACTACCACTCACCCGCAGAC	60.4
c-fos Rh	GGAATGAAGTTGGCACTGG	55.8
npas4 Fh	CAGGGTGACAGCATCTACGAC	59.0
npas4 Rh	ATCGGCCTCGAATAAGCAC	56.9
egr1 Fh	GACCGCAGAGTCTTTTCCTG	57.4
egr1 Rh	AGCGGCCAGTATAGGTGATG	57.4
gapdh Fh	GTCTCCTCTGACTTCAACAGCG	59.2
gapdh Rh	ACCACCCTGTTGCTGTAGCCAA	63.2

(15596026, Invitrogen) and reverse transcription of total RNA was carried out using RNA to cDNA EcoDry Premix (Oligo dT) (639549, Clontech) according to manufacturer's protocol. qRT-PCR was performed using a Bio-Rad CFX-96 quantitative thermocycler and SsoFast EvaGreen Supermix (172–5200, Bio-Rad). The relative mRNA transcript of genes (*c-fos*: NM_005252; *npas4*: NM_178864; *egr1*: NM_001964) was determined using the $2^{-\Delta\Delta Ct}$ method and *gapdh* was used as the reference (Madabhushi et al., 2015; Maheu et al., 2015; Nabokina et al., 2017; Tessier et al., 2012). Four biological replicates were performed for each sample. The information of primer sequences used for qRT-PCR can be found in Table 1, and the specific of the primers were also confirmed using Primer-BLAST.

2.6. Immunohistochemistry

For whole-mount zebrafish embryo immunostaining, 3 dpf zebrafish were fixed with HistoChoice® MB (H120; AMRESCO, LLC) for 3 h at room temperature, and then washed with PBS for 3 min × 3 times. The embryos were dehydrated with 50 %–70 %-90 %-100 %-100 % methanol and then rehydrated with 90 %-70 %-50 % methanol for 3 min at each concentration. Subsequently, the embryos were washed with PBST (0.5 % tween 20 and 0.5 % Triton X-100 in PBS) for 15 min × 3 times and then blocked with block buffer (5% BSA and 1% goat serum in PBST) for 2 h at room temperature. The samples were incubated with the primary antibody at 4 ℃ overnight and then washed with PBST for 15 min × 3 times, and subsequently incubated with the secondary antibody at 4 ℃ overnight. The embryos were washed with PBST for 15 min × 4 times, cleared with 50 % glycerol in PBS for 1 h at room temperature before imaging. The images of the brain were captured using a Zeiss Lightsheet Z.1 3D microscope as previously described (Xu et al., 2018). Briefly, zebrafish embryo was mounted with 1% low melting agarose in a small capillary glass tube after staining, then place the capillary tube into the chamber of the microscope, turn the sample almost vertical in the chamber and the back of the embryo head parallel to lens. Subsequently, the whole brain of the embryos was scanned with 3D Z-stack function and the images were exported after maximum projection was performed. The primary antibodies used in this study were: rabbit anti-phospho-Histone H3 (Ser10) (06–570; Millipore) and mouse anti-HuC/HuD Monoclonal Antibody (16A11) (A-21271; Invitrogen). The secondary antibodies used in this study were: Cy3-conjugated goat anti-mouse (A10521; Invitrogen) and FITC conjugated goat anti-Rabbit IgG Antibody (AP307 F; Sigma-Aldrich).

Neurons were exposed to phthalates and the solvent control as described above and then cells were washed with cold PBS, followed by fixation with 4% cold paraformaldehyde (PFA) in PBS. Cells were next permeabilized and blocked in PBS containing 0.2 % Triton X-100 and 10 % normal donkey serum at room temperature for 1 h, incubated with primary antibodies (γH2AX, Millipore, 05–636; MAP2, BioLegend, PCK-554 P and then diluted in blocking buffer at 4 ˚C overnight. Primary antibodies were visualized using the appropriate secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 647 antibodies A28175 and A-21449, respectively, Thermo Fisher Scientific, all diluted 1:500 in blocking buffer and nuclei were visualized with Hoechst 33,342 H1399, Thermo Fisher Scientific. Samples were washed three times in PBS after incubation with the primary and secondary antibodies. Images were acquired on a Zeiss LSM710 confocal microscope. Quantification of γH2AX intensity was analyzed with Imaris image analysis software Bitplane Ruprecht et al., 2019).

2.7. Statistical analysis

Statistical analysis was carried out using Student's two-tailed *t*-test and one way-ANOVA with Dunnett *post hoc* test performed using GraphPad Prism 8, and $p < 0.05$ was considered as a statistically significant difference. Data are presented as means ± S.D. (standard deviation).

3. Results

3.1. Phthalates affects the expression of estrogen receptors in zebrafish embryos

We wondered whether exposure to phthalates, such as DBP, DINP, and BBP, would disrupt the expression of estrogen receptors (*esr1, esr2a, esr2b*) in zebrafish during embryonic development. We found that no zebrafish embryo was dead at the concentration of 5 μM DBP and BBP. Even at the concentration of 20 μM of DINP, all zebrafish embryos were survival after 3 days exposure (Figure S1). Interestingly, our results showed that, like estrogen, 5 μM BBP induced the expression of *esr2b* and reduced the expression of *esr1* and *esr2a* at 3 dpf zebrafish (Fig. 1). Both DBP and DINP moderately, but not significantly ($p = 0.156$ and $p = 0.159$), increased the expression of *esr2b*; however, they also decreased the expression of *esr1* and *esr2a* (Fig. 1). Thus, phthalates DBP, DINP and BBP were capable of disrupting the expression of estrogen receptors (*esr1*, *esr2a*, *esr2b*) in zebrafish embryonic development. Previously it has been reported that the expression of *esr2a* was important for zebrafish neurogenesis (Mouriec et al., 2009; Tingaud-Sequeira et al., 2004). The results suggest that phthalates have potential estrogenic activity and might affect neurogenesis in the early development of zebrafish.

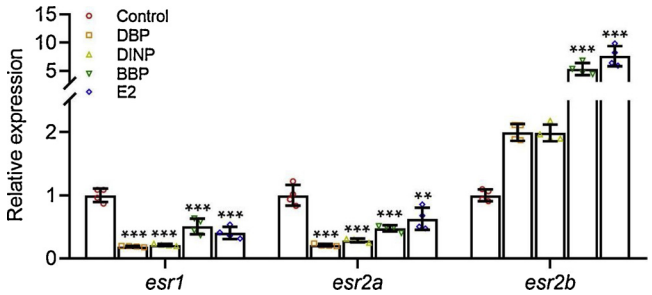

Fig. 1. The expression of *esr1*, *esr2a* and *esr2b* in 3 dpf zebrafish exposed to solvent control, DBP, DINP, BBP, and E2. Zebrafish embryos of 4–128 cell stage was exposed to 0.02 % methanol as solvent control, 5 μM DBP, 5 μM DINP, 5 μM BBP and 10 nM E2, respectively. After 3 days exposure, total RNA of the embryos was extracted and qRT-PCR was performed. An asterisk represents a significant difference was observed when compared with the control group at $p < 0.01$ (**) or $p < 0.001$ (***) level using one-way ANOVA with Dunnett *post hoc* test, $n = 4$.

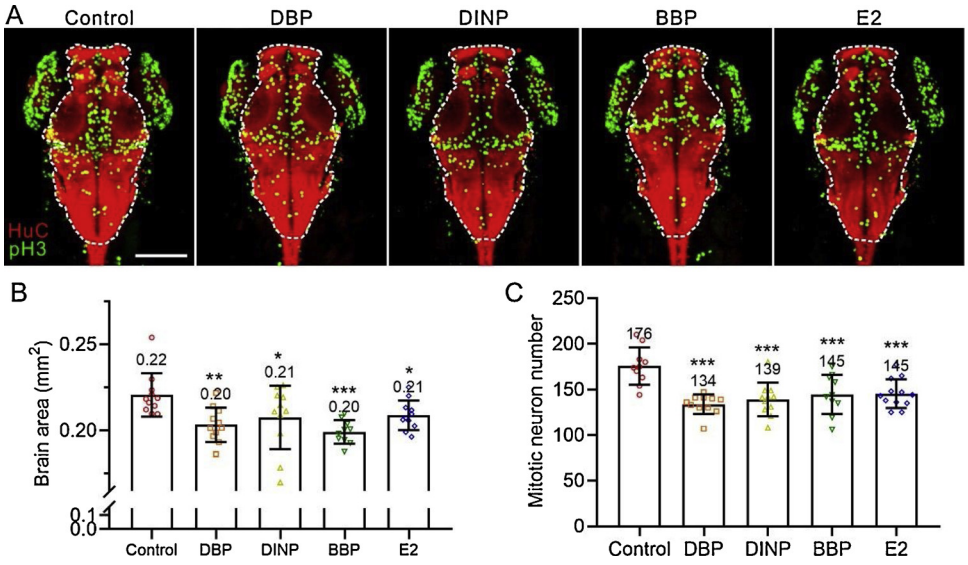

Fig. 2. Exposure to phthalates and estrogen reduced neurogenesis in zebrafish embryos. A. Representative images showing neurons and mitotic cells in the brain of zebrafish embryos exposed to the solvent control (0.02 % methanol), DBP (5 μM), DINP (5 μM), BBP (5 μM), and E2 (10 nM) from ~ 2 hpf to 3 dpf. The neurons (red) and mitotic cells (green) were immunolabelled with anti-HuC and anti-pH3, respectively. The brain area of zebrafish embryos was bounded with a white dashed line. Scale bar: 200 μm. **B, C**. Bar charts showing the quantification of brain area (B) and the number of mitotic neurons in the brain (C) in panel A. Asterisk represents significant difference was observed when compared with control group at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) level using one way-ANOVA with Dunnett *post hoc* test. n = 10 ~ 11.

3.2. Phthalates reduced neurogenesis in the brain of zebrafish embryos

To investigate whether phthalates affect neurogenesis in zebrafish during embryonic development, we immunolabelled elav/HuC, a marker of neurons (Kim et al., 1996; Lin et al., 2018a), and phospho-Histone H3 (pH3), a marker of mitotic cells, and visualized neurogenesis in the brain (*i.e.* forebrain, midbrain and hindbrain) of zebrafish embryos using light sheet microscopy, which is a powerful tool to study neurons in zebrafish embryos (Lin et al., 2018a). We next assessed brain size by measuring the forebrain, midbrain, and hindbrain area of zebrafish embryos with different treatment regimens. Surprisingly, our results show that exposure to DBP, DINP, BBP as well as E2 (positive control) significantly reduced the brain size compared with the control group (Fig. 2A, B). Additionally, we found that most of the mitotic neurons, which are double-labeled with HuC (red) and pH3 (green), located in the stratum periventricular (SPV) of the midbrain and the cerebellum of the hindbrain (Fig. 2A). Additionally, the number of mitotic neurons was significantly reduced in the brain of zebrafish embryos exposed to phthalates and E2 compared with control embryos (Fig. 2A, C). These results suggest that exposure to phthalates and estrogen at embryonic stage inhibits neurogenesis in zebrafish.

3.3. Tamoxifen increased the expression of estrogen receptors in zebrafish embryos

Tamoxifen is an antagonist of estrogen receptors and widely used in breast cancer therapy (Lumachi et al., 2013). We wondered if tamoxifen could reverse the reduction in *esr2a* of zebrafish embryos exposed to phthalates. qRT-PCR was performed to study the expression of estrogen receptors in zebrafish embryos exposed to tamoxifen only, DBP + tamoxifen, DINP + tamoxifen, BBP + tamoxifen, and the methanol solvent control. Compared with the solvent control, tamoxifen induced the expression of *esr2a* in zebrafish embryos (Fig. 3A). In addition, the expression of *esr2a* in zebrafish embryos was also induced by tamoxifen in the presence of DBP and BBP compared with exposure to DBP and BBP only (Fig. 3 B, D), though tamoxifen didn't significantly affect the expression of *esr2a* in zebrafish embryos exposed to DINP (Fig. 3C). In addition, tamoxifen didn't affect the expression of *esr1*, and increased the expression of *esr2b* in the solvent control and BBP group (Figure S2).

3.4. Tamoxifen rescued neurogenesis of zebrafish exposed to phthalates during embryonic development

Since tamoxifen increased the expression of *esr2a* in zebrafish embryos in the presence of DBP and BBP exposure (Fig. 3), we asked whether tamoxifen could rescue impaired neurogenesis in the brain of zebrafish embryos exposed to phthalates. Zebrafish embryos were exposed to estrogen and phthalates in the presence or absence of tamoxifen, and neurogenesis was assessed at 3 dpf. We found that the brain size of zebrafish embryos exposed to estrogen and DINP in the presence of tamoxifen was no different from the control group, though exposure to tamoxifen only didn't increase the brain size of zebrafish embryos (Fig. 4A, B). The brain size of the fish embryos exposed to DBP and BBP in the presence of tamoxifen were still smaller than that of the solvent control (Fig. 4B). Nevertheless, we found that tamoxifen could also partly increase the brain size of zebrafish embryos exposed to estrogen, DINP and BBP, but not DBP (Fig. 4A, C), when we compared the brain size of zebrafish embryos exposed to estrogen and phthalates (Fig. 2B) with those exposed to estrogen and phthalates in the presence of tamoxifen (Fig. 4B), and the brain size in figure 2B (0.22 mm^2) and figure 4B (0.22 mm^2) was no significant difference in solvent control group. To better understand the function of tamoxifen modulating neurogenesis in zebrafish embryos exposed to estrogen and phthalates, we investigated the mitotic neurons in the brain of zebrafish embryos with a different regimen. Consistent with the result of brain size in zebrafish embryos, we found that the number of mitotic neurons in the brain of zebrafish embryos exposed to estrogen and DINP in the presence of tamoxifen was not significantly different than that of the control group, albeit exposure to tamoxifen alone didn't increase mitotic neurons in brain of zebrafish embryos (Fig. 4A, D). We found the mitotic neurons in figure 2C (176) and figure 4D (173) was no significant difference in solvent control group. We next compared the number of mitotic neurons in fish embryos exposed to estrogen and phthalates (Fig. 2C) with those exposed to estrogen and phthalates in the presence of tamoxifen (Fig. 4D). The results revealed that tamoxifen increased the number of mitotic neurons in the brain of fish embryos exposed to estrogen, DBP and DINP (Fig. 4E).

Collectively, these data show that tamoxifen can partly (albeit not completely restore to a normal level) rescue impaired neurogenesis, as judged by brain size and number of mitotic neurons, in the brain of zebrafish embryos exposed in estrogen and phthalates.

3.5. Phthalates induced double-strand DNA break (DSB) in human neurons

It has been reported that estrogen can stimulate the expression of topoisomerase II which can generate breaks in double-stranded DNA cancer cells (Ju et al., 2006; Sasanuma et al., 2018). Additionally, DNA DSBs seem to be a neurotoxic event that is involved in several

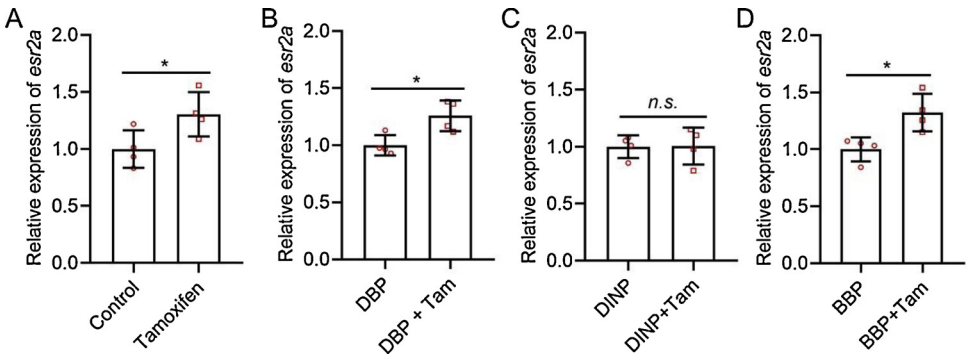

Fig. 3. The expression of *esr2a* in 3 dpf zebrafish with different regimens. A-D. Zebrafish embryos were exposed to the solvent control (0.03 % methanol) and tamoxifen (A, 10 nM), DBP (5 μM) and DBP in the presence of tamoxifen (B, 10 nM), DINP (5 μM) and DINP in the presence of tamoxifen (C), BBP (5 μM) and BBP in the presence of tamoxifen (D, 10 nM) from ~ 2 hpf to 3 dpf. The expression of estrogen receptors was investigated at 3 dpf using qRT-PCR. An asterisk represents a statistical difference at $p < 0.05$ (*) level with two-tail *t*-test, n = 4.

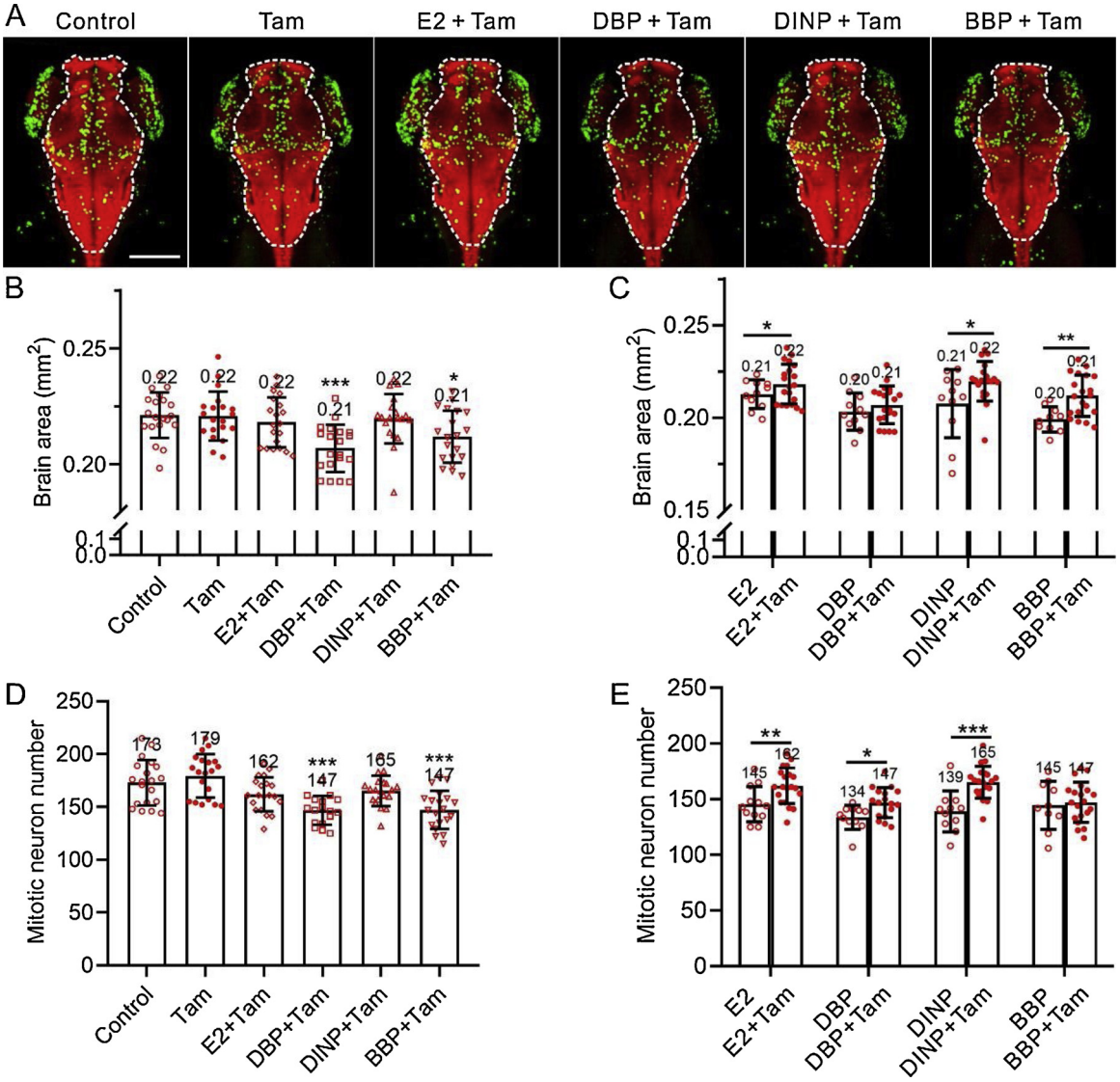

Fig. 4. Tamoxifen rescued impaired neurogenesis in the brain of zebrafish embryos exposed to estrogen and estrogenic phthalates. A. Representative images showing neurons and mitotic cells in the brain of zebrafish embryos exposed to the solvent control (0.03 % methanol), tamoxifen (10 nM), and E2(10 nM), DBP (5 μM), DINP (5 μM), BBP (5 μM) in the presence of tamoxifen (10 nM) from ~ 2 hpf to 3 dpf. The neurons (red) and mitotic cells (green) were immunolabelled with anti-HuC and anti-pH3, respectively. The brain area of zebrafish embryo was bounded with a white dashed line. Scale bar: 200 μm. **B**. Bar charts showing the quantification of a brain area in panel A. Asterisk represents significant difference was observed when compared with the control group at $p < 0.05$ (*) or $p < 0.001$ (***) level using one way-ANOVA with Dunnett *post hoc* test. n = 20. **C**, Bar charts showing the comparison of the brain area in Fig. 2B and 4B. An asterisk represents a significant difference was observed when compared with the control group at $p < 0.05$ (*) or $p < 0.01$ (**) level with two-tail *t*-test. **D**. Bar charts showing the quantification of the number of mitotic neurons in the brain in panel A. Asterisk represents significant difference was observed when compared with the control group at $p < 0.001$ (***) level using one way-ANOVA with Dunnett *post hoc* test. n = 20. **E**. Bar charts showing the comparison of the number of mitotic neurons in Fig. 2C and 4D. An asterisk represents a significant difference was observed when compared with the control group at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) level with two-tail *t*-test.

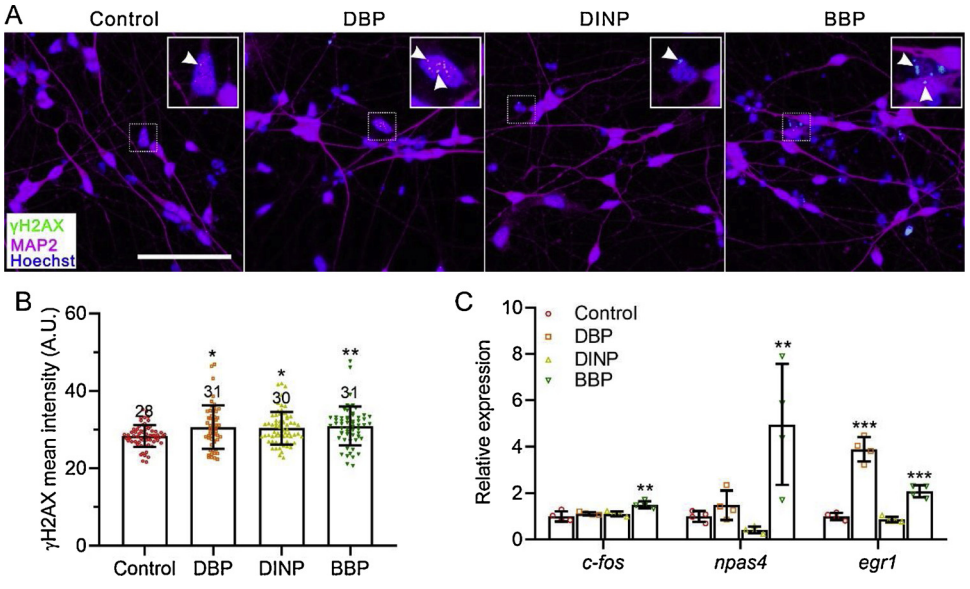

Fig. 5. Phthalates induced double-strand DNA breaks in human neurons. A. Representative images showing double-strand DNA breaks (γH2AX labeled, green) in the nucleus (Hoeches labeled, blue) of mature neurons (MAP2 labeled, pink) after exposure to the solvent control (0.001 % methanol), 1 nM DBP, 1 nM DINP, and 1 nM BBP. The white box in the right upper displays the magnification of the white dashed line bounded area in each panel. The white arrowhead demonstrates the expression of γH2AX in the nucleus of neurons. Scale bar: 100 μm. **B.** Bar charts showing the quantification of expression of γH2AX in panel A. An Asterisk represents a significant difference was observed when compared with the control group at $p < 0.05$ (*) and $p < 0.01$ (**) level using one way-ANOVA with Dunnett *post hoc* test. n = 54 ~ 68. **C.** Bar charts showing the relative expression of *c-fos, npas4,* and *egr1* in neurons exposed to the solvent control and phthalates (DBP, DINP, BBP). The asterisk represents a significant difference was observed when compared with the control group at $p < 0.01$(**) or $p < 0.001$ (***) level using one-way ANOVA with Dunnett *post hoc* test. n = 4.

neurodegeneration models in mice (Dobbin et al., 2013; Madabhushi et al., 2014). Thus, we wondered whether phthalates which showed estrogenic (BBP) or enhanced-estrogenic activity (DBP and DINP) could also induce double-strand DNA breaks (DSBs) in human neurons. After exposure to 1 nM phthalates for 30 min., the neurons were immunolabeled with anti-γH2AX, which is a well established DNA DSB signaling marker (Crowe et al., 2006; Madabhushi et al., 2015), and counterstained with MAP2 (labelling mature neurons) and Hoechst (labelling nucleus). Our results show that transient exposure to phthalates significantly induces DNA DSBs in neurons, as judged by the expression of γH2AX in the nucleus of the neurons (Fig. 5A, B). It was reported that the formation of DNA DSBs in the promoter early-response genes, including *c-fos*, *npas4*, and *egr1,* can induce the expression of these genes in neurons after stimulation (Madabhushi et al., 2015). Thus, we next investigated the expression of *c-fos*, *npas4*, and *egr1* in neurons after exposure to phthalates. qRT-PCR results indicate that the expression of these genes was significantly induced by BBP exposure (Fig. 5C). Additionally, exposure to DBP also induced the expression of *egr1*, though we didn't find that 1 nM DINP significantly induced the expression of these genes in neurons (Fig. 5C). Collectively, the data show that exposure to phthalates, especially DBP and BBP, induce DNA DSBs in human neurons.

4. Discussion

The adverse effect of phthalates, as a family of ubiquitous endocrine-disrupting chemicals in the environment, has attracted extensive concern from the whole world (Braun et al., 2013). It has been reported that phthalates (*i.e.* DBP, DINP, BBP) exhibit estrogenic activity for both in *vitro* and *in vivo* experiments (Cano-Nicolau et al., 2016; Harris et al., 1997), and some phthalates (such as di(2- Ethylhexyl) phthalate, showing estrogenic activity) are reported to affect reproductive disorder in adult fish (Golshan et al., 2015; Uren-Webster et al., 2010) and masculine play in boys (Swan, 2008; Swan et al., 2010). In the present study, we found that exposure to phthalates (*i.e.* DBP, DINP, BBP) disrupted the expression of estrogen receptors (*esr1*, *esr2a*, and *esr2b*) and impaired neurogenesis in the brain of zebrafish during embryonic development, while tamoxifen partly reversed these adverse effects. Additionally, our *in vitro* experiment revealed that exposure to phthalates induced DNA DSBs in human neurons.

Most of the research has shown that a high concentration of estrogen (such as 100 ~ 1000 nM E2) induces the expression of *esr1* in zebrafish embryos (Chandrasekar et al., 2010; Hao et al., 2013). Our results showed that exposure of ~ 2 hpf embryos to a low concentration of E2 (10 nM) moderately reduced the expression of *esr1* and *esr2a* while highly induced the expression of *esr2b* in 3 dpf zebrafish embryos (Fig. 1). Our finding were consistent with previous studies demonstrated that exposure to low dose E2 reduced the expression of *esr1* at 3 dpf (Ahi et al., 2016). In our previous study, we have shown that BBP exhibited estrogenic activity while DBP and DINP enhanced estrogenic activity in the presence of low concentration of estrogen (Chen et al., 2014). In the present study, we showed that 5 μM BBP and 10 nM estrogen shared similar characteristics that induced the expression of *esr2b* and reduced the expression of *esr1* and *esr2a*, while 5 μM DBP and DINP only reduced the expression of *esr1* and *esr2a* (Fig. 1). These results further confirmed that BBP and DBP/DINP exhibit varying estrogenic activity.

The expression of estrogen receptors was various during zebrafish embryogenesis. The expression of *esr1* started at 12 hpf and reached its peak level at 48 hpf and lasted to 120 dpf, while the expression of *esr2a* was extremely high as early as 3 hpf, and then decreased to a low level (Chandrasekar et al., 2010; Hao et al., 2013; Mouriec et al., 2009). At 24 hpf, *esr2a* was prominently expressed in the brain (Mouriec et al., 2009; Tingaud-Sequeira et al., 2004), suggesting that *esr2a* is involved in neurogenesis during development. Interestingly, our results showed that both phthalates and low concentration of E2 inhibited the expression of *esr2a*, and reduced brain size as well as the number of mitotic neurons (Fig. 2), indicating that phthalates, as well as E2, affect neurogenesis in zebrafish embryonic development. These results were consistent with reports that estrogen and phthalate (DBP) impair head development of zebrafish embryos (Ahi et al., 2016; Jergensen et al., 2019) and decrease the number of proliferating cells in the brain of adult zebrafish (Diotel et al., 2013; Makantasi and Dermon, 2014). Additionally, our findings were similar to reports that phthalates reduce the number of neurons in the rat brain (Kougias et al., 2018) and impair proliferating neurons (PC12 cells) *in vitro* (Chen et al., 2011). Tamoxifen is an important estrogen receptor modulator, which has been widely used in breast cancer therapy, and is known to be involved in a neuroprotective function in the brain (Baez-Jurado et al., 2018). Our data showed that tamoxifen increased the expression of *esr2a* in the

embryos exposed to DBP and BBP and rescued (at least partly) the impaired neurodevelopment in zebrafish embryos exposed to phthalates and estrogen (Fig. 3,4). Though we didn't find that tamoxifen significantly induced the expression of *esr2a* in the embryos exposure to DINP, tamoxifen rescued the brain size and mitotic neurons in the zebrafish embryos exposed to DINP to normal level (Fig. 3C, 4B, 4D). The different efficacy of tamoxifen rescuing the expression of estrogen receptors and neurogenesis in zebrafish embryos exposure to different phthalates might due to the different binding affinity of the phthalates to estrogen receptors (Takeuchi et al., 2005; Toda et al., 2004). Our data was also consistent with the finding that the estrogen receptor inhibitor ICI and tamoxifen promote neuron proliferation in zebrafish (Diotel et al., 2013) and protect neuron apoptosis after injury in the brain of rat (Lim et al., 2018), respectively.

It has been known that deficiency of the DNA repair factor BRCA1 occurring in Alzheimer's brains in both mice and human beings, leads to impaired cognitive functions (Suberbielle et al., 2015), suggesting that neurons in Alzheimer's might be more vulnerable to DNA damage. Thus, in the present study, we used iPSC derived-neurons generated from fibroblasts of Alzheimer's disease patient harboring an APP gene duplication to assess the neurotoxicity of phthalates at a low concentration (1 nM). Previous studies demonstrated that 0.1 mg/L phthalates did not show cytotoxicity in several types of human cells (Chen et al., 2011; Gutiérrez-García et al., 2019; Mankidy et al., 2013). However, using iPSC derived-neurons, we found that 1 nM phthalates (equivalent to 0.3 ~ 0.4 μg/L), especially DBP and BBP, can significantly induce DNA damage in cells and early-response genes (Fig. 5). Moreover, *c-fos* is known as an estrogen response gene in many cells, such as neurons (Cattaneo and Maggi, 1990; Garcia et al., 2000; Pfaus et al., 1993). However, the concentration of phthalates could be as high as ~1.25 – ~34.4 nM in water of the nature environment (Abtahi et al., 2019; Guo et al., 2013; Li et al., 2017). Thus, our results indicated that some environmental phthalates could impair the human neurons.

5. Conclusion

Collectively, our results demonstrate that phthalates (DBP, DINP, BBP) disrupt the expression of estrogen receptors and impair neurogenesis during zebrafish embryonic development, and induce DNA damage in human neurons. The adverse effect of phthalates on the neurodevelopment of zebrafish embryos can be partly rescued by tamoxifen. These findings suggest that the estrogen receptor pathway might be involved in one of the mechanisms underlying the neurotoxicity of phthalates.

Author contributions

XC and SHC designed and coordinated the study; SX designed, performed and analyzed the zebrafish experiments; SX and HZ wrote the paper; PCP, LHT, AL and JW performed and analyzed the experiments in neurons; SWC and FAM edited the manuscript. YSC contributed to zebrafish embryos exposure. All authors reviewed the results and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105469.

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